GENETIC VARIATION, FIBER PROCESSING AND USE PATTERN OF ALLO (*Girardinia diversifolia* (Link) Friis) IN NEPAL

A THESIS SUBMITTED TO THE

INSTITUTE OF SCIENCE AND TECHNOLOGY

THROUGH RESEARCH CENTRE FOR APPLIED SCIENCE AND TECHNOLOGY

TRIBHUVAN UNIVERSITY

NEPAL

FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

IN BIOTECHNOLOGY

BY

BIJAY RAJ SUBEDEE

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DECLARATION

Thesis entitled **"Genetic Variation, Fiber Processing and Use Pattern of Allo (*Girardinia diversifolia* (Link) Friis) in Nepal"** is submitted to the Institute of Science and Technology (IOST) through Research Centre for Applied Science and Technology (RECAST), Tribhuvan University, Nepal for the award of the degree of Doctor of Philosophy (Ph.D.), is a research work carried out by me under the supervision of Dr. Ram Prasad Chaudhary (Emeritus Professor), Research Centre for Applied Science and Technology, Tribhuvan University, Kirtipur, Nepal.

This research work is original and has not been submitted earlier in part or full in this or any other form to any university or institute, here or elsewhere, for the award of any degree.

…….

Bijay Raj Subedee

RECOMMENDATION

This is to recommend that **Mr. Bijay Raj Subedee** has carried out research entitled **"Genetic Variation, Fiber Processing and Use Pattern of Allo (*Girardinia diversifolia* (Link) Friis) in Nepal"** for the award of Doctor of Philosophy (Ph.D.) in **Biotechnology** under my supervision. To the best of my knowledge, this work has not been submitted for any other degree.

Mr. Subedee has fulfilled all the requirements laid down by the Institute of Science and Technology (IOST), Tribhuvan University, Kirtipur for the submission of the thesis for the award of Ph.D. degree.

……

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May 2022

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मितिः .....

LETTER OF APPROVAL

Date: 7/May/2022

On the recommendation of Emeritus Professor **Dr. Ram Prasad Chaudhary** this Ph.D. thesis submitted by **Bijay Raj Subedee**, entitled "**Genetic Variation, Fiber Processing and Use Pattern of Allo (*Girardinia diversifolia* (Link) Friis) in Nepal**" is forwarded by Research Centre for Applied Science and Technology (RECAST) Research Committee to the Dean, Institute of Science and Technology (IoST), Tribhuvan University, Nepal.

.....

Prof. Dr. Ram Nath Prasad Yadav
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Kirtipur, Kathmandu,
Nepal

ACKNOWLEDGEMENTS

With due respect, I would like to express my sincere gratitude to my thesis supervisor Dr. Ram Prasad Chaudhary, Emeritus Professor, Research Centre for Applied Science and Technology (RECAST), Tribhuvan University for the continuous support, motivation, and guidance in every step of research work. I also admire his continuous effort and endeavour for the completion of this Ph.D. thesis. I would like to express my sincere gratitude to Dr. Giri Raj Tripathi, Central Department of Biotechnology, for providing Molecular Laboratory facilities in Truffle Research Center, Tribhuvan University, Kirtipur without which this research work would not have been successfully accomplished. I am thankful to Prof. Dr. Ram Nath Prasad Yadav, Executive Director of RECAST, Prof. Dr. Rameshwar Adhikari, former Executive Director of RECAST, and Durga Prasad Pandey, former executive Director, RECAST for their continuous support and encouragement and faculties and staffs of RECAST for their kind support. I am very much thankful to Prof. Dr. Krishna Das Manandhar, Head of Central Department of Biotechnology, for his encouragement. I am indebted to Prof Dr. Haining Qin, Institute of Botany, Chinese Academy of Sciences (IBCAS), Beijing. Peoples Republic of China for providing me with space and laboratory facilities to carry out partial molecular research work. I would also like to thank Dr.-Ing. Ralf Lach, for providing an opportunity to carry out the characterization of samples at Polymer Service GmbH Merseburg, Germany.

I would like to thank Central Department of Chemistry, Tribhuvan University, Kirtipur and Department of Customs, Government of Nepal for helping with taking Fourier-Transform Infrared Spectroscopy (FTIR) patterns of some samples, and Nepal Academy of Science and Technology (NAST) for helping to conduct X-ray Powder Diffraction (XRD) of the samples.

I would like to acknowledge Kailash Sacred Landscape Conservation and Development Initiative (KSLCDI), Nepal, a collaborative programme between Ministry of Forests and Environment (MoFE) (Previously Ministry of Forests and Soil Conservation, MoFSC), Government of Nepal. ICIMOD and RECAST for providing scholarship (June 2015 to 2017) to carry out this research work. I also take this opportunity to express my appreciation to the respected teachers Prof. Dr. Bijaya

Pant, and Prof. Dr. Rajani Malla. I am also grateful to Mr. Achyut Nepal for providing me some required materials for carrying out the research.

I am thankful to my beloved friends, Sabin Dura, Amit Stapit, and all friends who helped me directly or indirectly, especially for conducting research with new ideas and positive attitudes. I also thank Mr. Komal Malla of Pokhara University for his valuable guidance.

I appreciate all the academic and administrative staffs of Research Centre for Applied Science and Technology for their constant stimulation and affection throughout my research at RECAST. Last, but not least, I am very much obliged to my father Prof. Dr. Raja Ram Subedi, my mother Hari Priya Subedi, my wife Pramila Poudel Subedee, my daughter Prabina Subedee, my son Pranav Subedee and all my family members for their everlasting moral support, inspiration to work in computer, and motivation with love and support.

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Bijay Raj Subedee

May 2022

ABSTRACT

Plants are the major source of natural fiber, which are greatly supporting the livelihood of Indigenous Peoples and Local Communities (IPLCs) of Nepal. *Girardinia diversifolia* (Link) Friis, a fiber yielding wild plant species, belonging to Family Urticaceae is commonly known as Himalayan giant nettle and locally as 'Allo' in Nepal. The plant is erect, 1.5- 3m tall, perennial herb, its leaves, and stems are covered with stinging hairs. *G. diversifolia* is widespread in South Africa and Madagascar. In Asia, the plant is widely distributed from subtropical to temperate regions of the Himalayas at altitudes of 1,000 m to 3,000 m above sea level.

Morpho-geographical studies were carried out in the samples collected from western, central, and eastern regions of Nepal. The study showed that *G. diversifolia* has an ample number of variations in leaf lobes and trichome numbers within individuals or among the populations with multiple synonyms that emphasized to conduct genetic variation analysis. However, *G. diversifolia* found in Panchthar District are tallest having cylindrical stems with small diameters and are considered in terms of good fiber quality. Morphologically this research also revealed the presence of two subspecies of *G. diversifolia* in Nepal: *G. diversifolia* subsp. *diversifolia* (having diagnostic features: leaves blade variable, 1-7 lobes, ovate with serrate margins, stipules 10-20 mm) and *G. diversifolia* subsp. *suborbiculata* (having diagnostic features: leaves blade unlobed, suborbicular or ovate-suborbicular with dentate margins, stipules 4-7 mm). Presence of *G. diversifolia* subsp. *suborbiculata* is probably a new record for Nepal and Bhutan.

Genetic variation was studied in samples collected from western, central, and eastern regions of Nepal. Genomic DNA was extracted using Doyle and Doyle (1987) with modifications which removed the high concentration of polyphenols and secondary metabolites contained in *G. diversifolia* that inhibit Polymerase Chain Reaction (PCR) amplification. Genetic variation analysis was performed using Inter Simple Sequence Repeat (ISSR) method. Out of sixteen primers of University of British Columbia (UBC), ten primers were screened for the ISSR analysis that produced 131 clear bands, of which 98.09% were found polymorphic. The mean effective number of alleles (ne), Nei's gene diversity (H), and Shannon's information index (I) were found

1.598, 0.349, and 0.523, respectively. The total genetic diversity Ht (0.350±0.049), low intrapopulation genetic diversity Hs (0.141±0.03), and low estimated gene flow Nm (0.355±0.11), reflected high genetic differentiation among population Gst (0.594±0.08). The analysis of genetic variance showed that among the population was found 60% whereas within the population was 40%. DNA sequencing has been done for phylogenetic study using *matK*, ITS, *rbcL*, and *trnL-F* primers. Among these markers, *matK* only divided *G. diversifolia* into two clades. Molecular marker *matK* has placed the populations from Darchula District and Panchthar District in one clade and the population of Kathmandu District in another clade.

Different parts of *G. diversifolia* have been traditionally used by IPLCs like Rai, Magar, and Gurung communities of Nepal. The plant is used in traditional medicine for the treatment of gastritis, joint pain, and headache. The fiber of this plant is one of the livelihood options for the IPLCs from which clothes, bags, coats, and many other textile products are prepared. Due to its multipurpose uses, we have further evaluated the retting processes and uses of this plant in Nepal.

In this study, sodium hydroxide, a commercial pectinase enzyme, and a partially purified enzyme extracted from papaya were used for fiber retting of *G. diversifolia* using the spray enzyme retting (SER) method. After the retting process, morphological and change in fiber composition were investigated using optical microscope, fourier transform infrared spectroscopy, change in the fiber crystallinity using X-ray powder diffraction, mass-related tensile strength, and mass-related elastic modulus were measured. The results showed that tensile strength (2.83±0.15) N mm/mg and elastic modulus (68.4±9.4) N mm/mg were found higher in alkali-treated fiber compared with commercial pectinase (2.75±0.51) N mm/mg and plant-based enzyme (2.79±0.86) N mm/mg. Results showed that fiber properties of *G. diversifolia* can be tailored using plant-based enzyme.The traditional fiber processing techniques, use of locally available materials, and medicinal value justify to conserve genetic diversity of *G. diversifolia* in Nepal. Nevertheless, the resource stock is declining from its natural habitat due to changes in traditional grazing practices, and rapid expansion of cash crops plantation like large cardamom (*Amomum subulatum*) and Chirayito (*Swertia chirayita*) where *G. diversifolia* grows naturally. Enhancing cultivation and sustainable harvesting from forest areas are important to conserve and protect from the exploitation of this important natural fiber producing species.

LIST OF ACRONYMS AND ABBREVIATIONS

AFLP	: Amplified fragment length polymorphism
AMOVA	: Analysis of molecular variance
CI	: Crystallinity index
EMR	: Effective multiplex ratio
FTIR	: Fourier transform infrared spectroscopy
GPa	: Gigapascal
Ib	: Band informativeness
IPLCs	: Indigenous peoples and local communities
KATH	: National herbarium and plant Laboratories
KIS	: Key informant survey
KSL	: Kailash sacred landscape
Mpa	: Megapascal
MR	: Multiplex ratio
NPB	: Number of polymorphic band
nrITS	: Nuclear ribosomal internal transcribed spacer
NTFPs	: Non-timber forest products
NTSYS	: Numerical taxonomy and multivariate system
PCoA	: Principal coordinates analysis
PE	: PE herbarium
PGR	: Plant genetic resources
PIC	: Polymorphism information content
PP	: Percentage of polymorphic
RAPD	: Random amplified polymorphic DNA
RFLP	: Restriction fragment length polymorphism
R_p	: Resolving power
SAHN	: Sequential, agglomerative, hierarchial and nested
SER	: Spray enzyme retting
TNB	: Total number of bands
UPGMA	: Unweighted pair group method of arithmetic average
XRD	: X-ray diffraction

LIST OF TABLES

LIST OF FIGURES

TABLE OF CONTENTS

CHAPTER 1

1. INTRODUCTION

1.1 Background

Genetic variation refers to the difference in DNA sequences among individuals or populations. Genetic variation is specific characteristic of each population because without it there would be no artificial selection, no genetic drift, no natural selection and no species adaptation to the changing environment (Flood *et al*., 2011). Small changes in genetic variation can lead to large-scale changes in the production of novel molecules and secondary metabolites with often unknown biological activity (Firn & Jones, 2000). Knowledge of the genetic variation of plant species is of great importance, because it contributes to the proper understanding of population of species, and allows for future use like selection of genotypes for conservation and sustainable utilization (Monfared *et al*., 2018).

Genetic diversity is defined as the total number of genetic traits in a species genetic makeup. It ranges widely from the number of species to differences within (intraspecific) and between (interspecific) species (Hamrick *et al*., 1992; Vellend & Geber, 2005). Genetic diversity is controlled by the following: migration, mutation, selection, and drift, each of which can be compared to the level of species diversity (Vellend & Geber, 2005).

Fiber is a fine slender thread that is many times longer than the width. Fiber is a natural or man-made substance that has been used from the beginning of human civilization. Natural fiber are obtained from plants, animals, insects, and man-made fibers are prepared through a process developed by human beings. Natural fibers are generally biodegradable, lightweight, neutral emitter of carbondioxide and are important sources of income for people living in rural areas (Fidelis *et al*., 2013). Plant fibers are natural, eco-friendly materials found in stems, leaves, fruits, and roots. The growing interest of consumers, industries, and scientists, in eco-friendly natural resources has attracted attention to the natural fibers (Silva *et al*., 2008).

More than 70 species of fiber producing plants are found in the tropical to temperate forests of Nepal. The main fiber plants used in Nepal are Hemp (*Cannabis sativa*), Jute (*Corchorus capsularis*), and Himalayan Giant Nettle (*Girardinia diversifolia*) (Singh & Shrestha, 1984). *Girardinia diversifolia* (Link) Friis belongs to the Urticaceae family. Nepal's indigenous peoples and local communities (IPLCs) have been using the plant for their home use since time of immemorial. Textile products made from the fiber of *G. diversifolia* are sold locally, as well as in national and international markets as niche product. The fiber of *G. diversifolia* has become an important source of livelihood for people living in remote rural areas of Nepal. But, very few studies are available on the social-ecological dynamics of this plant species.

Along with it's traditional use in the textile industry, currently it is used for manufacturing composite panels (Bajpai *et al*., 2013; Kumar & Das, 2017), but limited studies have been carried out on other properties regarding tensile strength, morphological and chemical alternation during fiber extraction processes. The fiber has the potential for high performance in applications like biocomposites and thermal insulation (Kumar & Das, 2017; Lanzilao *et al*., 2016).

Many studies are available in use of plants as medicine (Aggarwal & Mali, 2015; Bhattarai *et al*., 2010; Kunwar *et al*., 2019; Pangeni *et al*., 2020). Social-ecological systems are composed of multiple subsystems, internal variables at different levels within these subsystems show the relationship between economic, social, and political settings and related ecosystems (Ostrom, 2009). Since very limited studies have been done on the social-ecological and medicinal uses of *G. diversifolia*, this study has also attempted to look at its various uses in Nepal.

1.2 Taxonomy of *G. diversifolia*

1.2.1 Urticaceae

Urticaceae family comprises 54 genera and more than 2,000 species with the highest concentration of genera and species in tropical Asia (Friis, 1981; Kim *et al*., 2015; Wu *et al*., 2013). The majority of the genera and species of Urticaceae prefer to grow under forest canopies, streams, humid habitats, on moist soils of tropical montane forests at medium altitudes (Friis 1981; Friis 1993). Urticaceae includes six tribes, *viz.*

Boehmerieae Gaudich, **Forsskaoleeae** Gaudich., **Elatostemateae** Gaudich., **Parietarieae** Gaudich., **Cecropieae** Gaudich., & **Urticeae** Lam. & DC.; and are demonstrated to have monophyly of each tribe based on the DNA sequence analysis (Kim *et al*., 2015; Wu *et al*., 2013). Many genera and species of Urticaceae show significant morphological diversity and include members from herbs, shrubs, and small trees (Wu *et al*., 2013). The family is characterized by a pistil with a single stigma and a basal orthotropous ovule, with filaments inflexed in bud, and some of the species are armed with stinging hairs (Chen *et al*., 2003). The family comprises only wind pollinating species (Friis, 1993).

1.2.2 The tribe Urticeae

Urticeae comprises 12 genera and approximately 220 species found in humid habitats at forest margins (Deng *et al*., 2013; Friis, 1993; Hadiah *et al*., 2008; Kim *et al*., 2015). Urticeae is economically important for natural fiber and traditional medicine (Akbay *et al*., 2003; Shrestha *et al*., 2020). Urticeae shows diversity in habits, for example, from annual or perennial herbs such as *Girardinia*, *Urtica*, and *Nanocnide*; shrubs to woody climbers, and trees like *Dendrocnide*, *Urera*, and *Urtica* in which most of the species are herbaceous perennials (Friis, 1993). All Urticeae have stinging hairs on their stems or leaves except *Poikilospernum* (Kim *et al*., 2015). The longest stinging hair, i.e., more than 1 cm long hair, is the feature of *Girardinia* spp. (Friis, 1981). Two types of leaf arrangements are found within Urticeae, i.e., alternate leaves (e.g., *Dendrocnide*, *Laportea*, *Girardinia*, *Obetica*, *Nanocnide*, *Urera*, *Zhengyia*), and opposite leaves (*Hesperocnide, Urtica*). Leaf lamina is variously dentate and lobed. *Discocnide, Dendrocnide, Girardinia, Laportea*, and part of *Urera* and *Urtica* have intrapetiolar, fused stipules. Stipule number ranges from one to four. Male flowers usually have four to five tepals; female flowers have four tepals, mostly having one pair larger than the other (Friis, 1981; Friis, 1993; Kim *et al*., 2015). The genera *Dendrocnide*, *Girardinia*, and *Laportea* have linear stigma. Other genera such as *Hesperocnide*, *Nanocnide*, *Urtica*, *Urera* and *Gyrotaenia* have penicillate stigma (Friis, 1993). The chromosome numbers in *Urera* are *x= 10, 11, 12, 13,* where *Girardinia* has x = 10 (Friis, 1993; Shrestha, 1998). Molecular studies inferred five clades within the Urticeae: clade A (*Hesperocnide, Nanocnide, Laportea I, Urtica, Zhengyia*); clade B (*Discocnide, Dendrocnide*); clade C (*Girardinia*); clade D

(*Laportea II*); and clade E (*Poikiospermum, Urera, Obetia*) (Deng *et al*., 2013; Kim *et al*., 2015).

1.2.3 The genus *Girardinia*

Girardinia is from Urticaceae family. The name *Girardinia* is in honour of Jean Pierre Louis Girardin (1803–1884). He was a professor in Rouen and Lille (Burkhardt, 2018). *G. diversifolia* is a wild plant that grows abundantly in the hilly and mountainous territories of Nepal as well as in parts of the Hindu Kush Himalayan (HKH) region.

1.2.4 Description of *G. diversifolia*

G. diversifolia (Link) Friis is comprises of three subspecies (Kim *et al*., 2015), having a unique twice-divided tubular perianth on the female flower (Friis, 1993; Kim *et al*., 2015). *Girardinia* was considered to be close to *Laportea* but the study conducted shows *Girardinia* to be sister to *Dendrocnide* and *Discocnide* (Kim *et al*., 2015). Flora of China has documented three subspecies of *G. diversifolia* based on morphological variation in leaf: (i) subsp. *diversifolia* (with deeply (3-)5-7 lobed leaves, (ii) subsp. *triloba* (with 3-lobed leaves), and (iii) subsp. *suborbiculata* (with unlobed or rarely 3-lobed leaves) (Shu *et al*., 2003). In India, the species is comprised of two subspecies: (i) subsp. *diversifolia* (with deeply (3-)5–7-lobed leaves, and (ii) subsp. *suborbiculata* (with unlobed or rarely 3-lobed leaves) (Ambrish & Srivastava, 2016). However, in Nepal, only *G. diversifolia* with many synonyms (*G. heterophylla* Decne, *G. palmata* (forsk) Gaudich, *G. palmata* Gaudich) have been reported (Hara *et al*. 1982); Polunin and Stainton, 1984). Himalayan Giant Nettle is a common name and 'Allo' is the local name of *G. diversifolia* in Nepal. *G. diversifolia* is widely distributed in the subtropical and temperate Himalayas (Polunin & Stainton, 1984). This species is reported from eastern to central China, Myanmar, Nepal, India, Bhutan, Indonesia, Malaysia, and East Africa including Madagascar (Polunin & Stainton, 1984).

G. diversifolia is a tall and erect herb that reaches a length of 1.5 - 3 m high, with perennial root stock (Hooker, 1992; Polunin & Stainton, 1984; Malla *et al.*, 1986 Shrestha *et al.*, 2022). The aerial part is covered with stinging hair up to 9 mm long

(Friis, 1981). The plant surface consists of bristle and glandular hairs as well as punctiform cystoliths. Leaves are alternate, petiolate (3 - 15 cm), and vary in shape. The early leaves are undivided, and the later divided into three to seven lobes. The variety of leaf shapes present in plant species has led to the term polymorphic of the species with numerous synonyms (Friis, 1981; Friis, 1993). Leaves are 10 to 35 cm long, broadly ovate, acuminate, coarsely dentate or serrate, three nerved, and with cordate base. The stipules are linearly lanceolate, grow almost up to the tip, and fall during the flowering period (Friis, 1981). The species is monoecious. Male flowers are borne in long, slender, often panicled spikes, perianth four parted with four stamens (Polunin & Stainton, 1984). Female flowers are panicled spikes and usually forming short, dense, and bristly inflorescence up to 15 cm long. Perianth is 0.25 cm long, tubular, 3 lobed, and splitting when the fruit ripens. The achenes are broad, 2 to 3.05 mm long, and l.55 to 2.75 mm broad (Friis, 1981) (Figure 1).

Diagnostic characters of *G. diversifolia*

"Tepals without horn-like appendages, but the largest tepal of the female flower has a ± developed vertical crest on the outside; leaves elliptic to ovate in outline, entire, 3-, 5- or 7-lobed, usually less than 15 cm long, with serrate margin (sometimes doubly serrate, but then usually only in the lower part of the leaf); mature stems less than 2 cm diam" (Friis, 1981).

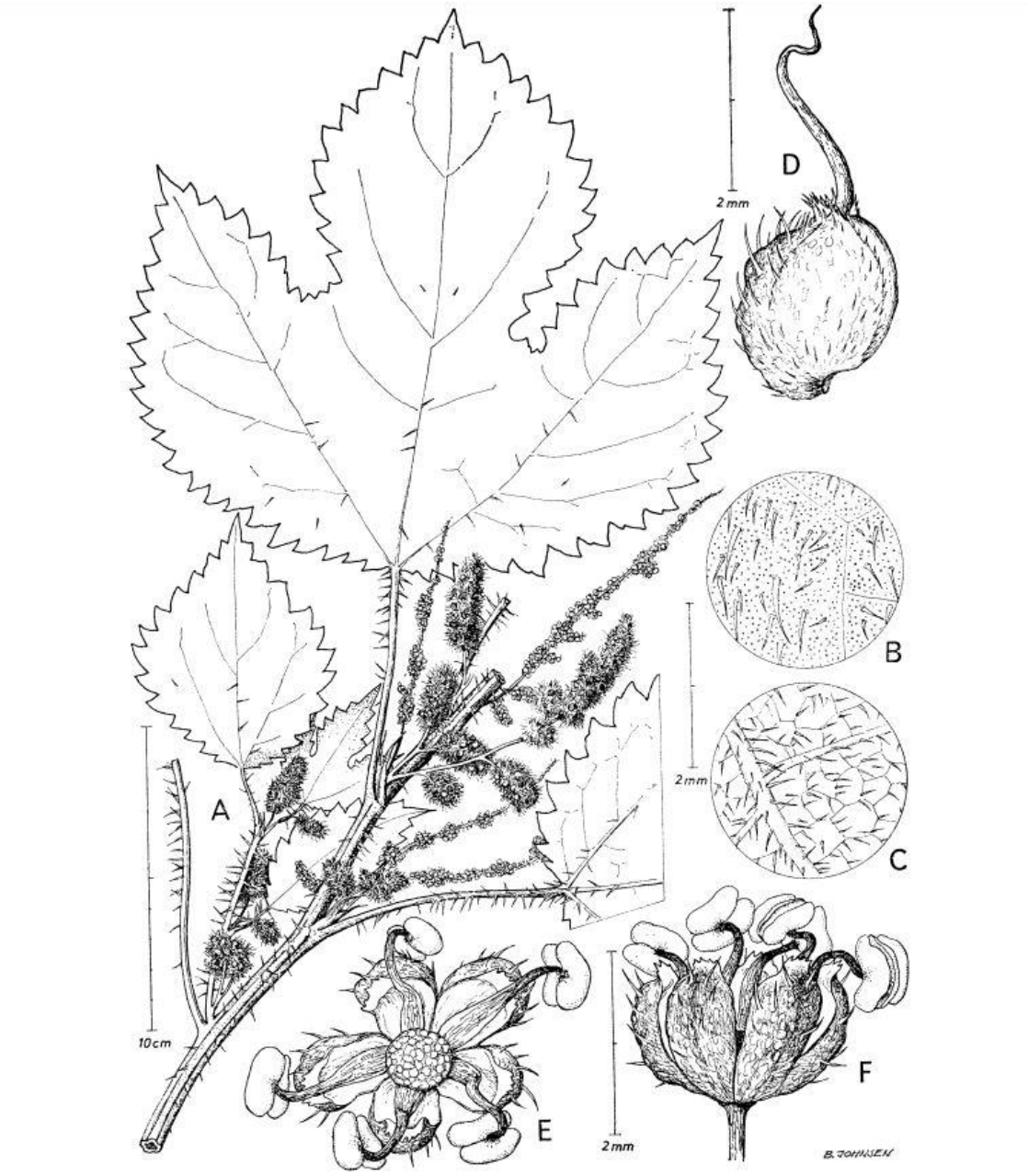


Figure 1: *G. diversifolia*. A. habit, B. upper surface of leaf, C. underside of leaf, D. female flower, E. open male flower from top view, F. male flower from side view. Source: Friis (1981).

1.3 Molecular markers for analysis of genetic variation in plants.

A molecular marker is a DNA sequence located in the known position of the chromosome. Molecular markers can show genetic polymorphism, which may be caused by mutations or alternations of nucleotides in the genome. Genetic markers represent the genetic differences between individual organisms or species (Collard *et al*., 2005). Genetic diversity can be determined based on biochemical, morphological,

and molecular information of the plant (Mohammadi & Prasanna, 2003). The plant fiber properties depend on the genetic characteristics of plant (Khalil *et al*., 2015). Molecular markers can detect genetic variation at the DNA level (Erzurumlu *et al*., 2018). Molecular markers show genetic variations on a much broader level without the intervention of environmental factors. It utilizes techniques that provide fast and detailed information on genetic diversity (Binneck *et al*., 2002; Souza *et al*., 2008). Different types of universal molecular markers are available for the study of genetic variation, with specific characteristics. Genetic variation within a species has three components: (i) genetic diversity (the amount of genetic variation); (ii) genetic distance (the amount of genetic variation between pairs of populations; and (iii)) genetic differentiation (the distribution of genetic variation among the populations) (Avise & Hamrick, 1996). A single molecular marker for that reason may not incorporate all aspects of genetic variance. The different forms of a DNA marker (e.g. different sized bands on gels) are called marker 'alleles'. Dominant marker only has two alleles (homozygotes). Codominant markers may have many different alleles (heterozygotes) (Collard *et al.,* 2005).

Molecular markers are commonly divided into two types:

I. Dominant markers- showing homozygosity eg. Inter Simple Sequence Repeat (ISSR), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphisms (AFLPs), DNA Amplification Fingerprinting (DAF).

II. Co-dominant markers- showing heterozygosity eg., Single Nucleotide Polymorphism (SNP), Sequence Tagged Sites (STS), Restriction Fragment Length Polymorphism (RFLP), Expressed Sequence Tag (EST), Microsatellites (SSR), Sequence Characterised Amplified Regions (SCAR).

DNA sequencing provides reproducible and informative datasets (Weising *et al*., 2005). Utilizing DNA sequencing technique, DNA barcoding has shown promising results for identification of taxon, ecological studies, and species level identification tools for biodiversity assessment (Valentini *et al*., 2009; Yuan *et al*., 2015). Various DNA regions from chloroplast genome (e.g., *matK*, *rbcL*), intergenic regions (*trnH-psbA*), and nuclear genome (e.g., ITS) have been utilized for many land plants (Dong *et al*., 2013; Kress *et al*., 2007).

1.3.1 Importance of molecular study in *G. diversifolia*

Molecular tools and techniques help in solving taxonomic problems and assigning plant taxa in their correct taxonomic hierarchy which is important for phylogenetic studies (Sarwat *et al*., 2012). Different molecular markers are used to analyze variation (Mondini *et al*., 2009). Molecular markers identify duplicates in germplasm collection which helps in its validation (Negawo *et al*., 2018; Sarwat, 2012). DNA-based markers have various advantages, as they are neutral and independent of environmental cues or development stage (Sarwat, 2012).

Morphological variations of leaves are present in the same plant of *G. diversifolia*. However, in some areas constant monomorphic division in leaves has created confusion at the taxa level (Chen *et al*., 2003; Friis, 1981; Singh & Shrestha, 1988). Despite the remarkable morphological variation of *G. diversifolia* (Chen *et al*., 2003; Friis, 1981; Singh & Shrestha, 1988; Wu *et al*., 2013) subspecies level of genetic variation is still unknown in Nepali species. The assessment of genetic variation within and among the populations of *G. diversifolia* present in Nepal has not yet been conducted at the molecular level. Thus, this study investigates the molecular variation in *G. diversifolia* which is an important species for the conservation and sustainable utilization of the plant genetic resources.

1.4 Classification of plant-based fibers

The natural fibers found in plants are lignocellulosic biomass which contains cellulose, hemicellulose, waxy substances, lignin, and pectin (Kumar *et al*., 2019). The schematic structure, structural organization, and chemical composition of the three main components of natural fiber are presented (Figure 2). Mostly classification of natural fiber is by botanical type. The following basic types of natural fiber are listed below (Rowell, 2014).

1. Bast fibers such as jute (*Corchorus* sp.), flax (*Linum* sp.), hemp *(Cannabis sativa),* Allo (*Girardinia diversifolia*), kenaf (*Hibiscus* sp.) and ramie (*Boehmeria nivea*).
2. Leaf fibers such as banana (*Musa* sp.), sisal (*Agave* sp.).

3. Grass and reed fiber such as bagasse of sugarcane **(***Saccharum* sp.), and bamboo (*Bambusa* sp.).
4. Seed fibers such as coir (*Cocos* sp.), cotton (*Gossypium* sp.).
5. Core fibers such as kenaf (*Hibiscus* sp.) and hemp *(Cannabis sativa).*

1.4.1 Characterization of cellulose fiber

Cellulose fiber

Cellulose fibers are ethers or esters of cellulose. Cellulose fibers are plant-based materials, mostly present in bark, leaves or wood of plants.

Bast fiber is a natural fiber presents around the stem in some of the dicotyledonous plants. Bast fibers support the conductive cells of the phloem. It provides strength to the stem. Some bast fibers are obtained from herbs such as flax (*Linum* sp.), hemp *(Cannabis sativa)*, ramie (*Boehmeria nivea*), and stinging nettle (*Urtica dioica*) (Kozlowski *et al*., 2005). Cell wall structure of natural fiber (Figure 2).

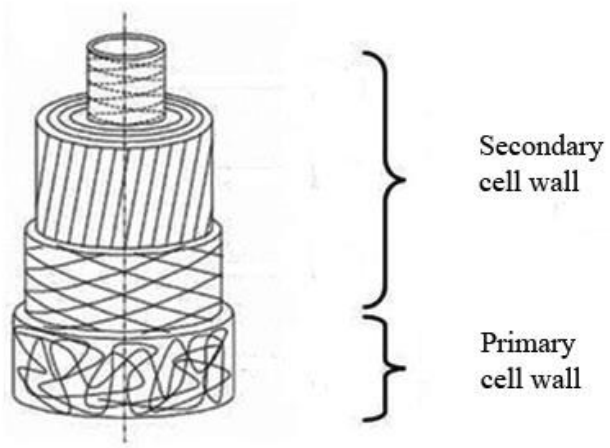


Figure 2: Structure of natural fiber (Source: Célino *et al*., 2014)

The section is polygon but is generally considered circular for calculating mechanical properties. It consists of hollow polyhedrons in many layers. The outer wall is primary wall. The primary cell wall is made of pectin, hemicellulose, low crystalline cellulose, and less wax (Gorshkova *et al*., 2000). The secondary wall, occupies about 90% of the total section. It is composed primarily of cellulose microfibrils that are parallel to each other and is included in the amorphous matrix composed of pectin,

lignin, and hemicellulose (Célino *et al*., 2014). The three layers differ from each other due to their differences in thickness and structural organization.

Cellulose is the main structural element of bast fiber which is covered by hemicellulose, lignin, pectin, and waxy material (Bismarck *et al*., 2002). Other than cellulose, high proportion and amount of hemicellulose, lignin, pectin, and waxy materials create alternation in the quality of textiles and applications like biocomposites (Kozlowski, 2012).

Bast fibers can be modified through different separation techniques; mechanical, chemical, enzymatic, and microbiological to produce high-quality fiber (Dreyer *et al*., 2002; Kozlowski, 2012). These separation techniques change the composition and structure of hemicellulose, lignin, pectin, waxy material, etc. (Bismarck *et al*., 2002; Mwaikambo & Ansell, 2002). A certain degree of disruption of the fiber and other changes in the properties too may alter the fineness and strength of the fiber.

Reinforcement material in polymer biocomposites is an emerging sector for the use of natural fibers (Aslan *et al*., 2011; Paridah *et al*., 2011). *G. diversifolia* fibers are used in the textile industry and also for the manufacturing of composite panels (Bajpai *et al*., 2013; Kumar and Das, 2017) but limited studies have been carried out regarding properties of the fiber of *G. diversifolia*. Even the basic properties like tensile strength, morphological, and chemical alternation during fiber extraction processes are still a matter of research. Despite having the potential for high performance in biocomposites applications, high wear-resistance, and thermal insulation (Kumar & Das, 2017; Lanzilao *et al*., 2016), very limited information is available about the mechanical and morphological changes during the degumming process of this fiber, which affects the quality of the product. Thus, the present study has been carried out to analyze the changes in fiber morphology, tensile strength, and elastic modulus during the separation process.

1.4.2 Social-cultural value

G. diversifolia has social and cultural values for indigenous peoples and local communities (IPLCs) of Nepal like Rais, Gurungs, Tamangs, Sherpas, etc. Kulung Rai, an ethnic community of Nepal, use clothes made of *G. diversifolia* in their

religious ceremonies. Kulung Rai community from eastern Nepal, offers cloth to god during Nagi Puja and presents *G. diversifolia* clothes to their daughters during wedding ceremonies (Barakoti & Shrestha, 2008). Leaf, stem, and root of the plant are traditionally utilized as medicine.

The bast fiber of *Urtica* sp. and *Girardinia* sp. have been used for textile and string production (Friis, 1993). IPLCs of Nepal have been utilizing fiber extracted from stems of *G. diversifolia* for their domestic use. From aeons ago to the present day, the fiber of this plant is used to produce fishing nets, coats, bags, etc. (Deokota & Chhetri, 2009; Singh & Shrestha 1988; Subedee *et al*., 2020). Products of *G. diversifolia* are in great demand in national as well as international markets (Pandey *et al*., 2020). But very limited studies have been conducted about the socio-economic, ecological impacts, and genetic diversity of this plant. This study also explores the economic, social, cultural, medicinal values, and traditional uses of *G. diversifolia* in Nepal, and identifies the opportunities, strengths, weaknesses, and threats of the species. This research also documents the technology used to produce the market sellable products of this plant.

1.5 Rationale

G. diversifolia shows remarkable morphological variation (Chen *et al*., 2003; Friis, 1981; and Wu *et al*., 2013). Three subspecies have been documented in the Flora of China. Similarly, two subspecies have been documented in India (for details see subsection 2.1). Including *G. diversifolia,* molecular phylogeny of Urticaceae from China were analysed on the basis of *rbcL* exon, *trnL-F* spacer, and (nrITS) (Wu *et al*., 2013). Unlike that a detailed study of morphological variation as well as molecular phylogeny of *G. diversifolia* in Nepal Himalaya have not been undertaken in Nepal yet.

The genetic study of plants provides extensive information about their diversity and the important metabolites they produce (Fernie & Klee, 2011). High-quality of DNA is required to evaluate genetic diversity in plants (Sá *et al*., 2011) for the polymerase chain reaction (PCR) process in which *G. diversifolia* presented a great challenge. Members of Urticaceae family produce large amounts of exudate, which interferes in DNA extraction (Sarrazola & Alzate, 2019). Different methods of DNA extraction

have been used for several species of Urticaceae (Bharmauria *et al*., 2009), indicating that modifications are needed to get good quality DNA for PCR-based analysis (Aboul-Maaty & Oraby, 2019). Therefore, modifications to the DNA extraction protocol are essential to obtain sufficient quantity and quality DNA.

Bast fiber grows in bundles and is modified by mechanical, chemical, enzymatic, and microbiological separation techniques (Dreyer *et al*., 2002; Kozlowski, 2012). Different separation techniques cause different degree of impurities in hemicellulose, lignin, pectin, and waxy material along with structural disruption (Bismarck *et al*., 2002; Mwaikambo & Ansell, 2002). Similarly, fineness and strength of the fiber are also affected because of the degree of disruption. Usually, for the separation of the natural fibers, chemical separation with NaOH and mechanical separation using steam explosion are involved. These processes are not sustainably feasible due to high fuel consumption, large variation in fiber quality, high labor cost, and longer time for processing (Amaducci & Gusovius 2010; Jankauskienė *et al*., 2015). Here, to mitigate these shortcomings of other processing, enzymatic processing could be an alternative. Though it too has issues related to cost effectiveness (Akin *et al*., 2002). Considering these factors, for this study exploring of improved enzymatic process that is cost effective is also essential.

The IPLCs of Nepal have been involved in processing and product development from the fiber of *G. diversifolia*, since time immemorial (Deokota & Chhetri 2009; Singh & Shrestha 1988). Even though use of *G. diversifolia* for the production of handicraft stuffs in traditional way is familiar; its use in textile industry of Nepal is not promising yet.

One of the reasons for that lag is the absence of social-ecological analysis regarding the use of the fiber. Thus, this study has investigated the economic, medicinal, social, and traditional usages of *G. diversifolia* with an attempt to identify the technological and policy related gaps.

1.6 Research questions

Research questions

- What are the morpho-geographical variations or characteristics of *G. diversifolia* in Nepal?
- What are the genetic variations in *G. diversifolia* of Nepal at different spatial scale?
- How does chemical and enzymatic retting method affect the fiber properties during processing of fibers from *G. diversifolia?*
- What are the traditional uses and practices of *G. diversifolia* in Nepal?

1.7 Objectives

The general objectives of the present research are to analyze the morpho-geographical and genetic variation of *G. diversifolia*, its fiber processing, and document its use pattern in Nepal.

1.7.1 Specific objectives

The specific objectives are to:

- study the morpho-geographical characters of *G. diversifolia* in Nepal;
- analyze genetic variation and phylogenetic study of *G. diversifolia;*
- study the effect of chemical and enzymatic retting on tensile strength, and elastic modulus of the fiber *G. diversifolia;* and
- document social-ecological aspect of *G. diversifolia* in Nepal.

CHAPTER 2

2. LITERATURE REVIEW

2.1 Nomenclature and description of *G. diversifolia*

Gaudichaud (1830) established the genus *Girardinia* and clearly states that the genus has more than one species. Blume (1855) published a synopsis of *Girardinia* and reinstated the name '*Girardinia palmata* Gaud'. Blume validated Latin description, citing *Girardinia leschenaultiana* Decne. as a synonym. Decaisne adopted the name *Girardinia heterophylla* for Forsskal's taxon. IB Friis (1981) again published a synopsis of *Girardinia* (Urticaceae), from the proposed name *Girardinia vahlii* from Forsskal's taxon and published specific epithet *Urtica diversifolia* Link and proposed the combination *Girardinia diversifolia* (Friis, 1981).

IB Friis (1981) conducted a detailed study on the genus *Girardinia* and published "A synopsis of *Girardinia* (Urticaceae)". The taxonomy of the species is well covered by Friis 1981.

"Tall, short-living herbaceous plants covered with long, stinging hairs, monoecious or dioecious by abortion. Leaves alternate, petiolate, elliptic to ovate in outline, but very variably divided, with various degrees of division of the leaves on the same plant, always with three major nerves from the base of the leaf blade. Cystoliths dot-like. Stipules intrapetiolar, fused almost to the apex, caducous. Flowers in unisexual, axillary inflorescences. Male inflorescence ± branched, slender, almost spike-like, with dense clusters of flowers. Female inflorescence ± branched, elongated, sometimes almost spike-like, sometimes contracted, with very dense cymules of flowers and fiercely armed with stinging hairs. Male flowers pedicellate, with 4 or 5 tepals and the same number of stamens, a rudimentary ovary always present. Female flowers apparently sessile, with 3 tepals almost completely fused into a hollow structure ± enclosing the reflexed ovary; the fourth, free tepal is ± rudimentary or lacking. Staminodia absent. Ovary laterally compressed, asymmetrical, reflexed, with filiform stigma. Fruit a flattened, broadly ovate or subcordate achene. Seeds with very little endosperm, and embryo with broad cotyledons."(Friis, 1981).

Key to subspecies of *Girardinia diversifolia* following Shu *et al*. (2003)-

1a. Leaf blade usually not lobed, rarely 3-lobed, margin coarsely dentate or double-dentate, teeth gradually larger toward leaf apex; stipules 6-10 mm; male inflorescence unbranched, 1-2 cm; female inflorescence often in same axil as male, 2-6 cm ..... G. diversifolia subsp. *suborbiculata*

1b. Leaf blade 3-7-lobed, rarely lowermost leaves not lobed and then margin regularly serrate or double-serrate, teeth gradually smaller toward leaf apex; stipules 12-30 mm; inflorescences not as above, often more than 6 cm.…….. 2

2a. Leaf blade 3 lobed each sublobe further divided into 2 to 3 lobes, often deeply (3-)5-7-lobed, variable in overall shape but often broadly ovate; petiole and major leaf veins greenish; inflorescence 10-28 cm, branched or not, rarely 2-5 cm and scorpioid ..... G. diversifolia subsp. *diversifolia*

2b. Leaf blade usually 3-lobed, often obovate; petiole and veins on abaxial leaf surface usually purplish; inflorescence unbranched, 4-8 cm.. G. diversifolia subsp. *triloba*

Three subspecies of *G. diversifolia* have been described in Flora of China.

2.1.1 *G. diversifolia* (Link) Friis subsp. *diversifolia*

Herbs perennial, monoecious, stipules oblong-ovate, leaf blade elliptic, oblate or ovate, base subtruncate, 3, 5, or 7-lobed, regularly or doubly serrate at leaf base. Male inflorescences: cymose-racemose, 5-11 cm; female inflorescences, 10-28 cm, 2.5-3 mm in diameter (Shu *et al.* 2003) (Figure 3).

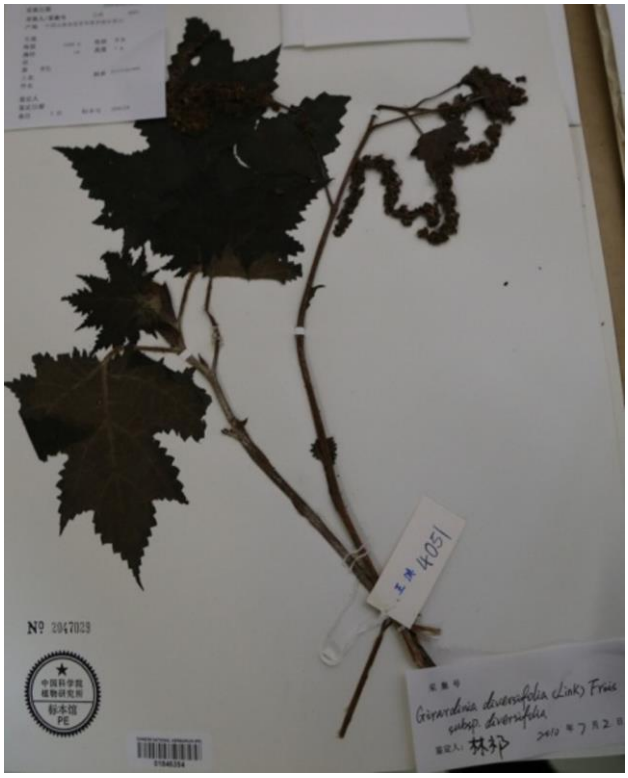


Figure 3: *G. diversifolia* (Link) Friis subsp. *diversifolia.* (Source: PE Herbarium, China).

2.1.2 *G. diversifolia* (Link) Friis subsp. *suborbiculata* (C. J. Chen) C. J. Chen & Friis

Herbs monoecious, suborbicular leaf blade, rarely 3-lobed, base truncate or rounded, margin dentate. Male inflorescences: spicate, 1-2 cm. Female inflorescences: 1-6 cm (Figure 4).

Figure 4: *G. diversifolia* subsp. *suborbiculata* (C. J. Chen) C. J. Chen & Friis (Chen, 2003.) (Source: PE Herbarium, China).

2.1.3 *Girardinia diversifolia* (Link) Friis subsp. *triloba* (C. J. Chen) C. J. Chen & Friis.

Herbs monoecious, leaf blade broadly ovate, often 3-lobed, triangular lobes, regularly dentate, petiole and veins on abaxial leaf mostly purplish. Male inflorescences: spicate, 1-2 cm. Female inflorescences: 1-6 cm. (Shu *et al.* 2003) (Figure 5).

Figure 5: *G. diversifolia* subsp. *triloba* (C. J. Chen) C. J. Chen & Friis (Chen, 2003) (Source: PE Herbarium, China).

2.2 Distribution

2.2.1 Global distribution

G. diversifolia is widespread in tropical Africa, Southern Sudan, Ethiopia, Angola, South Africa, Zimbabwe, and in Madagascar (Brink, 2009). In Asia, natural habitat of *G. diversifolia* is distributed in subtropical and temperate Himalayas from Kashmir to Sikkim, in Assam and Khasi Hills to Burma, Nepal, Bhutan, Srilanka, Indonesia, and China (Hara *et al*. 1982; Hooker, 1992; Polunin & Stainton, 1984) (Figure 6). Its distribution also extends from Marwar, central India, Travancore, and Ceylon (Hooker, 1992).

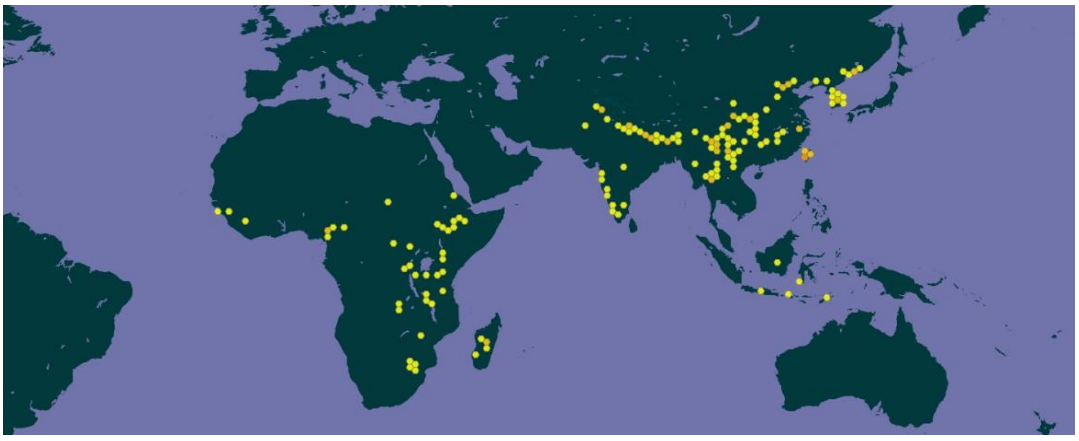


Figure 6: Global distribution of *G. diversifolia* (Source: gbif.org; accessed on 10th December, 2021)

2.2.2 Distribution of *G. diversifolia* in Nepal

In Nepal *G. diversifolia* is distributed from eastern to far-western region at altitudes ranges from 1,000 to 3,000 meter above sea level (Hooker, 1992; Polunin & Stainton, 1984).

G. diversifolia has been documented from eastern region to far-western region of following districts of Nepal: Taplejung (Bhandari *et al*., 2021), Panchthar, Therathum, Illam (Singh & Shrestha 1988), Dhankuta, Solukhumbhu, Sankhuwasabha (Deokota & Chhetri 2009), Dolakha (Singh & Shrestha 1988), Ramechhap (Sigdel *et al*., 2013), Sindulpalchok, Sindhuli, Kavre, Makawanpur, Rasuwa, Dhading, Chitwan, Gorkha, Lamjung, Kaski, Baglung, Myagdi, Parbat, Sallyan, Jajarkot, Pyuthan, Rukum, Jumla, Surkhet, Rolpa, Jumla, Dailekh, Kalikot, Mugu, Achham, Surkhet, Doti, Dadeldhura, Darchula in National Herbarium and Plant Laboratories (NHPL)/KATH as shown in the (Figure 7).

The potential forest areas and estimated production capacity of *G. diversifolia* in Nepal are given in the map provided by the Department of Small and Cottage Industry of Nepal (Figure 7).

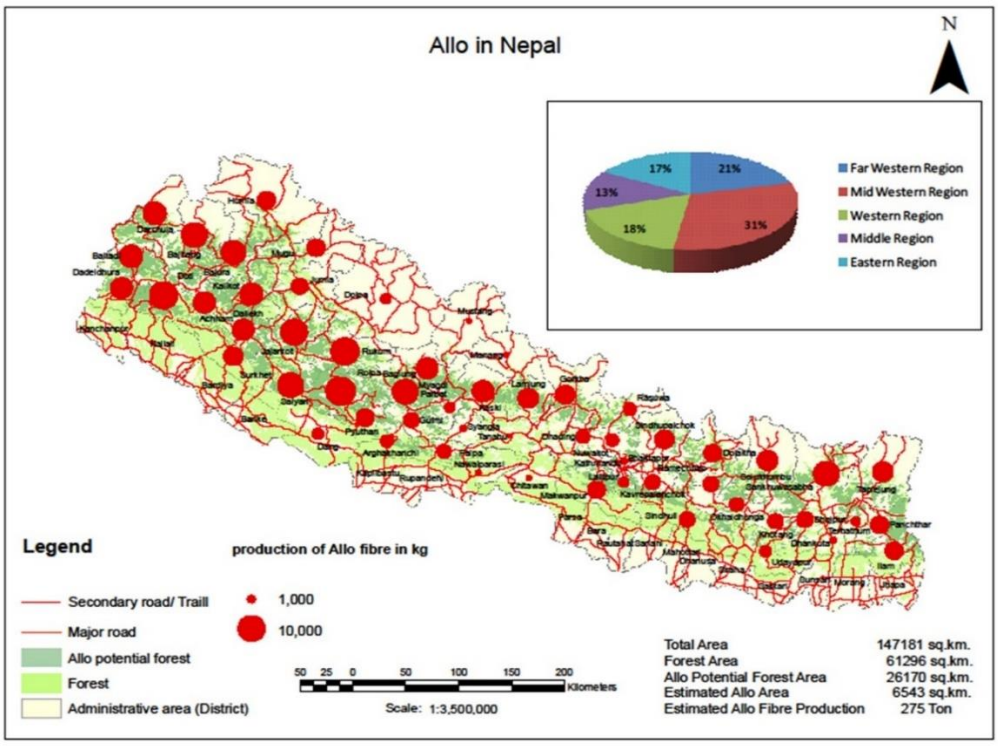


Figure 7: Distribution and estimation of potential forest area with tentative production capacity of fiber of *G. diversifolia* in Nepal. Source: The Department of Small and Cottage Industry.

According to the Department of Cottage and Small Industries of Nepal, the abundancy of *G. diversifolia* ranges from high to low in the given order: mid-western region (31%), far-western region (21%), western region (18%), eastern region (17%) and central region (13%). The total 'Allo' potential forest area of Nepal is 26,170 sq km where as estimated 'Allo' production area is 6,543 sq km. The total estimated production of Allo fiber in Nepal is 275 tons per year (Figure 7).

2.3 Morphological variation in leaf

The most striking variation was found in the leaf morphology of *G. diversifolia* as shown in (Figure 8). Leaf serrates are uniformly found in the specimens from the Himalayas (Friis, 1981), but at the same time, very deep incisions were found in the same areas of leaf (12, 13, 15, 22).

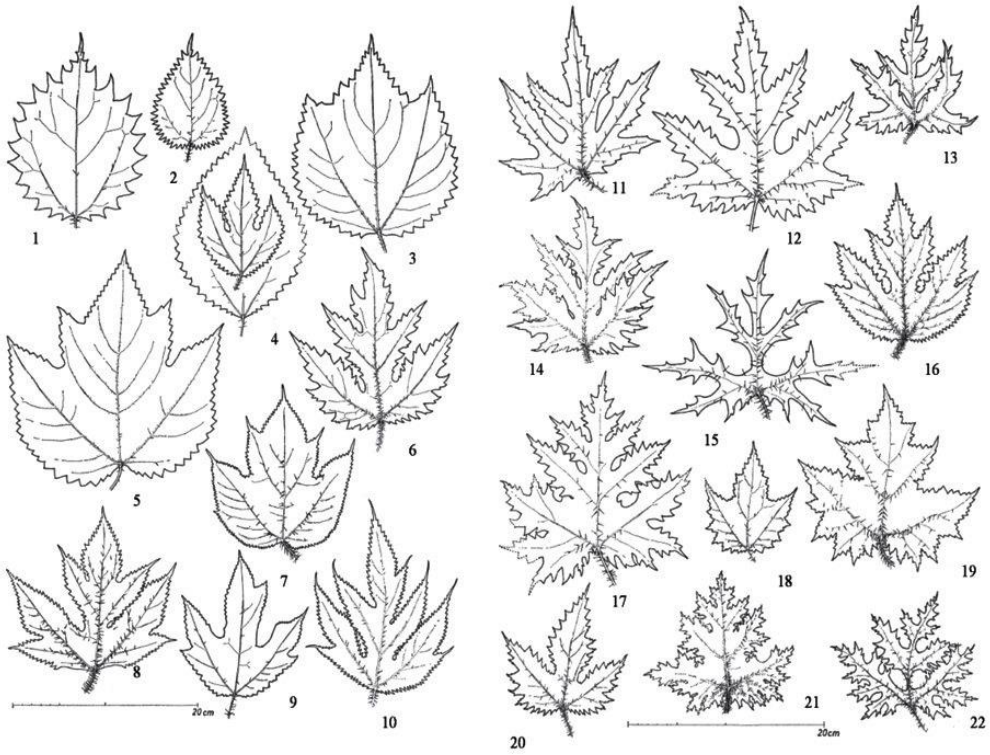


Figure 8: Variations in leaf morphology of *G. diversifolia*. Source: Friis (1981)

2.4 Cytological studies in *G. diversifolia*

Karyomorphological and micromorphological studies were performed on samples of *G. diversifolia* collected from Kathmandu and Dolakha Districts of Nepal (Shrestha,

1998). The study was based on two collections (9200K and 92005J) deposited at National Herbarium and Plant Laboratories (KATH).

Collection no: 9200K. In this sample, the chromosome number was 2n = 20, similarly, the karyotype formula was used 2n = 20 = M8 + M8 + sm 2 + st2. Karyotype of this taxon comprised four types of chromosomes and chromosome length ranges from 0.7 to 1.7 μm and absolute length was 9.4 μm.

Collection no: 92005J also had the same chromosome number 2n = 20. The karyotype formula of this was 2n = 20 = M 4 + m14 + sm2. Here three types of chromosomes were observed. In this sample the chromosome length varied from 0.8 μm to 1.3 μm and absolute length was 9.8 μm. A karyomorphological and micromorphological study of these two collections showed that *G. diversifolia* has two different ecotypes (Shrestha, 1998).

2.5 Genetic variation using Inter-Simple Sequence Repeat (ISSR)

Molecular markers are the most effective, efficient, and accurate tools and techniques capable of detecting genetic variation in plants and animals (Marwal & Gaur, 2020). ISSR is a commonly used molecular marker, however, such marker has not been used in *G. diversifolia.*

ISSR markers detect genomic polymorphisms and provide high ability to resolve the intra-specific and inter-specific levels of variation in a wide range of organisms (Zietkiewicz *et al*., 1994). ISSR uses 16–25 bp long microsatellite core unit bearing oligonucleotide primers, anchored or non-anchored at the 5' or 3' prime site with 1–4 nucleotides. The ISSR technique is fast, cost-efficient, and does not require initial sequence knowledge. ISSR markers are dominant markers and are inherited in simple Mendelian fashion (Ratnaparkhe *et al*., 1998). ISSRs are polymorphic and highly reproducible than RAPDs because, they use longer semiarbitrary SSR primers at stringency PCR conditions (Reddy *et al*., 2002). Genetic variations are studied in many plants including *Urtica dioica* (Haghpanah *et al*., 2016); *Balanites aegyptiaca* (Abdelaziz *et al*., 2020) by calculating different parameters such as: (i) Polymorphic information content (Nagy *et al*., 2012; Roldan *et al*., 2000); (ii) Primer resolving power (Prevost & Wilkinson, 1999); (iii) Marker Index (Varshney *et al*., 2007); (iv) Similarity and dissimilarity indices; (v) Nei's genetic diversity (Nei and li, 1979); (vi)

Shannon's diversity index (Shannon, 1948); and (vii) Analysis of molecular variance (Excoffier, Smouse & Quattro, 1992).

2.6 Genetic marker using sequencing technique

The barcoding method has been very useful in species identification, biodiversity studies, and phylogenetics. DNA barcoding detects taxonomic uncertainty and determines the erroneous taxonomy (Surya & Hari, 2017). It follows the basic principle of taxonomic practice, which is associated with a specific reference collection in conjunction with species concepts. This is a tool to be used to construct phylogenies and for species identification purposes (Kress *et al*., 2005). Since this barcoding method has not been used anywhere in *G. diversifolia* present in the Himalayan region, thus the use of DNA barcoding is important.

2.6.1 Internal transcribed spacer

Internal transcribed spacer (ITS) of nuclear ribosomal DNA is often used as DNA markers in plant phylogenetic reconstructions at the species level or even below. ITS1 or ITS2 are the important markers in evolution and molecular systematics that show significant sequence variability at the species level or provide accuracy in the reconstruction of phylogenetic trees (Keller *et al*., 2010). The selected ITS region or a part of it was suitable for over 95% of most plant groups, such as angiosperms, gymnosperms, etc. (Cheng *et al*., 2016). Due to its high resolution and inter- and intra-specific relationships, ITS region is mostly used DNA fragments in plant molecular systems at the generic and species levels (Álvarez *et al*., 2003). ITS1 and ITS2 exhibited high universality 82.2% to 91.7% in angiosperms, gymnosperms, and bryophytes (Cheng *et al*., 2016, Liu *et al*., 2014). The markers ITS1–5.8S–ITS2, were used where phylogeny was well resolved in the *Urtica* sp. (Grosse-Veldmann *et al*., 2016). ITS region for species of all genera of Urticeae was used in order to assess monophyly and generic relationships within Urticeae (Kim *et al*., 2015; Wu *et al*., 2013).

2.6.2 Chloroplast maturase K (*matK*) gene

The chloroplast maturase K (*matK*) gene is a highly variable coding gene that has been suggested for barcoding and distinguishing land plant species. The *matK* gene is the most promising marker for identifying or distinguishing plant species. The length of *matK* gene is about 1570 bp and codes for a maturase protein. It is located within an intron of the chloroplast *trnK* gene (Yu *et al*., 2011). *MatK* gene has higher evolutionary rate, so it is used to study phylogenetic reconstructions at high taxonomic levels, such as Order, Family also for Genus or Species (Chase *et al*., 2007; and Yu *et al*., 2011). The universality of this primer pair showed a strong amplification (93.1%) and sequencing (92.6%) successes in the 58 species from 47 families of angiosperm plants (Yu *et al*., 2011).

2.6.3 *rbcL* gene

The *rbcL* gene encodes a large subunit of ribulose- 1, 5-bisphosphate carboxylase / oxygenase (RUBISCO). The *rbcL* has been sequenced from many plant taxa, such as 19 Saxifragales and Saxifragales related taxa (Chase *et al*., 1993; Dong *et al*., 2013). Kim *et al*. (2015) comprised data set for species of all genera of Urticeae in order to assess monophyly and generic relationships within Tribe Urticeae.

2.6.4 Molecular phylogenetic relationships of Urticaceae

The phylogenetic relationships and morphological evolution of twelve genera based on the analysis of two plastid DNA regions (*trn*L-F spacer, *rbcL* exon) and (nrITS) of Urticaceae has been investigated by Kim *et al*. (2015). Inferred phylogeny five clades in Urticaceae are namely: **clade A** Urtica (with *Hesperocnide*), *Laportea* I, *Zhengyia*, and *Nanocnide*; **clade B** *Discocnide* and *Dendrocnide*; **clade C** consisting only *Girardinia*; **clade D** consisting *Laportea* II; and **clade E** consisting *Obetia, Poikilospermum* and Urera I, II, III, as illustrated in (Figure 9).

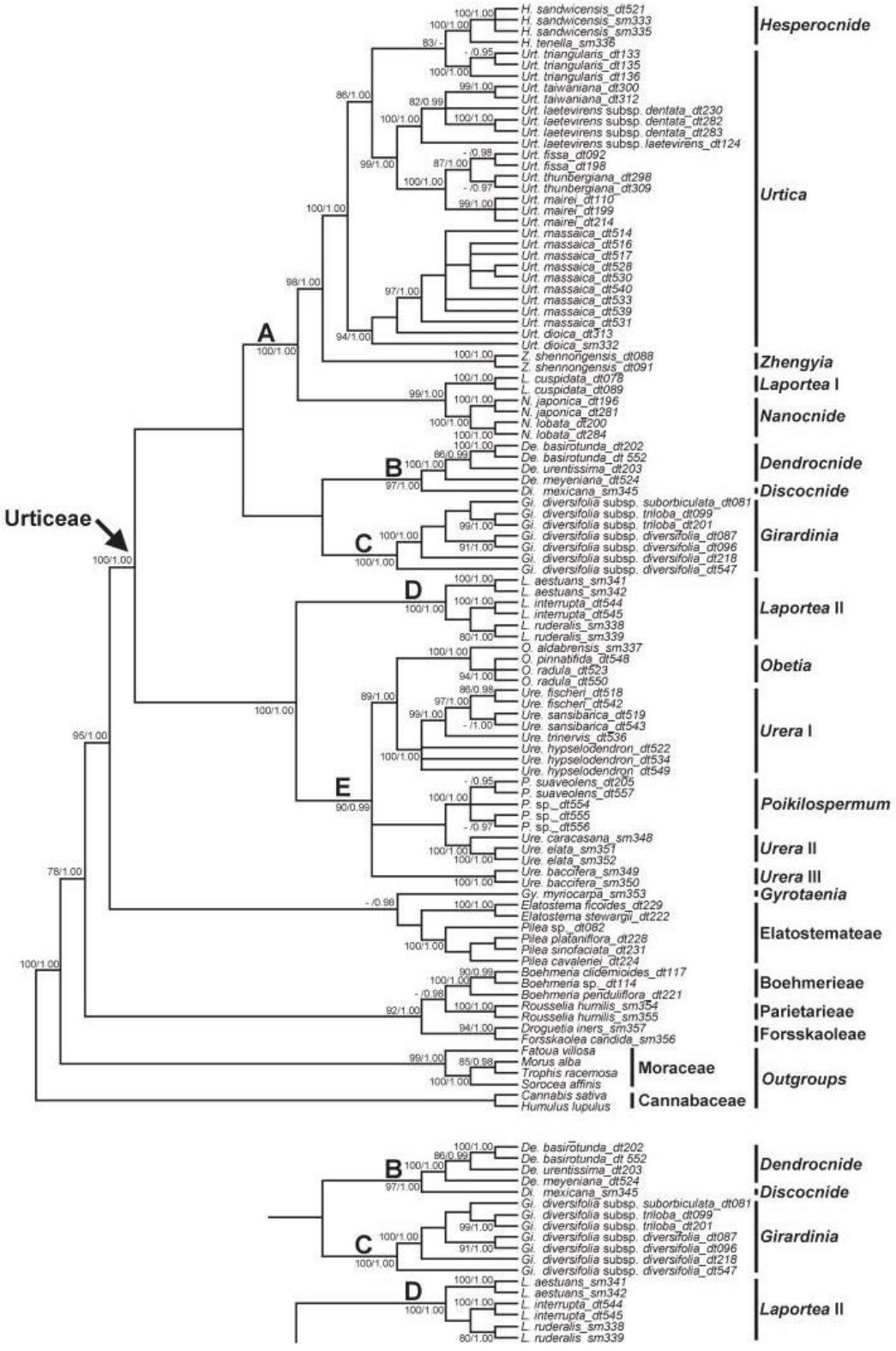


Figure 9 : Strict consensus of the 70 most parsimonious trees Source: (Kim *et al*., 2015).

Urticaceae has been divided into four clades by Wu *et al*. 2013. According to Wu *et al*., Clade III comprises eleven genera, which is largely consistent with Friis's (1993) tribe Urticeae. Among the five subclades, the three genera (*Nanocnide*, *Laportea*, *Girardinia*) consist of one monophyletic genus where each one contains two sister genera (*Dendrocnide* with *Discocnide*). Morphologically, stinging hairs are mostly present in Urticaceae, usually unisexual flowers may be both monoecious and dioecious, the leaves are often entire and bear stipules. Urticaceae are wind pollinated and spread their pollens as the stamens mature (Wu *et al*., 2015).

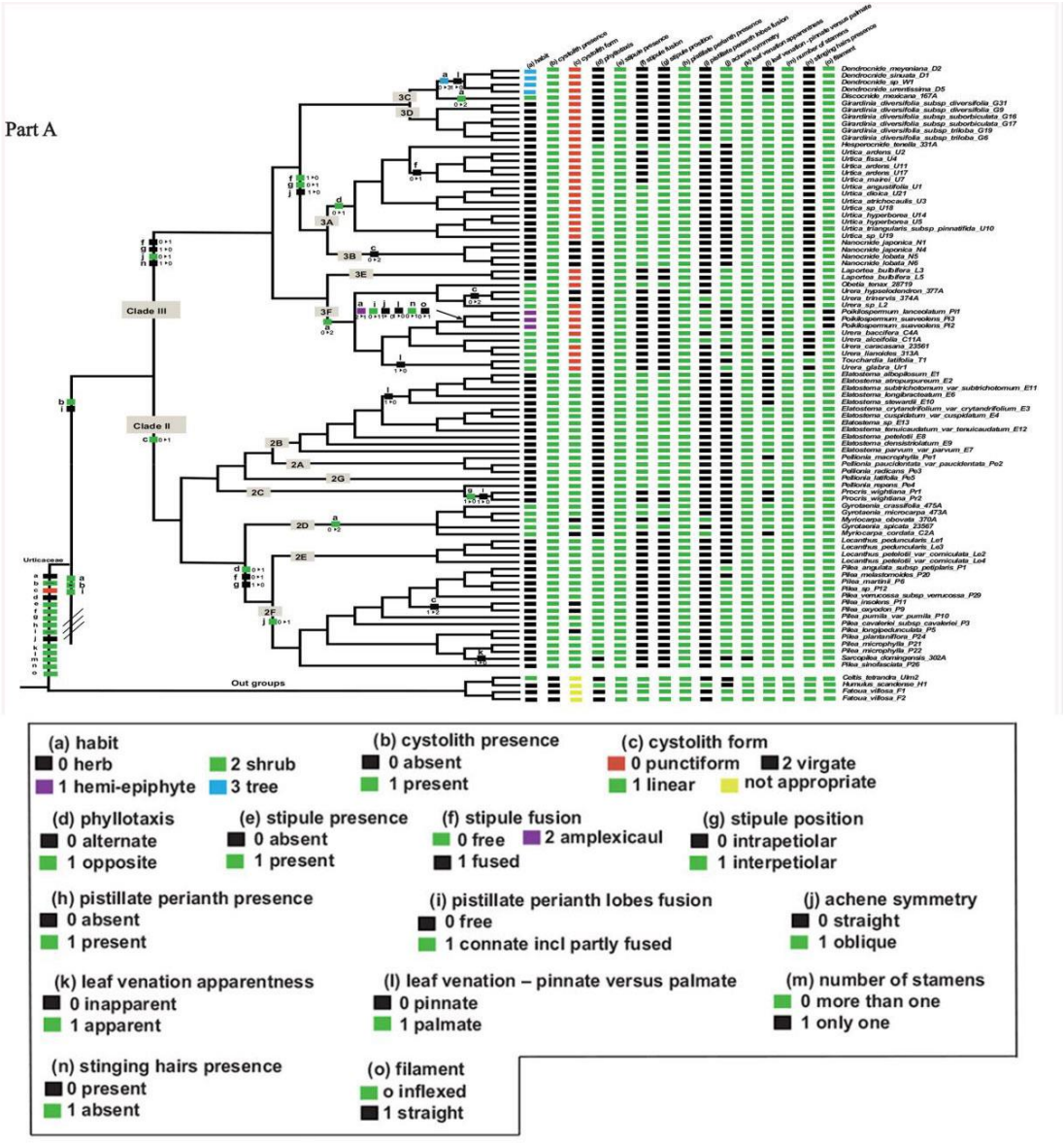


Figure 10: Maximum parsimony analysis of fifteen selected morphological characters in Urticaceae. (Source: Wu *et al*., 2015)

By mapping the character of 19 traits, the phylogenetic tree was made by Wu *et al*., 2015 including: habit, stipule presence, stigma form, phyllotaxis, cystolith form stipule fusion, perianth presence, stipule position, achene symmetry, types of palmate venation, leaf venation apparentness, external morphology of achene, number of stamens, presence of stinging hair and filament.

Capitate stigmas arose at least once in each of Clades I, II and III. Synapomorphy of two genera: *Laportea,* and *Girardinia* appeared. Oblique achenes are also a synapomorphy in Clade III, within which three reversals to the straight state have occurred, i.e., *Poikilospermum, Touchardia,* and the common ancestor of *Urtica* and *Nanocnide.*

Since many overlapping morphological variations have been found in *G. diversifolia,* Kim *et al*., (2015) and Wu *et al*., (2013) suggested for further molecular studies on this plant.

2.7 Biochemistry of natural fiber containing cellulose

Natural fibers are made up of lignin, cellulose, and hemicellulose (Mohanty *et al*., 2001). Some chemical compositions from different natural fibers are shown in (Table 1). The physical properties of some natural fibers are presented in (Table 2).

Table 1: Chemical compositions of some natural fiber

	Cellulose %	Hemicullose%	Lignin %	Ash%	Pectin%	Wax%	References
Hemp	70.2-74.4	17.9-22.4	3.7-5.7	2.6	0.9	0.8	Srinivasa *et al*., 2011
Kenaf	31-39	15-19	21.5	4.7	-	-	Srinivasa *et al*., 2011
Jute	61-71.5	13.6-20.4	12-13	-	0.2	0.5	Srinivasa *et al*., 2011
Flax	71-78.5	18.6-20.6	2.2	1.5	2.2	1.7	Srinivasa *et al*., 2011
Cotton	82.7	5.7	-	-	-	0.6	Srinivasa *et al*., 2011
Sisal	67-78	10-14.2	8-11	-	10	2.0	Srinivasa *et al*., 2011
Coir	36-43	0.15-0.25	41-45	-	3-4	-	Srinivasa *et al*., 2011
Allo	68.10	14.12	10.45	3.90	2.12	1.31	Sett *et al*., 2016

The quantity of cellulose found in natural fibers can vary depending on the age and species of the plant. Cellulose consists of linear chain of 1,4-β-bonded anhydroglucose units having alcoholic hydroxyl groups (Figure 11).

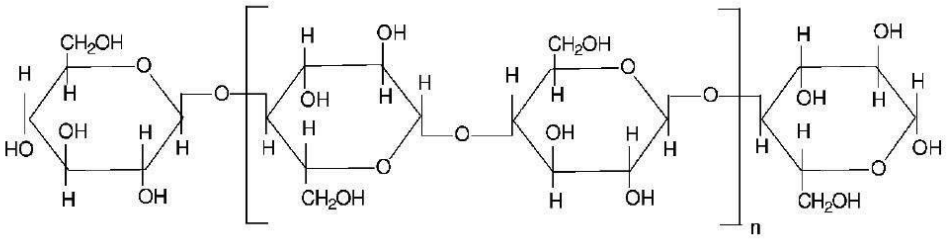


Figure 11: Cellulose structure. Source: Bledzki *et al*., 1996

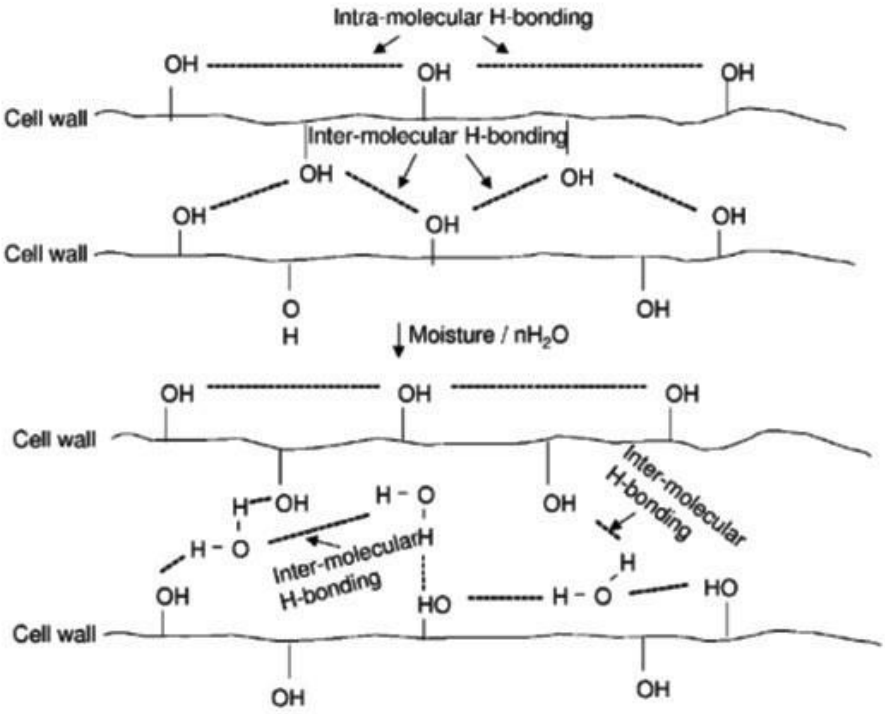


Figure 12: Hydrogen bonding in natural fiber (Source: Bledzki *et al*., 1996).

Hydroxyl groups form intramolecular hydrogen bonds inside the macromolecule. Intermolecular hydrogen bonds form among other cellulose macromolecules. They also form Hydroxyl groups from the air (Figure 12). Natural fibers have moisture content from 8% to 12.6% (Bledzki *et al*., 1996).

Table 2: Physical properties of some natural fibers

Fiber	Density (g/cm^3)	Strain break (%)	Tensile strength (Mpa)	Young's Modulus (Mpa)
Hemp	1.48	1.6	550-900	70
Flax	1.4 - 1.5	1.2 - 3.2	345 – 1500	27.6 - 80
Kenaf	1.2	2.7 - 6.9	295 – 930	22 - 60
Ramie	1.5	2-3.8	220-938	44-128
Jute	1.3 - 1.46	1.5 - 1.8	393 – 900	10 - 30
Nettle	**1.51**	**1.7**	**650**	**38**
Banana	1.35	5 – 6	529 – 914	27 - 32
Coir	1.2	15 – 30	175 – 220	4 - 6
Sisal	1.33 - 1.5	2 – 14	400 - 700	9 - 38
Abaca	1.5	2.9	430-813	33.1-33.6
Pineapple	1.5	1 – 3	170 – 1627	60 - 82
Cotton	1.21	3 – 10	287 – 597	5.5 - 12.6
Bamboo	0.6 - 1.1	1.3 – 8	140 – 441	Nov - 36

Source: Koohestani, 2019; Lanzilao *et al*., 2016; Papadopoulou *et al*., 2015.

2.7.1 Fiber of *G. diversifolia*

The average length of the fiber is 350 mm - 483.5 mm and diameter is 70 µm. In fresh weight, the minimum fiber yield per plant was 1.58% and maximum yield was 2.8%. The minimum yield on dryweight form was 3.49% and maximum yield was 13.18% (Lanzilao *et al*., 2016; Sethmann, 2001; Singh & Shrestha. 1988). Simplified diagram of stem section is shown in (Figure 13).

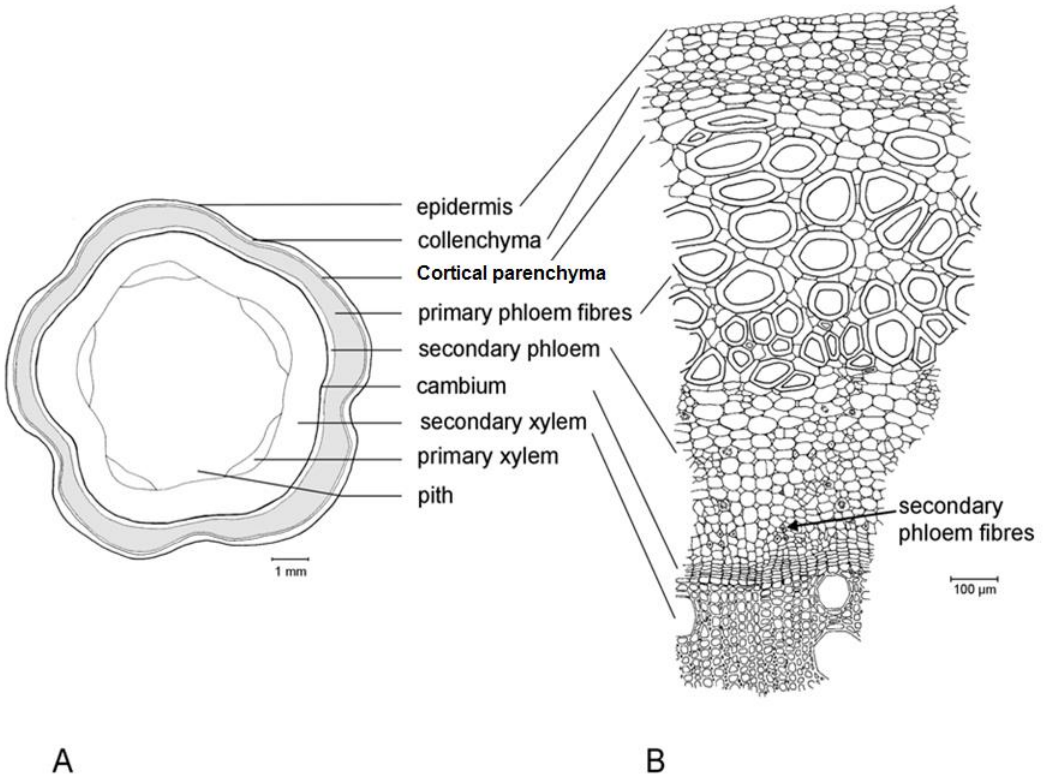


Figure 13: (A) Simplified diagram of stem section, (B) Stem section (a part enlarged) of *G. diversifolia (*Source: Sethmann, & Dreyling, 2001).

2.7.2 Fiber properties of *G. diversifolia*

Lanziola *et al*. (2016), studied about morphological characters and physical properties of *G. diversifolia*. The study compared morphological characters and physical properties with (*Urtica dioica* L.) fiber. The average length of *G. diversifolia* was found to be longer than that of *U. dioica*. It was reported that *G. diversifolia* fiber is the longest fiber among other natural bast fibers studied. *G. diversifolia* showed greatest strain at failure during tensile test (Lanziola *et al*., 2016).

Tensile test of *G. diversifolia* was compared to that of *U. dioica* which showed that tensile strength of *G. diversifolia* fiber is greater than that of other common bast fibers (Lanziola *et al*., 2016). Tensile strength of *U. dioica* was about three times that of flax and eight times that of cotton. The tensile strength values calculated according to lanziola (5099.8 MPa) were higher than those of some industrial fibers such as S – glass (4570 MPa) and carbon (4000 MPa) (Hargitai *et al*., 2008). Young's modulus of

G. diversifolia has (73 GPA) is generally higher than that of common bast fiber due to its extensibility (Lanziola *et al*., 2016).

2.8 Fiber processing

2.8.1 Mercerisation

Mercerisation is a treatment of fiber containing cellulose, the treatment improves dye uptake , reduces fabric shrinkage, tear strength, and provides a silk-like luster. This procedure was devised in 1844 by John Mercer. John Mercer treated cotton by washing it with a solution at sodium hydroxide (20–30%) at 55–65 ° C temperature. The most important chemical treatment for the good properties of bast fiber is treatment with alkali. The effect of treating bast fiber with sodium hydroxide varies from fiber to fiber, but usually, the fiber becomes swollen, increasing the consumption of dyes. The fibers with a high lignin content increased crystallinity due to the removal of lignin (Kozlowski *et al*., 2012).

2.8.2 Alkali treatment in natural fibers

Alkaline treatment in natural fiber disrupts hydrogen bonding in the network structure and enhances the roughness of the surface. Alkaline treatment removes some of the lignin, wax and oil covering the outer surface of the fiber cell wall (Kozlowski *et al*., 2012).

During alkali treatment, when hemicelluloses are removed, the inter fibrillar region becomes less dense, less likely to harden, and thus enables the fibrils to rearrange in the direction of tensile deformation. Alkaline treatment changes the crystallization of natural fiber (Mohanty *et al*., 2001).

An increase in the percentage of crystallinity index of alkali treated fibers indicates the removal of cementing materials, which shows the better packing of the cellulose chains. Sodium hydroxide treatment leads to an increase in molecular orientation and decrease in spiral angle. The elastic modulus of natural fibers is expected to increase with an increasing degree of molecular orientation. A high alkali concentration may depolymerize the native cellulose. It also delignifies the fiber excessively, which can unfavorably affect the strength of the fiber.

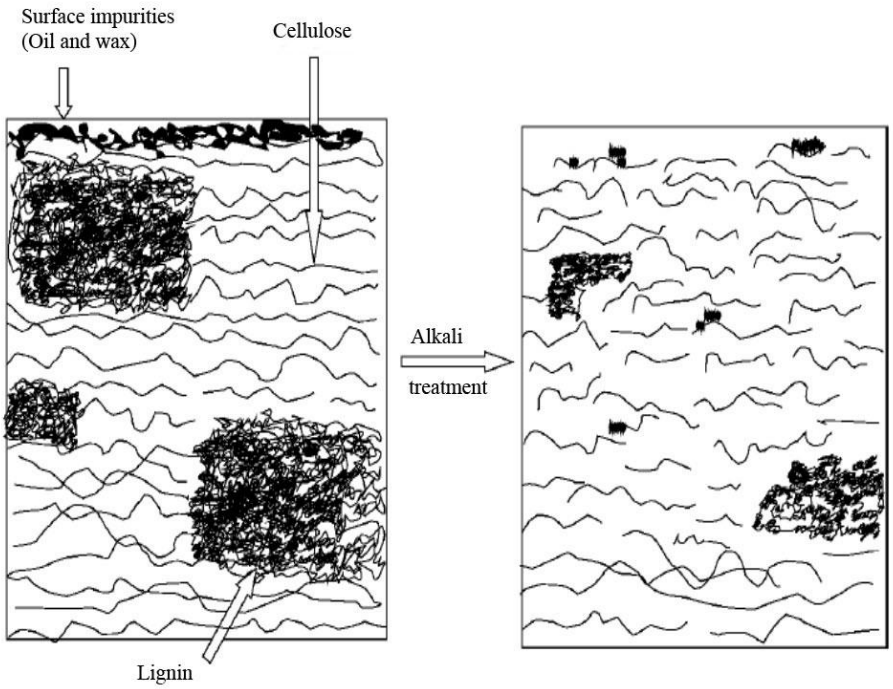


Figure 14: Schematic representation of raw and alkali-treated natural fiber (Source: Mohanty *et al*., 2001)

The crystalline structure of cellulose changes after the treatment with alkali at a lower concentration of NaOH, breaking the intermolecular hydrogen bonds between neighboring cellulose chains. By increasing the concentration of NaOH, intramolecular hydrogen bonds within individual cellulose chains break down. The hydrogen bonds reform the fiber to form Cellulose II and this treatment causes changes in the molecular structure of cellulose which leads to differences in mechanical performance and shape.

2.8.3 Enzymatic treatment of natural fiber

Natural fibers are mostly processed by chemical separation with NaOH and steam explosion, but these processes are not sustainable due to high fuel consumption, large variation in fiber quality, high labor cost, and longer fiber processing time (Amaducci & Gusovius, 2010; Jankauskienė *et al*., 2015). Among these processes, the enzymatic process tends to be more sustainable due to its less energy consumption (Kozlowski, 2012).

Enzyme plays an important role in degumming of bast fiber, improving cleanliness, homogeneity, fineness, and softness. They are used for retting of natural fiber, degumming decorticated fibers and in finishing textile products. For this reason, it is important to choose appropriate enzyme for bast fiber (Kozlowski, 2012).

Enzyme mixtures with chelators enhance the efficiency of enzyme retting of natural fiber. Chelators such as ethylenediaminetetraaceticacid (EDTA) remove Ca^{2+} from pectin bridges and destabilize structure of cell wall (Adamsen *et al*., 2002; Akin *et al*., 2002).

One of the limiting factors of enzyme treatment is the cost. Cost is a major obstacle to commercial processing. So, it is a challenge to decrease the amount of enzyme in retting process. Spray Enzyme Retting (SER) was developed that uses smaller amounts of the enzyme (Akin *et al*., 2000).

Different types of enzymes used in treatment of bast fibers are cellulases, catalases, α-amylase, pectinase, proteases, and laccase. Among the enzymes, pectinase enzymes are the most used enzymes for retting of bast fiber. Pectinase enzymes contribute to the break down of pectic contents. Pectinolytic enzymes are used in the retting and extraction of bast fibrous plants, such as hemp, kenaf, jute, ramie and flax, (Bruhlmann *et al*., 1994).

2.9. Use of *G. diversifolia* in Nepal

2.9.1 *G. diversifolia* and enterprise development

The livelihood of the highland Nepali people could be improved by developing microenterprises having their own traditional skills (Dunsmore, 1998). In the eastern Hills from 1980 to 1986, a program called Koshi Hill Area Rural Development Programme (KHARDEP) was implemented, which was based on *G. diversifolia* related activities. The project established *G. diversifolia* weaver club in Sisuwatar of Sankhuwasabha District. According to Dunsmore (1998), skilled craftspeople living in the highlands are good at knitting, basketry, and developing products that can compete in the international market. Dunsmore (1998) emphasized that inorder to drive this sector forward; training programs with new skills should be conducted from

time to time. Market identification needs to be done and suitable labour saving equipments should be used to improve the socio-economic situation (Dunsmore, 1998).

2.9.2 Medicinal use and traditional practice of *G. diversifolia* in Nepal

Indigenous peoples and local communities of Nepal use different parts of *G. diversifolia* as herbal medicine. They commonly use *G. diversifolia* for joint pain, headache, treating gastritis, and tuberculosis. Some of its medicinal uses and practice are listed as follows (Table 3).

Table 3: Medicinal uses of *G. diversifolia*

Parts used	Traditional uses
Root	Juice of roots about 6 teaspoons twice a day is taken orally for remedy of gastritis, stomach ache, and constipation (Manandhar, 2002). In case of joint pain and swelling, root paste is applied. For the treatment of gastritis, the juice of the root is mixed with the plant Ghodtapre (*Centella asiatica*) and boiled for 10 minutes, strained and the liquid (about 4 teaspoons) is served twice a day.
Leaf	The leaf juice is used in the treatment of headaches, joint pains, and tuberculosis. Leaf is also used as vegetable (Barakoti 2008; Gurung *et al*., 2012; Malla *et al*., 2014).
Stem	To treat a fractured bone, stems are heated and wrapped around the leg or arm (Pandey *et al*., 2007).
Inflorescence	Inflorescence is used as soup and vegetable for its high nutritional value (Malla *et al*., 2014).

G. diversifolia has traditional veterinary uses by Gujjar and Bakarwal tribes of Poonch, Jammu & Kashmir of India, where the root powder is dried and given along with milk to cattle to treat retention of the placenta (Dutta *et al*., 2021). In Uttarakhand, its root paste is used for the treatment of pimples and boils in domestic animals (Dutta *et al*., 2021). *G. diversifolia* has ultraviolet protection properties and thus can be utilized to protect the skin from UV radiation (Pargai & Gahlot, 2020).

2.9.3 Cultural use of *G. diversifolia*

An extensive study has been conducted in Sisuwa, Bala, Mangtewa, and Tamku VDCs of Sankhuwasabha District of Nepal (Dunsmore, 1998) where a commercial enterprise of this species was established in 1999 using traditional knowledge. Kulung Rai community uses *G. diversifolia* in their religious ceremonies and offer its cloth to God in their Nagi Puja (Barakoti & Shrestha, 2008; Subedee *et al*., 2017).

Traditional knowledge related to *G. diversifolia* has been documented in other places of Nepal such as Bhedetar of Sunsari District where indigenous peoples (Rai, Magar, and Tamang) use *G. diversifolia* traditionally. *G. diversifolia* is used for food, fodder, and medicinal purpose. People use *G. diversifolia* fabric to make sacks, bags, coarse clothes, fish nets, etc. (Deokota & Chhetri, 2009).

Traditional knowledge of processing and use of *G. diversifolia* among Gurung, Magar, Rai, and Tamang communities of Sikles, Kaski District has been documented by Gurung *et al*. (2012). The study explored the processing methods and practices in Sikles Village for the production of mats, sacks, traditional dresses, etc. The study also observed the market network and showed that incentives or subsidies from government and donor agencies are needed to promote *G. diversifolia* as an income source.

2.9.4 Fiber extraction from *G. diversifolia*

The primary cell wall of *G. diversifolia* is called 'Allo lokta' (Chanysi, 2010). Allo's lokta is processed by different methods in different parts of Nepal. First outer layer of the stem are peeled out, and tied up in bundles to set sun dry. The sun-dried bark is soaked into water for one or two nights. Then it is cooked in wood ash or caustic soda (Chanysi, 2010; Deokota & Chhetri, 2009; Singh & Shrestha 1988; Subedee *et al*., 2020) for 3 to 4 hours and then washed in running water (Gurung *et al*., 2012). The washed raw fiber is mixed well in whitish clay "Kamero Maato" and dried in the sun for 7 to 8 days (Chanysi, 2010) that softens the Allo fibers as well as makes spinning easier. During fiber spinning process dried white clay as a form of dust is removed. Fiber processing by this method takes a lot of time and firewood for cooking therefore, the development of new technologies is deemed necessary

(Chanysi, 2010; Deokota & Chhetri, 2009; Singh & Shrestha 1988; Subedee *et al*., 2020).

2.9.5 Chemical constituents of *G. diversifolia*

Many bioactive compounds are present in *G. diversifolia* such as; 7-hydroxysitosterol, 3-hydroxystigmast-5-en-7-one and β-sitosterol (Njogu *et al*., 2011); β-sitosterol, sitostanol, campestrol, fucosterol, phytol, 5-0-feruloylquinic acid, ascorbic acid, trans syringin, linolenic and linoleic acid (Shrestha *et al*., 2020).

2.9.6 Challenges and threats to the natural resources

A study in eastern hills of Nepal that was based mostly in Tamku, Sisuwa, Bala and Mangtewa VDCs of Sankhuwasabha District indicated the decline of Allo populations from the natural forests. The study suggested for the cultivation of Allo for its sustainable availability in its natural habitat. The study recommended the exploration of the different types/varieties of this plant and its potential fiber yield. The study prioritized the use of appropriate technology which requires less fuel and environmental friendly to increase the income generation of rural communities (Barakoti & Shrestha, 2008).

A study conducted in Bhedetar (Sunsari District) urged the importance of maintaining the constant fiber quality stating it as an essential step for long term sustainability of Allo sectors (Deokota & Chhetri, 2009).

CHAPTER 3

3. MATERIALS AND METHODS

3.1 Study area

Nepal is a Himalayan country in South Asia bordering India to the south, east, west and China to the North. It is located parallel to the Himalayan ranges and extends 800 km from 80°05′ to 88°10′E, with an average width of about 180 km, with latitudes ranging from 26° 20′N in the east to 30° 25′N in the west. Collection sites were selected on the basis of uniform distribution of *G. diversifolia* in selected districts: eastern, central, and western regions of Nepal, where *G. diversifolia* is found in abundance in the forest.

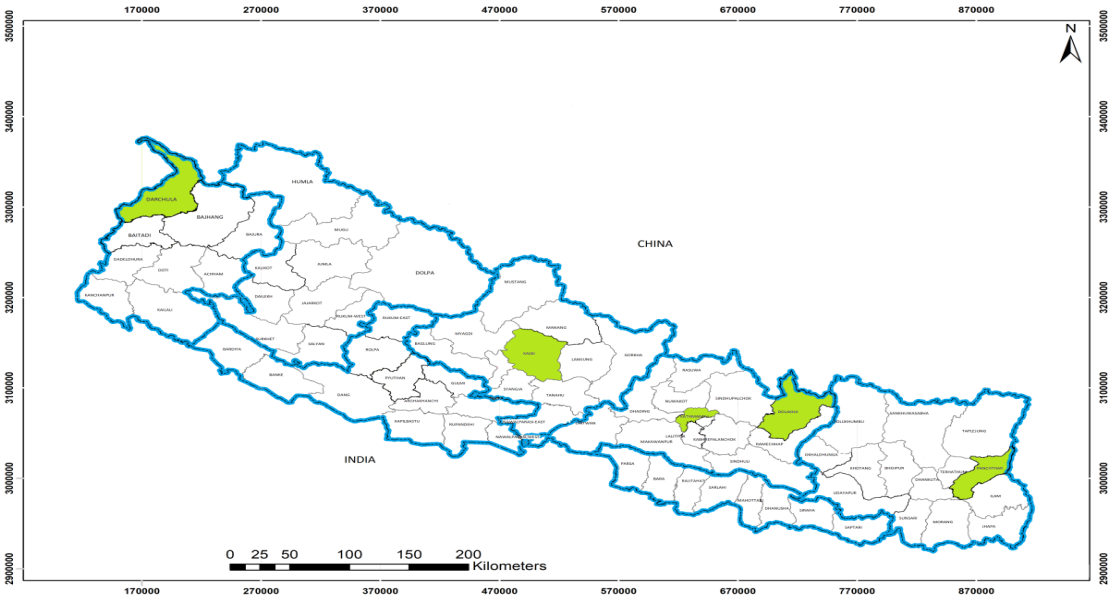


Figure 15: Map of Nepal showing sample locations sites from eastern to western region: Panchthar District, Dolakha District, Kathmandu District, Kaski District, and Darchula District.

3.1.1 Field survey and data collection

In this study, three regions of Nepal (western, central, and eastern) were selected for collecting samples and social-ecological study. Field visit was conducted in different seasons from 2017 to 2020 AD.

3.1.1.1 Material collection

Plant materials

Plant materials were collected for morpho-geographical study. A total of forty-five germplasm were collected from the population of *G. diversifolia* ranging from 1,000 m asl (above sea level) to 2,700 m asl elevation. These forty-five samples represent five different populations of the *G. diversifolia* collected from different districts of Nepal representing three regions: western region (Darchula District); central region (Kaski District, Kathmandu District, and Dolakha District); and eastern region (Panchthar District) (Table 4). Rhizomes, and bast fibers were also collected for plantation and fiber study.

Table 4: Sample summary of *G. diversifolia* collected from eastern, central, and western Nepal.

SI. No	Collection site	District	Plant samples code number	Voucher number	Latitude/ Longitude
1	Siddin	Panchthar	Phi1	191011	27° 09' 41" N 87° 54' 01" E
2	Siddin	Panchthar	Phi2	191012	27° 09' 25" N 87° 53' 43" E
3	Siddin	Panchthar	Phi3	191013	27° 09' 08" N 87° 53' 28" E
4	Siddin	Panchthar	Phi4	191014	27° 08' 47" N 87° 53' 08" E
5	Siddin	Panchthar	Phi5	191015	27° 08' 31" N 87° 52' 47" E
6	Siddin	Panchthar	Phi6	191016	27° 08' 19" N 87° 52' 35" E
7	Prangbung	Panchthar	Phi7	191017	27° 10' 15" N 87° 54' 59" E
8	Prangbung	Panchthar	Phi8	191018	27° 10' 36" N 87° 56' 07" E
9	Prangbung	Panchthar	Phi9	191019	27° 10' 11" N 87° 58' 15" E
10	Prangbung	Panchthar	Phi10	191020	27° 10' 01" N 87° 58' 26" E
11	Jiri	Dolakha	Jir1	191001	27° 36' 57" N 86° 12' 48" E
12	Jiri	Dolakha	Jir2	191002	27° 37' 03" N 86° 12' 34" E
13	Jiri	Dolakha	Jir3	191003	27° 37' 13" N 86° 11' 59" E
14	Jiri	Dolakha	Jir4	191004	27° 37' 36" N 86° 11' 30" E
15	Jiri	Dolakha	Jir5	191005	27° 37' 47" N 86° 11' 24" E
16	Jiri	Dolakha	Jir6	191006	27° 38' 06" N 86° 11' 19" E
17	Malung	Dolakha	Jir7	191007	27° 31' 05" N 86° 02' 56" E
18	Malung	Dolakha	Jir8	191008	27° 30' 59" N 86° 02' 42" E
19	Dandakharkha	Dolakha	Jir9	191009	27° 31' 08" N 86° 00' 28" E
20	Dandakharkha	Dolakha	Jir10	191010	27° 31' 10" N 86° 00' 11" E
21	Machhegau	Kathmandu	Kat1	191026	27° 39' 22" N 85° 14' 51" E
22	Machhegau	Kathmandu	Kat2	191027	27° 39' 21" N 85° 14' 45" E

SI. No	Collection site	District	Plant samples code number	Voucher number	Latitude/ Longitude
23	Machhegau	Kathmandu	Kat3	191028	27° 39' 08" N 85° 14' 45"E
24	Machhegau	Kathmandu	Kat4	191029	27° 39' 07" N 85° 14' 34" E
25	Machhegau	Kathmandu	Kat5	191030	27° 39' 05" N 85° 14' 23" E
26	Machhegau	Kathmandu	Kat6	191031	27° 39' 02" N 85° 14' 22" E
27	Machhegau	Kathmandu	Kat7	191031	27°39' 02" N 85° 14' 14" E
28	Nagarjun	Kathmandu	Kat8	190901	27° 44' 10" N 85° 15' 56" E
29	Nagarjun	Kathmandu	Kat9	190902	27° 44' 23" N 85° 15' 37"E
30	Nagarjun	Kathmandu	Kat10	190903	27° 44' 30" N 85° 15' 14" E
31	Ghandruk	Kaski	Kas1	191021	28° 23' 14" N 83° 48' 45" E
32	Ghandruk	Kaski	Kas2	191022	28° 22' 31" N 83° 48' 12" E
33	Ghandruk	Kaski	Kas3	191023	28° 22' 29" N 83° 48' 08" E
34	Panchase	Kaski	Kas4	191024	28° 14' 03" N 83° 49' 27" E
35	Panchase	Kaski	Kas5	191025	28° 13' 51" N 83° 49' 08" E
36	Hopari,	Darchula	Dar1	191101	29° 46' 4.5" N 80° 39' 31" E
37	Dhulighad	Darchula	Dar2	191102	29° 46' 51" N 80° 38' 40" E
38	Okhaldhar	Darchula	Dar3	191103	29° 47' 0.6" N 80° 37' 28" E
39	Malephar	Darchula	Dar4	191104	29° 47' 13" N 80° 37' 02" E
40	Godhyan	Darchula	Dar5	191105	29° 47' 06" N 80° 36' 52" E
41	Godhyan	Darchula	Dar6	191106	29° 47' 12" N 80° 36' 37" E
42	Pangdhunga	Darchula	Dar7	191107	29° 47' 17" N 80° 36' 21" E
43	Pangdhunga	Darchula	Dar8	191108	29° 47' 16" N 80° 36' 11" E
44	Pangdhunga dhar	Darchula	Dar9	191109	29° 47' 20" N 80° 36' 08" E
45	Pangdhunga dhar	Darchula	Dar10	191110	29° 47' 34" N 80° 36' 23" E

3.1.1.2 Reagents and extraction buffer

Chemical reagents: CTAB, Tris HCl, EDTA. NaCl, Polyvinylpyrrolidones (PVP) (Sigma, Sintra, Portugal), Phenol:Chloroform:Isoamyl Alcohol (25:24:1),β-mercaptoethanol, Ammonium acetate, Tris base, Ethanol, Glacial acetic acid, dNTPs (Vivantis, Malaysia), *Taq* DNA polymerase (Vivantis, Malaysia), template DNA, primers (University of British Columbia, Canada), $MgCl_2$ (Vivantis, Malaysia).

Enzyme pectinase: (17389- 10G, Pectinase from *Aspergillus niger* from Sigma-aldrich); NaOH: Fisher scientific; Ethylenediaminetetraacetic acid (EDTA):

Qualigens. Sodium hydroxide (NaOH), crude papaya enzyme, and distilled water. All the chemicals having analytical grades were used.

TAE (Tris-Acetate-EDTA) buffers

Stock solution of (50 X) buffer was prepared by dissolving 242 grams of tris base into distilled deionized water. Glacial acetic acid of 57.1 mL was added, 100 mL of 0.5 M EDTA (pH 8.0) solution and bringing the final volume up to 1L. The working solution of 1X TAE buffer was made by diluting the stock solution (50 X) in deionised water.

Cetyl Trimethyl Ammonium Bromide (CTAB)

The CTAB extraction buffer was prepared by mixing 100 mL of 1M Tris buffer at pH 8.0 and 280 mL of 5M NaCl; 40 mL of 0.5 M EDTA and 20 gm of CTAB in sterile Schott bottle, the final volume was made with deionized water up to 1L (Purohit *et al*., 2012).

1Molar Tris Buffer, pH 8.0: for 1 L

For the preparation of 1 M Tris buffer; 121.1 g Tris base was dissolved in 700 mL of distilled water. Tris base dissolved to bring 900 mL. For adjusting pH (8.0) concentrated HCL approximately 50 mL was added and the final volume was made up to 1 L (Sambrook *et al.*, 1989)

0.5 Molar, EDTA pH 8.0: for 1 L

186.1 g of disodium EDTA. $2H_2O$ was added and dissolved into 800 ml of distilled water. NaOH pellets was used to adjust pH 8.0. Final volume the solution was made 1L by adding distilled water, autoclaved, and stored at room temperature until needed (Sambrook *et al.*, 1989)

5 Molar NaCl: for 1 L

For the preparation of 5 M NaCl; 292 g of NaCl was dissolved in 800 mL of H_2O. The volume was adjusted to 1 L with addition of H_2O. NaCl solution was stored at room temperature (Sambrook *et al.*, 1989).

7.5 Molar Ammonium acetate: for 250 mL

Ammonium acetate was prepared for 250 mL: 144.5 g of ammonium acetate was dissolved in 200 mL of distilled water and a final volume of 250 mL was made with addition of distilled water (Sambrook *et al.,* 1989).

1.8%Agarose Gel

Agarose 1.8 gm was dissolved in TAE buffer (100 mL, 1X) by heating in microwave. The gel was cooled approximately at 55°C and was poured into the gel casting tray with comb fixed in place. Gel was further cooled at room temperature before further use (Sambrook *et al.,* 1989).

Sodium hydroxide

Fifty grams of fiber sample was treated in 5% sodium hydroxide (NaOH) of Fisher scientific and immersed in 165 mL distilled water

Enzyme pectinase

Fifty grams of dry *G. diversifolia* fiber was soaked in 50 mM EDTA in 165ml distilled water with pH 5.0 and then 0.3% pectinase enzyme (17389- 10G, Pectinase from *Aspergillus niger* from Sigma-aldrich) was added (Akin *et al*., 2000)**.**

3.2 Methods

3.2.1 Herbarium preparation

Collection sites were selected on the basis of uniform distribution of the plants in selected districts of eastern, central and western regions of Nepal. Herbarium specimens were collected, kept between newspaper sheets, pressed by herbarium press and dried in sunlight (Forman & Bridson, 1989). The collected herbarium specimens were reaffirmed by descriptions given in taxonomic literatures (Hooker 1992; Grierson & Long 1983; Shu *et al*., 2003) as well as by comparing morphological characteristics of herbarium specimens deposited at National Herbarium and Plant Laboratories (KATH), Department of Plant Resources, Nepal.

3.2.2 Plantation

Rhizomes collected from field were planted at Truffle Research Centre's orchard, Tribhuvan University, Kirtipur, Kathmandu, Nepal (27°40’50”N, 85°17’26.5”E). Leaf samples were collected from the planted plants for DNA extraction.

3.2.3 Social-ecological aspect of *G. diversifolia* in Nepal

Focus group discussions, semi-structured questionnaires, key informant interviews, informal meetings, and field observations were conducted as primary methods of data collection mainly for socioecological study using Participatory Rural Appraisal (PRA)/Rapid Rural Appraisal (RRA) tools and Key Informant Survey (KIS). Non-timber forest products (NTFPs) collectors, traders, “Allo” processers, and community members were invited to informal meetings and focus group discussions. A total of 110 informants (30 male, 80 female) representing various IPLCs including Gurung, Kulung Rai, Dhami, Manyal, Bohora, and Thagunna communities were participated in the discussion.

3.2.4 Morpho-geographical study of *G. diversifolia* in Nepal

Herbarium specimens of *G. diversifolia* deposited at KATH, Godawari was studied using the morpho-geographical method. Study of morphological characteristics of *G. diversifolia* was carried out by examing over 90 herbarium specimens. Date of collection, specimen numbers and place of collection from Nepal were noted during the study (Appendix 7). Information about distribution, habit, habitat, vegetative (stem, leaf, trichome) and reproductive (flowering, and fruiting) characters were studied from herbarium specimens. Herbarium specimens data confirmed consistency in morphological characters of leaf such as: number of trichomes at adaxial surface of leaf (cm^2); average length of trichome at adaxial surface of leaf (mm); leaf shape, leaf margin, number of trichomes at abaxial surface on the vein of leaf (cm^2); average length of trichome at abaxial surface of leaf (mm); average length of leaf (cm); average breadth of leaf (cm); and number of leaf lobes, stamens, and seeds. Carl Zeiss Binocular Microscope, Primostar magnification 10×, 40× and 100× and USB

microscope magnification 20× to 800× were used to examine the stem, leaf, and flowers of *G. diversifolia*.

3.2.5 Plant DNA extraction

Young, fresh and healthy leaf tissues were preferred to extract DNA. About five to ten young leaves per individual (up to 1 g) was gathered, labelled, air dried, and stored separately. All the samples were packed with silica gel into air-tight zip-lock bags for drying and interim storage.

G. diversifolia leaf samples were taken and evenly powdered in liquid nitrogen and then genomic DNA was extracted following the modification of Doyle and Doyle (1987) protocol.

3.2.5.1 Modification of Doyle and Doyle (1987) protocol.

Prior to DNA extraction, 5% β-mercaptoethanol and 5% PVP (Polyvinlypyrrolidone) were added to the CTAB buffer. Leaf samples of *G. diversifolia* were grinded in liquid nitrogen in a pre-chilled mortar and pestle. About 100 mg of grinded leaf powdered was taken into pre-chilled 2 mL centrifuge tube and added 500 µL of CTAB buffer then mixed well by vortexing. Tubes were incubated at 55°C for an hour in heating block with shaking at an interval of 10 min. The tubes were centrifuged at 13,000 rpm for 10 minutes at room temperature and transferred the supernatant into new fresh tubes. 500 µl of Phenol:Chloroform:Isoamyl Alcohol (25:24:1) was added and mixed well. The tube was centrifuged at 13000 rpm for 10 minutes at room temperature. The top supernatant containing DNA was transferred into a new labeled 1.5 mL tube. The Phenol:Chloroform:Isoamyl Alcohol (25:24:1) step was repeated for twice. The volume of the aqueous phase was estimated approximately 350 µl and 0.08 volume of cold 7.5 M Ammonium acetate was added which was approximately 28 µl. 0.54 volumes of cold isopropanol which was approximately 204 µl was added and mixed well. The tube was set in freezer at -20 °C for an hour and centrifuged for 3 minutes at 14,000 rpm. The supernatant was decanted carefully without disturbing the resultant DNA pellet and washed with 700 µl of 70% ice cold ethanol by inverting the tubes few seconds to mix and centrifuged at 14,000 rpm for 1 minute at maximum speed. This step is repeated twice. The ethanol was decanted and DNA pellet was air-dried at a clean beanch for 5 minutes to

evaporate remaining ethanol in the tube. Finally, the DNA pellet was resuspended in 100 µL of TE buffer (Cullings, 1992; Doyle & Dickson, 1987; Doyle & Doyle 1987)

The yield of extracted DNA was measured at 260 nm on a nanospectrometer (Bio-spec nano, SHIMADZU, Japan). The purity of DNA was measured by estimating the absorbance ratio from 260 nm to 280 nm.

3.2.6 Polymerase chain reaction optimization

PCR was performed using PCR Master Mix (5X PCR Master Mix) of New England Biolab and separate *Taq* polymerase, dNTPs, from VIVANTIS, Malaysia.
Gradient PCR reaction was performed to determine the optimal annealing temperature on a DNA thermocycler (Biorad T100). A total volume of 15 µl PCR reaction mixture was prepared containing 1X buffer, (1.5-4) mM $MgCl_2$, (0.1-0.6) mM dNTPs, (0.1-0.6) unit Taq polymerase, (0.1-0.9) µM primer, and (25-100) ng of DNA template. Optimized PCR program was as follows: 93°C for 3 min, followed by 45 cycles of denaturing at 93°C for 30 s, annealing of primers at (45-52°C) for 45 s, extension at 72°C for 2 min, final extension at 72°C for 10 min and holding temperature at 4°C. Electrophoresis of the PCR products was performed in 1.8% agarose prepared in 1X TAE buffer with added 10 mg/ml of ethidium bromide. PCR products were loaded in pre-cast wells of a gel submerged in 1X TAE buffer, 80V electric current was applied for 1 hour. A DNA ladder of 100 base pair was used to determine the molecular weight. The DNA bands were observed and analysed using Unified Gel Documentation System, (WiseDoc WGD-30, DAIHAN South Korea).

3.2.7 Genetic diversity assessment based on PCR profiling

Samples from the eastern, central and western regions of Nepal were used for ISSR analysis. Clear bands were used for scoring purposes, while vague bands were discarded.

An absence (0) and presence (1) binary matrix were generated in excel sheet. The total number of bands obtained for ISSR marker, primer banding characteristics namely, number of polymorphic bands and percentage of polymorphic (P%), resolving power (Rp), band informativeness (I_B), for each of the primers were

calculated utilizing Microsoft office excel 2007. In addition, the effectiveness of different primers for ISSR was calculated by using different indices, such as- Polymorphism Information Content (PIC).

$$PIC = 2f_i(1- f_i)$$

PIC_i is the PIC of the marker $_i$. frequency of the amplified allele f_i , and $(1 - f_i)$ was the frequency of the null allele. Average heterozygosity is obtained by averaging the PIC value obtained for all the markers and was calculated as follows:

$$Hav = \sum[2fi(1 - fi]/ N$$

For the dominant marker the PIC value less than 0.25 indicates lower polymorphism. 0.5 is the maximum value of PIC for dominant marker, since two alleles per locus are considered and are affected by number and frequency (Nagy *et al*., 2012).

Primer resolving power (RP)

Resolving power (Rp) demonstrates the ability of the most informative primers to detect differences between genotypes (Prevost &Wilkinson, 1999).

Rp was calculated by using: Rp = $\sum$ Ib

Ib is the band informativeness with Ib =1 - [2 x (0.5-p)], p is the proportion of clones containing the band.

Multiplex Ratio (MR)

Total number of loci analyzed per assay is multiplex ratio (MR). MR is calculated by dividing total number of amplified bands by total number of primers used in a particular marker system (Bhatt *et al*., 2017).

Multiplex ratio was calculated by using (MR) = TB/TP

TB is total number of amplified bands and TP is total number of primers used.

Marker Index (MI)

Marker Index (MI) is a product of two functions of each primer - polymorphic information content (PIC) and effective multiplex ratio (EMR) (Varshney *et al*., 2007).

Marker index was calculated by using MI = PIC × EMR.

3.2.8 Genetic diversity analysis using similarity matrices and phenograms

The binary data matrix created for bands obtained from the ISSR-PCR profiles generated by 10 primers and 45 samples were analyzed using Numerical Taxonomy and multivariate system (NTSYS-PC, version 2.02i, Exeter software, Setauket, Newyork, USA). DNA bands were scored as 0 for absence and 1 for presence (Transue *et al*., 1994). Similarity indices were calculated using (Similarity for Qualitative Data) SIMQUAL computational algorithm based on the similarity matrices. Sequential, Agglomerative, Hierarchial and Nested (SAHN) clustering was performed using the Unweighted Pair Group Method of Arithmetic Average (UPGMA) algorithm. Similarity estimates were calculated based on three measures:

1. Simple Matching coefficient (SM) (Sokal & Michener, 1958) calculated as

$$\text{Sij} = \frac{a+d}{a+b+c+d}$$

2. Dice's coefficient of similarity (D) (Dice, 1945; Nei and li, 1979) calculated as

$$\text{Sij} = \frac{2a}{2a+b+c}$$

3. Jaccard's coefficient (Jaccard, 1908) calculated as

$$\text{Sij} = \frac{a}{a+b+c}$$

where

Sij = Similarity between two individuals, i and j;

a = Number of bands present in both i and j;

b = Number of bands present in i and absent in j;

c = Number of bands present in j and absent in i

d = Number of bands absent from both i and j

NTSYS-PC was used to construct 3D-plot of the distribution of the collected samples of *G. diversifolia*. The variation was compared to phenogram using suitable similarity matrix with the analysis of Eigen vector.

Principal coordinate analysis (PCoA)

Principal Coordinate Analysis (PCoA) is a popular multivariate analysis method that allows the analysis of proximity matrix, regardless of whether it is a variation matrix.

The group of the population was constructed using principal coordinate analysis (PCoA) based on Nei's genetic distance matrix of populations using the software GenALEx 6.5.

Analysis of molecular variance (AMOVA)

Genetic variation was evaluated using the binary data matrix created for ISSR loci by analysis of molecular variance (AMOVA) (Excoffier *et al*., 1992) using statistical program GENEALEx 6.5 (Peakall &Smouse, 2001).

The AMOVA based on Eucildean metric (Excoffier *et al*., 1992) is given by:

$$E = (\varepsilon^2 xy) = n\,[1\text{-}2n_{xy}/2N],$$

$2n_{xy}$ is the number of markers shared by two individuals (X and Y) and n is the total number of polymorphic markers (Tsuda *et al*., 2004). Molecular variances within the population were calculated as an indicator of intra-population genetic variation. Using the GENALEx program, based on 999 random permutations, the significance of the variance components was tested by calculating their probabilities (Peakall and Smouse, 2001).

3.2.9 DNA sequencing

Polymerase chain reaction (PCR) amplification was carried out using the universal marker that includes ITS, *matK*, *rbcL*, and *trn*l-F regions for DNA barcoding. The DNA barcoding - sequencing has become a standardized and largely used molecular approach for sample identification and species differentiation (Hebert *et al*., 2004). The PCR profile for *matK*, *trn*H-psbA, ITS, *rbcL*, *trn*L-*trn*F were included initial step

at 94 ^{0}C for 2 min was conducted, followed by 40 cycles of 30sec at 94 ^{0}C, 30 Sec 52 ^{0}C and 72 ^{0}C for 1min and final extension for 72^0 C for 5 min.

Selected primers for the amplification of genomic regions are presented in (Table 5).

Table 5: Primers used in the amplification of *matK*, *trn*H-psbA, ITS, *rbcL*, *tran*L-*trn*F regions of *G. diversifolia*

Locus	Primer name	Primer sequence (5'-3')	References
matK	*matK*472F	5'- CCCRTYCATCTGGAAATCTTGGTTC -3'	Yu *et al*., 2011
	*matK*1248R	5'- GCTRTRATAATGAGAAAGATTTCTGC -3'	Yu *et al*., 2011
ITS	ITS-p5	5′- AAG TCGTAACAAGGTTTCCGTAG-3′	Kollipara *et al*., 1997
	ITS-u4	5'- RGTTTCTTTTCCTCCGCTTA -3'	Cheng *et al*., 2016
	ITS-u1	5'- GGAAGKARAAGTCGTAACAAGG -3'	Cheng *et al*., 2016
rbcL	CPG03F	5'- ATGTCACCACAAACAGAGACTAAAGC-3'	Dong *et al*., 2013
	Cp063R	5'- TTCCATACTTCACAAGCAGCAGCTAG -3'	Dong *et al*., 2013
Trnl-F	trnL-c	5′- CGAAATCGGTAGACGCTACG -3′	Taberlet *et al*., 1991
	Cp054R	5'- CTGCTCTACCAGCTGAGCTATCCCGAC -3'	Dong *et al*., 2013
trnH-psbA	trnH	5'- ACTGCCTTGATCCACTTGGC -3'	Kress *et al*., 2007
	psbA	5'- CGAAGCTCCATCTACAAATGG -3'	Kress *et al*., 2007

Confirmed PCR products were purified and sent to Beijing Genomic Institute (BGI; Shenzhen, China) for sequencing. Sequences were analyzed by Sequencher 4.7 (Gene Codes, Ann, MI, USA).

3.3 Data analysis of DNA sequencing

Maximum parsimony (MP) analyses were conducted using PAUPv4b10 (Swofford, 2002). All characters were equally weighted, character states were treated as randomly, and gaps were treated as missing. The maximum likelihood (ML) analyses were performed using IQ-Tree 1.4.3 (Nguyen *et al*., 2015), with 1000 replicates of ultrafast likelihood bootstrap (Minh *et al*., 2013) to obtain bootstrap branch support values. Bayesian inference (BI) was conducted with Mrbayes v3.2 (Ronquist *et al*., 2011). Trees were sampled at every 1,000 generations with the first 25% discarded as burn-in. The remaining trees were used to build a 50% majority-rule consensus tree.

The DNA sequences were assembled using SeqMan tool in the DNASTARLasergene v.7.1 and aligned using CLUSTAL W (Thompson *et al*., 1994) implemented in MEGAX (Kumar *et al*., 2018).

The *matK* gene (736 bp) sequences were used for establishing the phylogenetic relationship of *G. diversifolia* with other species from the family Urticaceae. Two sequences, each representing one haplotype from Nepal's population of the species (One from Kathmandu and the other from Panchthar District) were used for testing phylogenetic relationship. Homologous sequences from other species were accessed from the GenBank (Table 6). The corresponding sequences of *Urera caracasana* was downloaded from NCBI GenBank and used as the outgroup for phylogenetic analyses. The phylogenetic analysis was performed using the maximum-likelihood (ML) algorithm in RAxML v.8.2.11 (Stamatakis, 2014). The ML analysis was conducted with 1000 bootstrap replicates using the best partition scheme (DNA, P1 = 1-736\3, 2-736\3; P2 = 3-736\3) determined by using the GTR+Γevolutionary model and Bayesian Information Criterion (BIC) in Partition Finder v.1.0.1 (Lanfear *et al*., 2012). The phylogenetic tree was annotated and visualized in the software FigTreev.1.4.3 (Rambaut, 2014).

Table 6: GenBank accession numbers of the *matK* sequences used in the phylogenetic analysis

S.N.	Species	Accession Number (NCBI)
1	*G. diversifolia*	This study
2	*G. diversifolia*	This study
3	*G. diversifolia* subsp. *diversifolia*	MK931205
4	*G. diversifolia* subsp. *diversifolia*	KF138001
5	*G. diversifolia* subsp. *triloba*	KF138006
6	*G. diversifolia* subsp. *triloba*	MK931208.
7	*G. diversifolia* subsp. *suborbiculata*	KF138003
8	*G. diversifolia* subsp. *suborbiculata*	MK931207
9	*Discocnide Mexicana*	MK931199
10	*Dendrocnide meyeniana*	MK931202
11	*Laportea* sp.	MK931215
12	*Urera caracasana*	MH358024

3.4 Fiber processing

3.4.1 Sample preparation and treatment

Stem fiber from *G. diversifolia* was harvested from the samples of Darchula District, far-western region of Nepal (29° 47' 34.9" N, 80° 36' 23.5" E) in September, 2019. Extracted fiber was dried in safe shade for a week, chopped into about 30 cm segments and stored inside the room. Sodium hydroxide (NaOH), crude papaya enzyme, Ethylenediaminetetraacetic acid (EDTA) plus Pectinase enzyme, Pectinase enzyme (Akin *et al*., 2000) were used for the treatment as follows.

Table 7: Symbols of the samples and their treatment conditions.

Symbols	Chemical constituents and conditions
NAT	Natural fiber without retting
NaOH	Sodium hydroxide
ENZ	Pectinase enzyme only
ENZ/CH	Pectinase enzyme+ Ethylenediaminetetraacetic acid
PENZ/ CH	Enzyme extracted from papaya pericarp + Ethylene diamine tetra-acetic acid

3.4.1.1 Enzyme treatment

Manually chopped 30 cm fibers of *G. diversifolia* were sprayed with enzyme formulation 0.3% pectinase enzyme (17389-10G, pectinase from *Aspergillus niger* from Sigma-Aldrich) at a ratio of 165 mL per 50- gram fiber with hand-held sprayer Enzyme treated *G. diversifolia* fibers were soaked for 60 seconds, drained for 30 seconds excess liquid was removed, sealed in a plastic bag. Incubated at 40° C for 24 hrs for retting, and rinsed for 60 seconds under running water (Akin *et al.,* 2000). Air dried fiber was hand carded and FTIR, XRD and tensile strength measurement were performed.

3.4.1.2 NaOH treatment

The fiber was chopped about 30cm long and was sprayed with 5% sodium hydroxide (NaOH) at a ratio of 165 ml per fifty-gram fiber with a hand-held sprayer. Sodium hydroxide treated *G. diversifolia* fibers were soaked for 60 seconds, drained for 30 seconds. Sealed in a plastic bag. Incubated at 40° C for 24 hrs for retting. The retted

material, after incubation was immersed in water for 60 seconds, rinsed for 60 seconds under running water and dried in air. Air dried fiber was hand carded. Fiber properties were analyzed using FTIR, XRD, microscopy, and tensile strength test.

3.4.1.3 Enzyme plus EDTA treatment

Fibers of *G. diversifolia* (30 cm) were sprayed with enzyme formulation 0.3% pectinase enzyme (17389-10G, pectinase from *Aspergillus niger* from Sigma-Aldrich and 50 mmol EDTA as a chelator) 165 mL per 50-gram fiber with a hand-held sprayer. Enzyme treated *G. diversifolia* fibers were soaked for 60 seconds, drained for 30 seconds, sealed in a plastic bag. Incubated at 40° C for 24 hrs for retting, and rinsed for 60 seconds under running water. Air dried fiber was hand-carded and FTIR, XRD and tensile strength measurement were performed.

3.4.1.4 EDTA plus Crude papaya extract treatment

Fifty grams of ripened papaya pericarp was ground in a 50 ml NaCl solution of 0.9% (w / v) using a blender. The homogenate concoction was subjected to sonication (4° C) using a bandelin sonoplus HD 2200 for 10 min at 50-60 Hz frequency and then centrifuged at 10,000 rpm for 30 min at 4°C. (Martial *et al*., 2017). The supernatant was filtered from cotton wool, refrigerated, and was used as the crude extract.

Chopped fibers of *G. diversifolia* were sprayed with enzyme formulation (crude papaya extract and 50 mmol EDTA as a chelator) at a ratio of 165 mL per 50-gram fiber with hand-held sprayer. Enzyme treated *G. diversifolia* fibers were soaked for 60 seconds, drained for 30 seconds excess liquid was removed. Sealed in a plastic bag. Incubated at 40° C for 24 hrs for retting, and washed for 60 seconds under running water. Air dried fiber was hand-carded and FTIR, XRD, and tensile strength measurements were performed.

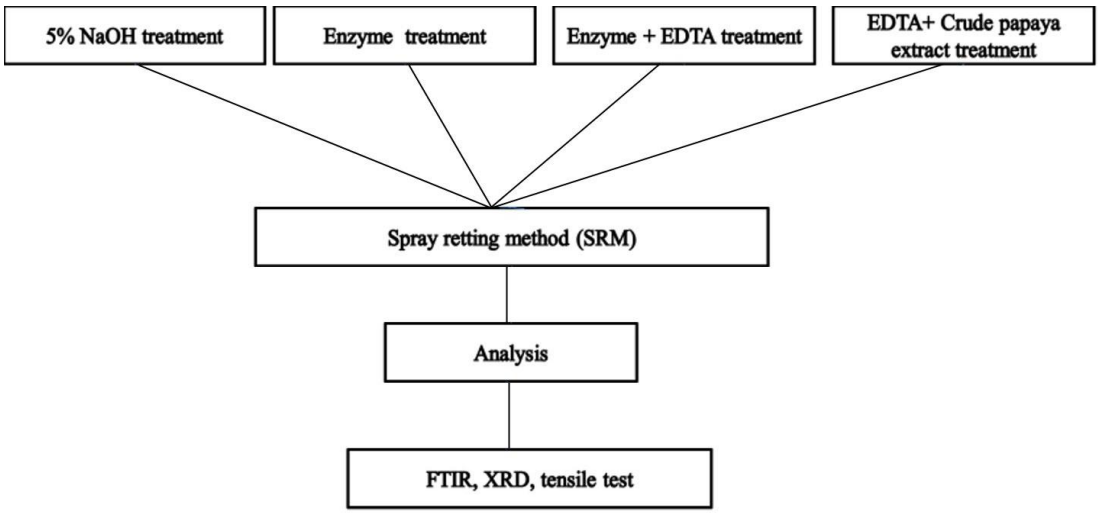


Figure 16: Spray retting method for processing fibers (Akin *et al*., 2000; Adamsen *et al*., 2002; Kumar *et al*., 2017; Sawpan *et al*., 2011).

3.5 Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) was performed on treated and untreated fibers. The FTIR analysis was carried out using SHIMADZU IR Affinity-1S model transmission spectrometer with KBr pellet method over the range of 4000 - 500 cm^{-1} at Department of Customs, Kathmandu, Nepal.

3.6 X-ray diffraction

Powder X-ray diffraction (XRD) technique (Bruker 2, Germany) was used to determine crystallinity of untreated and treated fiber samples with Cu Kα radiation (λ = 0.15418 nm) at a scanning speed of 0.02^0/s, 2θ mode between 10° and 80° at National Academy of Science and Technology (NAST), Lalitpur, Nepal. Segal equation was used for rapid analysis of crystallinity index (CI) of fiber (Segal *et al*., 1959).

$$\mathrm{CI}(\%) = \frac{(I002 - \mathrm{Iamp})}{\mathrm{I002}} \times 100$$

3.7 Microscopy and tensile testing

Macrophotography and optical microscopy were done by using a stereomicroscope Stemi 2000-C (Zeiss, Germany). For uniaxial tensile testing five fiber bundles of the given length L_1 of 50 mm were cut from each fiber type and the mass *m* of these fiber

bundles was measured using a precision balance CP124S-OCE from company Sartorius. The fiber bundles were glued on rectangular frames (sizes: 40×40 mm^2) made of paper (free length of the fiber bundles: 20 mm). The uniaxial tensile testing was conducted at room temperature (23° C), a universal testing machine (Zwicki Z2.5 from ZwickRoell GmbH & Co. KG, Ulm/Germany; see Figure 17) equipped with 500 N load cell was used (crosshead speed: 50 mm/min, span $L0$: 25 mm, preload: 0.5 N). The rectangular frames including the fiber bundles were clamped as shown in (Figure 17). Before the testing, the paper was scissor-cut so that the fiber bundles are freestanding. From the raw data, i.e., the load (F in N) vs. length (L in mm) diagrams (measured by traverse), the mass-related load (in N mm/mg) vs. strain (in %) diagrams were calculated:

$$\text{Mass related load } = \frac{\text{F}.L_1}{\text{m}} \text{ and strain} = \frac{\text{L}}{L_0} \times 100\%.$$

From these diagrams two material parameters were collected: the mass-related tensile strength and the mass-related elastic modulus. Fiber bundles were tested and the mass per fiber bundle length were measured. The load has been related to the mass per fiber bundle length value, designated as mass-related load.

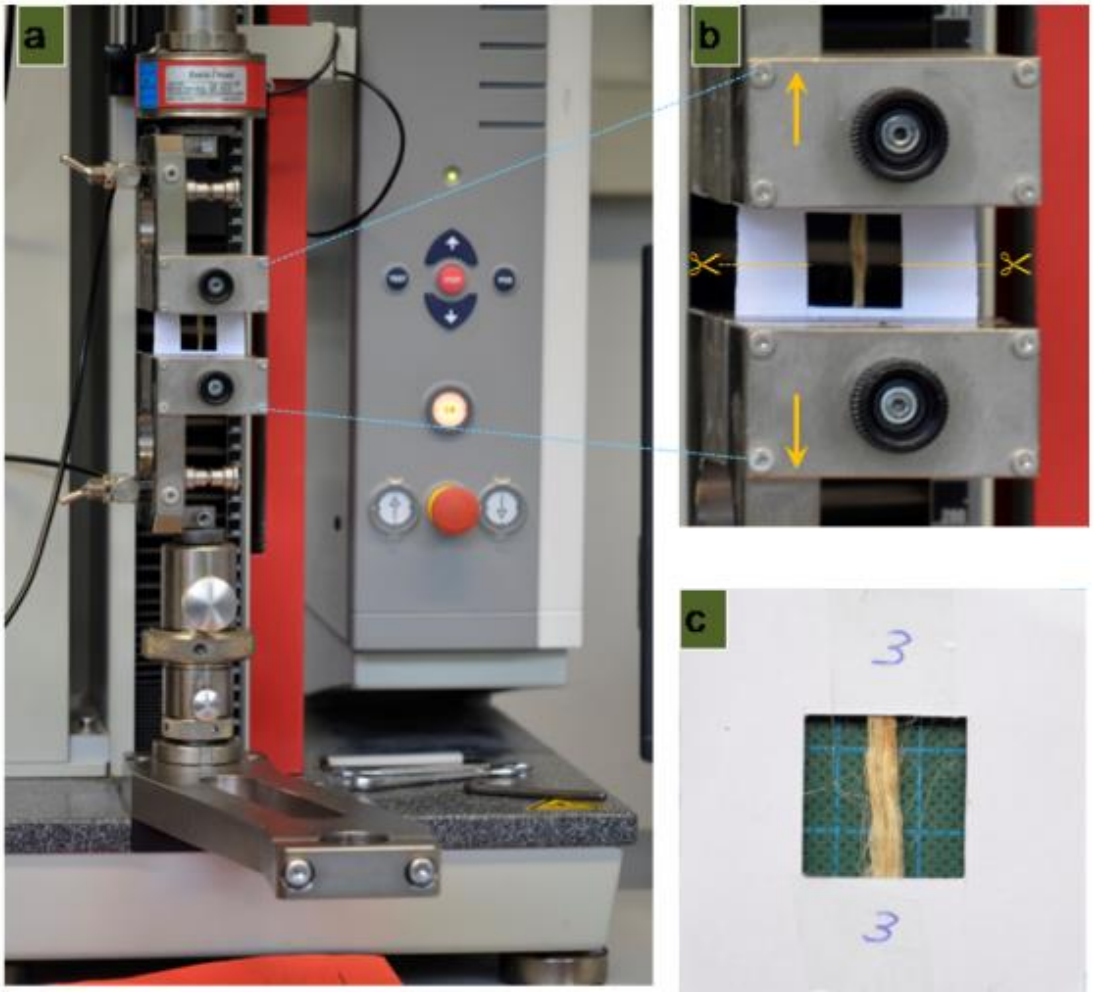


Figure 17: (a) Universal testing machine for tensile tests of the fiber bundles, (b) detail of clamping, (c) fiber bundle fixed inside a frame made of paper.

CHAPTER 4

4. RESULTS AND DISCUSSION

4.1 Results

4.1.1 Morpho-geographical study of *G. diversifolia* in Nepal

G. diversifolia is tall and erect herb, 1.5 to 3 m high, having perennial root stock. Young leaves are ovate or suborbicular having one lobe and mature ones are 3-7 lobed, petiolate, petioles 5-15 cm long with stinging trichomes, length of leaves is 10-14 cm on average and as broad as the length, base obtuse to truncate, 3 nerved with bristly stinging hairs, apex in general acuminate, rarely acute, coarsely dentate to dentate, or serrate. Stipules ovate, interpetiolar, about (7mm) 1 to 2 cm long. Plant is monoecious. Male flowers are long slender, perianth 4, stamens 4. Female flowers are simple dense and bristly inflorescence up to 18 cm long; perianth 0.25 cm long, tubular, 3 lobed, and splitting when the fruit ripens. Achenes are broad, 2 to 3 mm long, and 1.5 to 2.7 mm broad (Figure 18).

Figure 18: Morphology of *G. diversifolia*. A seeds; B germination of seed ; C-E Habit before flowering; F Mature plant with male inflorescence; G Clumps; H Female inflorescence with fruit.

G. diversifolia is found in Nepal from eastern to far-western region at an altitude of 1,000 to 3,000 meters above sea level. Based on the study of herbarium specimens deposited at the National Herbarium and Plant Laboratories (KATH), my own field study and herbarium collection during 2017 to 2021, key informants interviews with 110, and feasibility assessment conducted by the Department of Small and Cottage Industry, Government of Nepal, the availability of *G. diversifolia* in different districts of Nepal is reflected in Figure 19. Dark green color represents high amount of *G. diversifolia* available in those districts, such as Taplejung, Panchthar, Therathum, Illam, Solukhumbhu, Sankhuwasabha, Dolakha, Ramechhap, Sindulpalchok, Kavre, Rasuwa, Dhading, Gorkha, Lamjung, Kaski, Baglung, Myagdi, Parbat, Salleyan, Jajarkot, Pyuthan, Rukum, Jumla, Surkhet, Rolpa, Dailekh, Kalikot, Achham, Doti, Dadeldhura, Darchula Districts; light green shows lower amount of *G. diversifolia* in the districts, such as Dhankuta, Udayapur, Sindhuli, Makwanpur, Chitwan, Arghakhanchi, Dang, Manang, Mustang, Dolpa, Mugu, Humla Districts); and districts marked with white colour such as Morang, Jhapa, Saptari, Sunsari, Siraha, Dhanusha, Mahottari, Bara, Parsa, Rupandehi, Kapilbastu, Banke, Kanchanpur, Kailali Districts do not have *G. diversifolia*.

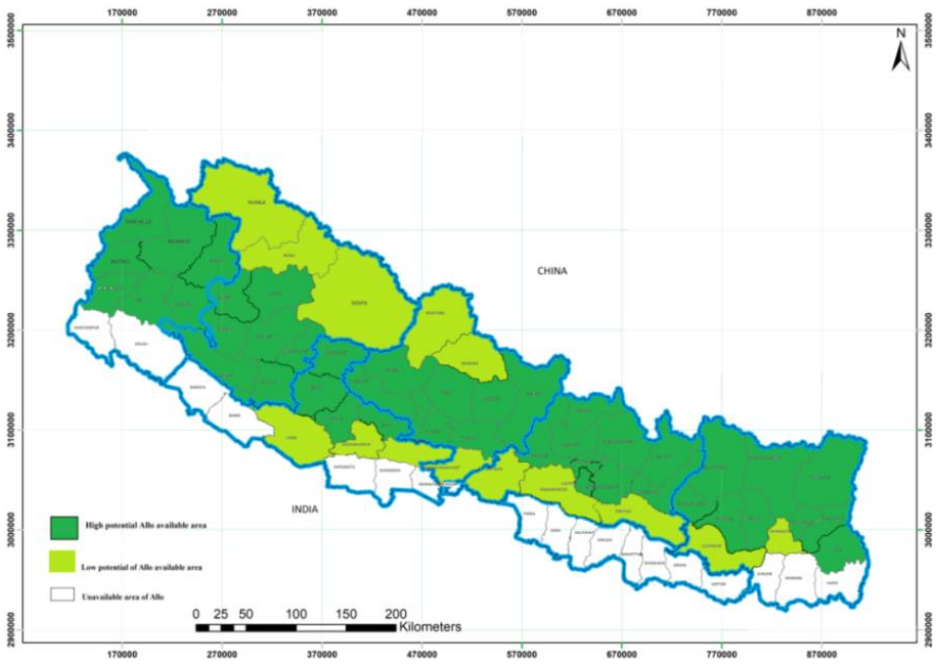


Figure 19: Distribution map showing the availability of *G. diversifolia* in Nepal

Key to the infraspecies of *G. diversifolia*

1a. Leaves blade unlobed, suborbicular or ovate-suborbicular with dentate margins, stipules 4-7 mm …….subsp. *suborbiculata*

1b. Leaves blade variable, 1-7 lobes, ovate with coarsely serrate rarely coarsely dentate or serrate margins, stipules 10-20 mm ……....subsp. *diversifolia*

4.1.1.1 Major characters of *G. diversifolia* subsp. *diversifolia*

The study of herbarium specimens, observation from germination stage to fruiting stage of the plant, 16 morphological characters were selected as possible delineating characters: (i) height of plant (cm); (ii) diameter of stem (mm); (iii) leaf length (cm); (iv) number of leaf lobes; (v) breadth of leaf (cm); (vi) length of trichome at abaxial surface (mm); (vii) number of trichome in abaxial surface per cm^2 of leaf; (viii) length of trichome at adaxial surface (mm); (ix) number of trichome at adaxial surface per cm^2 of leaf; (x) length of stipules (cm); (xi) length of petiole (cm); (xii) number of nodes; (xiii) length of male flower bud (mm); (xiv) number of stamens; (xv) Length female flower; and (xvi) size of seeds.

4.1.1.2 Height of plant

Among the plant samples collected from Nepal, the average height of *G. diversifolia* from Kathmandu District was shortest, i.e. 240 cm whereas, the tallest height was 330 cm from Panchthar District (Figure 20). The average height of *G. diversifolia* from Darchula District, Kaski District, Dolakha District are 270cm, 285cm, 300cm respectively (Figure 20). Local communities involved in processing and manufacturing of textile products from *G. diversifolia* prefer smaller stem diameters and longer lengths of stem for quality fibers.

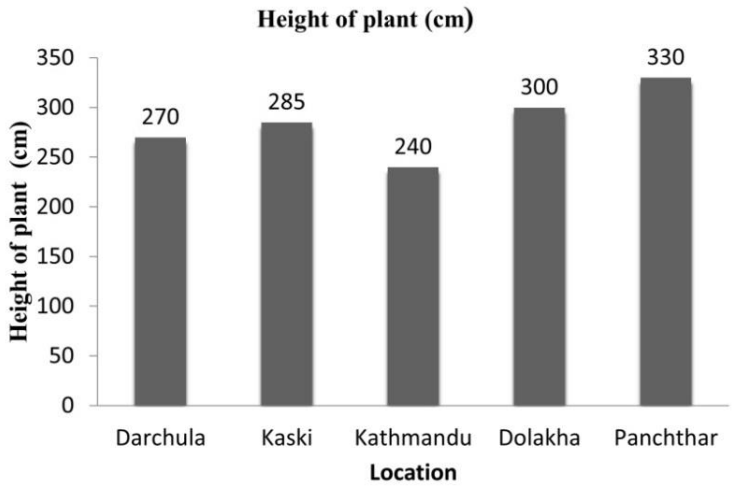


Figure 20: Comparison of plant height (cm) of *G. diversifolia* from different districts of Nepal.

4.1.1.3 Stems

Stems sympodial, upper stem often zigzag, woody at base, straight, branched, 5-angled, 1-3 m tall. The woody forms have white-brown bark in the stem. Stems are herbaceous, erect, and cylindrical, with layers in ring form in brown colour. Stems are pubescent with stinging hairs (Figure 21). Stems grow in clump, and each clump has many stems. The number of stems in a clump varied from 5 to 15. The number of nodes varies from 35 to 56. Stem of *G. diversifolia* has five rounded bulge separated by furrows. Figure 21 shows a cross-section of stem showing stinging hairs, epidermis, collenchyma, primary phloem fibers, seconday phloem fibers, secondary xylem and primary xylem.

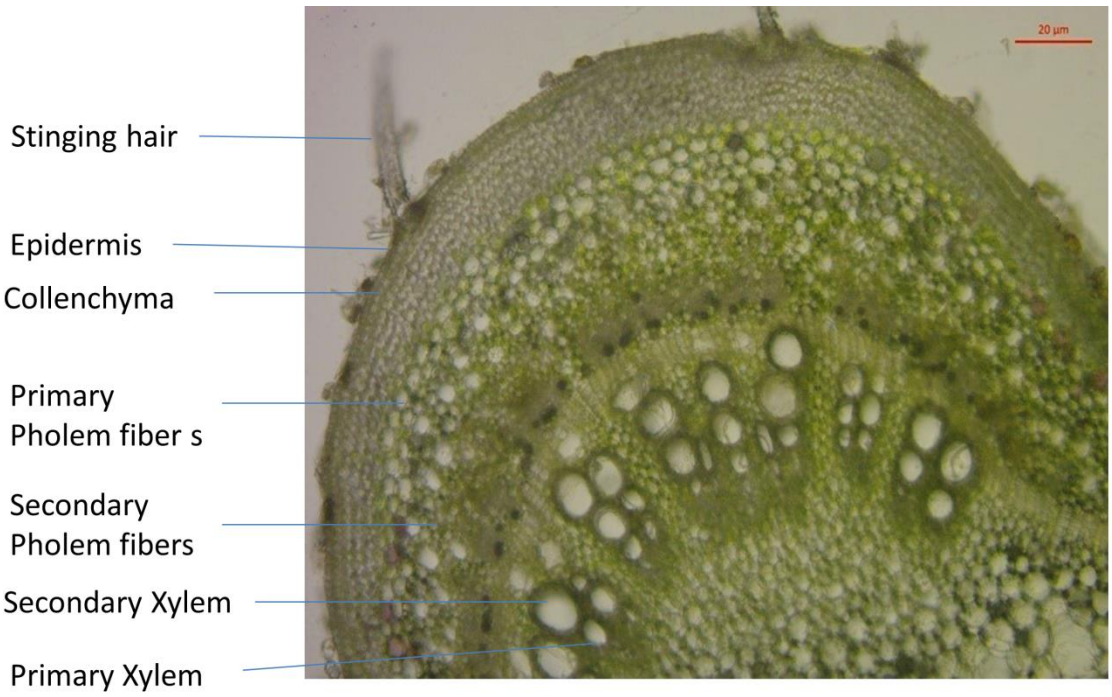


Figure 21: Transverse section of stem of *G. diversifolia* observed under 40×.

Among the plant samples collected from Nepal, the average stem basal diameter of *G. diversifolia* from Kathmandu population was 37.5 mm, however, the stem of *G. diversifolia* from Panchthar District had the smallest diameter 18.5 mm (Figure 22). The average stem basal diameter of *G. diversifolia* from Darchula District, Kaski District, Dolakha District are 24.5mm, 25mm, 30.1mm, respectively (Figure 22).

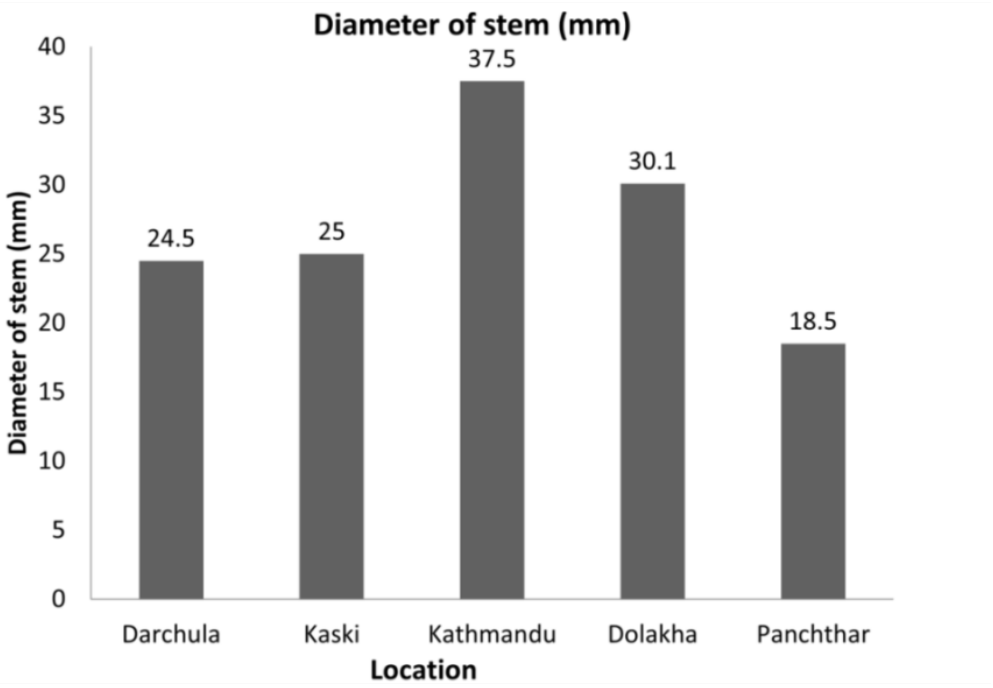


Figure 22*:* Comparison of stem basal diameter in (mm) of *G. diversifolia* from different districts of Nepal**.**

4.1.1.4 Leaves

The leaves are alternate, petiolate (petiole 4 to 18 cm long); leaves lobed, leaf lobes vary from 1 to 7 lobes; in general, young leaves are undivided, and the mature ones are 3 to 7 lobed ovate, 10 to 14 cm long and often are as broad as the length or slightly smaller (Table 8; Figure 23); leaf base obtuse in populations from Darchula, Kaski, Dolakha and Panchthar Districts; wheras, obtuse to truncate from Kathmandu District (Figure 24); margin serrate from the population of Darchula, Dolakha and Panchthar District or coarsely serrate rarely coarsely dentate from the population of Kaski and Kathmandu District (Figure 24), and veins 3, with bristly stinging hairs on the adaxial and abaxial surface (Figure 24). Stipules are about 1 cm long from the population of Darchula, Kaski, Kathmandu and Panchthar District upto 2 cm from populations of Dolakha District, ovate, and interpetiolar, found almost up to the tip and fall during the flowering period.

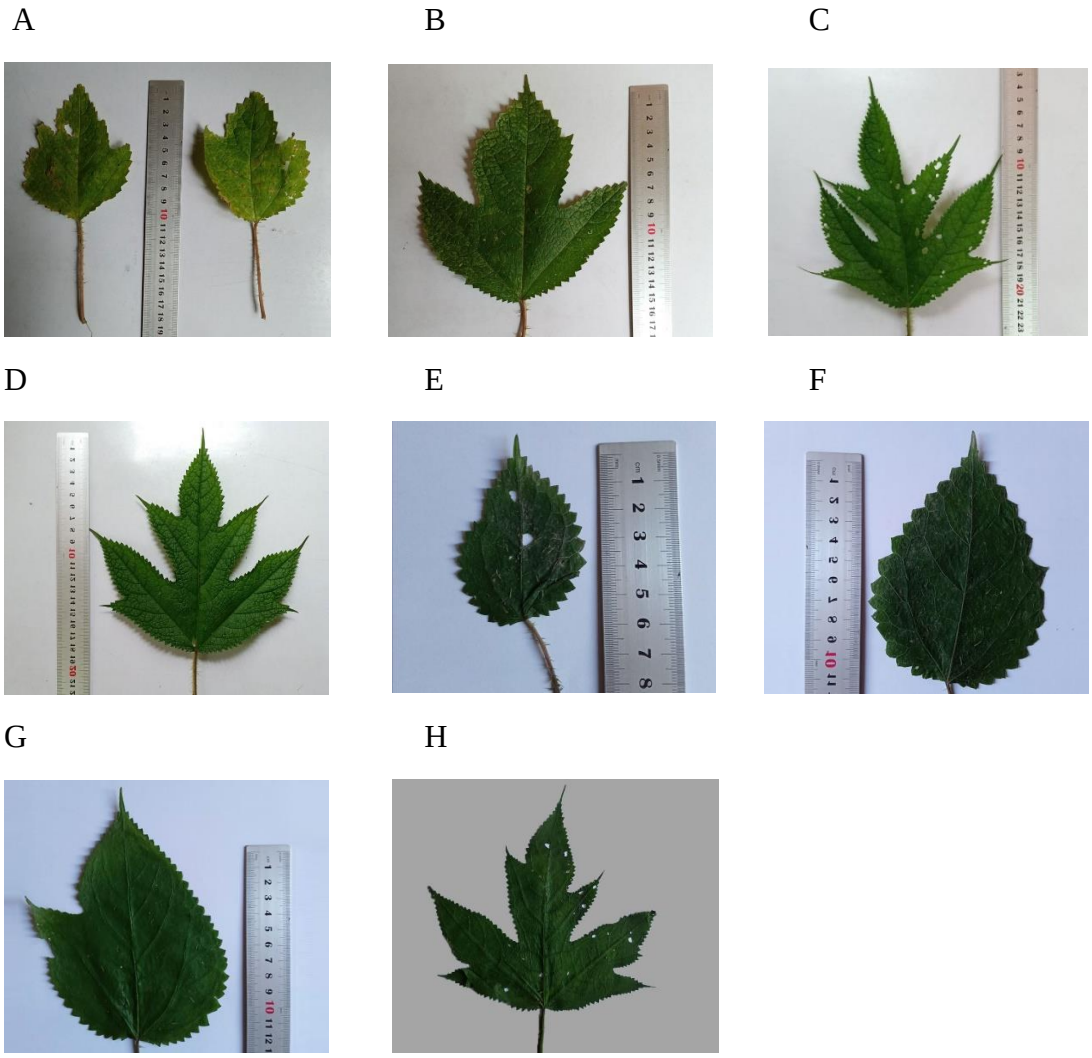


Figure 23: Difference in the number of leaf lobes found in *G. diversifolia* in the same plant. (A), (B), (C), (D). lobes with 3-7 lobes; (E). (F). lobes with single lobe; (G). (H). 2-7 lobes.

Leaf lobes and sub-lobes: Characters of leaf lobes and sublobes vary in the leaves. The leaves from Darchula District have 3 lobes, each sublobe divided into 2 lobes; Kaski District has 3 lobes each sub-lobes divided into 2 deeply trilobes; leaves from Kathmandu District are unlobed to 3(5) lobed; leaves from Dolakha District and Panchthar District has 3 lobes each lobe further divided into 2 to 3 sublobes (Table 8). Figure 24 shows the leaves of *G. diversiolia* from Kathmandu, Darchula, Panchthar, Kaski and Dolakha District respectively.

A

B

C

D

E

F

G

H

I

J

Figure 24: A and B, adaxial and abaxial surface of leaf from Kathmandu District; C and D, adaxial and abaxial surface of leaf from Darchula District; E and F, adaxial and abaxial surface of leaf from Panchthar District; G and H, adaxial and abaxial surface of leaf from Kaski District; I and J, adaxial and abaxial surface of leaf from Dolakha District**.**

Table 8: Morpho-geographical variation in leaf lobes of *G. diversifolia*

Districts	Leaf shape	Number of leaf lobes	Leaf margin	Leaf base	Average length of leaf (cm)	Average breadth of leaf (cm)
Darchula	Ovate	3 lobed each sub lobe divided into 2 lobes	Serrate	Obtuse	14	14
Kaski	Ovate	3 lobed each sublobe divide into 2 lobes deeply trilobed.	Coarsely serrate	Obtuse	13	10
Kathmandu	Ovate	Unlobed to 3(5) lobed	Coarsely dentate	Obtuse to truncate	13	11
Dolakha	Ovate	3 lobed each sublobe further divided into 2 to 3 lobes	Serrate	Obtuse	12	12
Panchthar	Ovate	3 lobed each sublobe further divided into 2 to 3 lobes	Serrate	Obtuse	14	10

The average length of stipules of *G. diversifolia* from Dolakha District is 2 cm, which is longest as compared to the samples from Darchula, Kaski, Kathmandu, and Panchthar Districts which is 1 cm long.

Table 9: Average length stipules of *G. diversifolia* from different districts of Nepal.

Districts	Average length of stipules (cm)
Darchula	1
Kaski	1
Kathmandu	1
Dolakha	2
Panchthar	1

4.1.1.5 Trichomes

The study of trichomes at adaxial surface of leaf shows that population of *G. diversifolia* from Kaski District had highest number i.e. 3 trichomes per cm^2 of leaf. The number of trichomes at adaxial surface in other districts such as Darchula, Kathmandu and Dolakha had 1 trichomes per cm^2 of leaf and population of Panchthar

District had 2 trichomes per cm^2 of leaf, whereas number of trichomes on abaxial surface of Kaski District has 4 trichomes per cm^2 of leaf. The number of trichomes at abaxial surface in other districts such as Darchula, Kathmandu and Panchthar had 2 trichomes per cm^2 of leaf and population of Dolakha District had 1 trichomes per cm^2 of leaf. Population of *G. diversifolia* from Panchthar District has longest trichome 3.7 mm at adaxial surface, and 4 mm at abaxial surface among the collected samples of Nepal (Table 10). Length of trichomes at abaxial surface of leaf in other districts such as Darchula, Kathmandu and Dolakha Districts have 3.9, 3.6 and 1.9 mm respectively given in Table 10. Population of *G. diversifolia* from Kaski District have highest number of trichomes at adaxial surface compared to population from Darchula, Kathmandu, Dolakha and Panchthar Districts.

Table 10: Number and length of trichomes of *G. diversifolia* observed in morpho-geographical study.

Districts	Number of trichomes		Average length of trichome (mm)	
	Adaxial surface	Abaxial surface	Adaxial surface	Abaxial surface
Darchula	1	2	3.3	3.9
Kaski	**3**	**4**	3.4	3.8
Kathmandu	1	2	3.6	3.6
Dolakha	1	1	2.3	1.9
Panchthar	2	2	3.7	4

4.1.1.6 Inflorescences

Axillary cyme, panicles or spikes, both male and female flowers are found in same plant.

4.1.1.7 Flowers

Male flowers: Perianth- 4, stamens- 4 and pistilode globose and rudimentary ovary conspicuous (Figure 25).

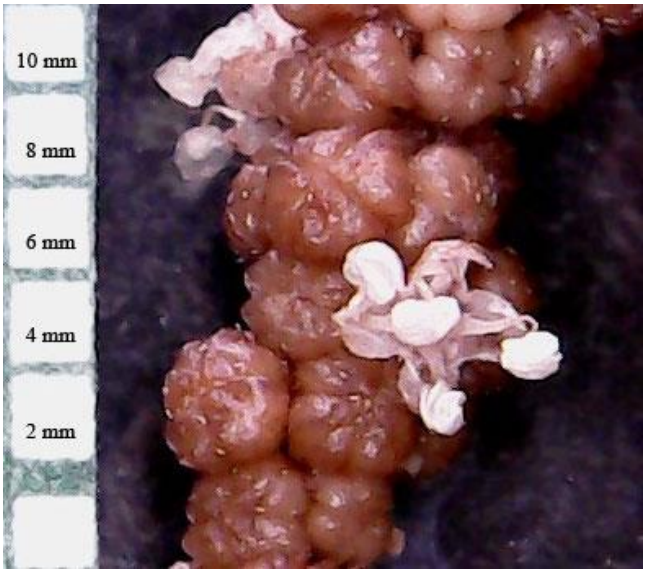


Figure 25: Male flower of *G. diversifolia*

Female flowers: 3 lobes, tubular, 2 or 3-toothed, split to base on one side, (Figure 26).

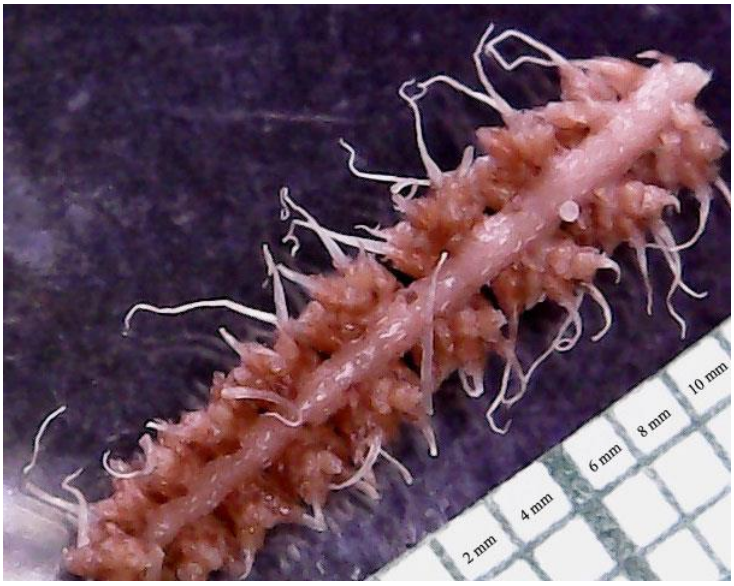


Figure 26: Female flower of *G. diversifolia*

4.1.1.8 Achenes

The achenes are broad, compressed, 2 to 3.05 mm long, 1.55 to 2.75 mm broad; cotyledons broad, persistent stigma usually reflexed (Figure 27).

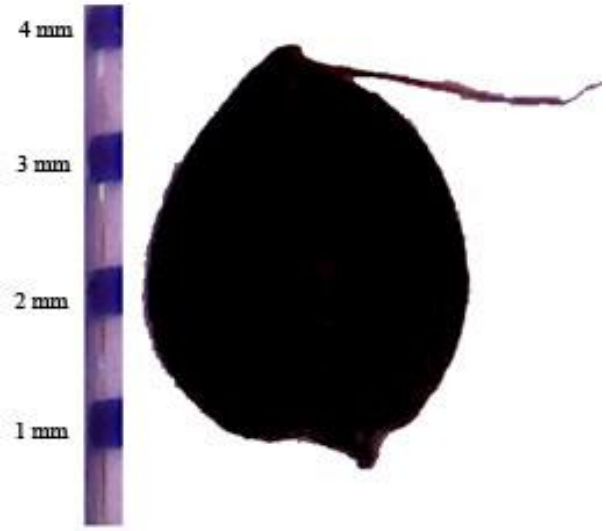


Figure 27: Seed of *G. diversifolia*

Table 11: Comparison of main morphological traits of *G. diversifolia* found in different districts of Nepal.

Group	Samples code number	Main morphological traits
Panchthar	Phi1, Phi2, Phi3, Phi4, Phi5, Phi6, Phi7, Phi8, Phi9, Phi10	**Smallest basal stem diameter (18.5mm), longest tricomes on adaxial surface (3.7mm) and abaxial surface (4mm). When compared with other samples, the height of the *G. diversifolia* from Panchthar District was the tallest (330 cm).** Leaf margin serrate.
Kathmandu	Kat1, Kat2, Kat3, Kat4, Kat5, Kat6, Kat7, Kat8, Kat9, Kat10	**Longest diameter of the stem at the base (37.5mm)** When compared with other samples, the height of the *G. diversifolia* from **Kathmandu District was the shortest (240cm). Leaf margin serrate, rarely coarsely dentate.**
Dolakha	Jir1, Jir2, Jir3, Jir4, Jir5, Jir6, Jir7, Jir8, Jir9, Jir10	**The lowest number of trichomes i.e.,1 per cm^2 of leaf on abaxial and adaxial surface of the leaf**. **Longest length of stipules 2 cm**. Leaf margin serrate.
Darchula	Dar1, Dar2, Dar3, Dar4, Dar5, Dar6, Dar7, Dar8, Dar9, Dar10	Moderate length of trichome at the adaxial 3.3 mm and abaxial surface 3.9 mm. Leaf margin serrate.
Kaski	Kas1, Kas2, Kas3, Kas4, Kas5	**Highest number of trichomes at adaxial surface (3 trichomes per cm^2 of leaf) and at abaxial surface (4 trichomes per cm^2 of leaf).** Leaf margin coarsely serrate

While selecting 16 morphological characters of *G. diversifolia*, differences were found in main 7 characters viz. (i) height of plant (cm) (Figure 20); (ii) Stem diameter (mm) (Figure 22); (iii) length of trichome in adaxial surface (mm) (Table 10) (iv) trichome numbers in adaxial surface per cm^2 of leaf (Table 10); (v) length of

trichome in abaxial surface (mm) (Table 10); (vi) trichome numbers in abaxial surface on the vein per cm^2 of leaf (Table 10); (vii) length of stipules (Table 9). A cumulative table of main morphological characters are given in Table 12.

Table 12: A cumulative table of main morphological characters

Group	Height of plant (cm)	Stem (diameter) mm	Length of trichome in adaxial surface (mm)	Length of trichome in abaxial surface (mm)	Trichome numbers in adaxial surface of leaf	Trichome numbers in abaxial surface of leaf	Length of stipules (cm)	Leaf margin
Darchula	270	24.5	3.3	3.9	1	2	1	Serrate
Kaski	285	25	3.4	3.8	3	4	1	Coarsely serrate
Kathmandu	**240**	**37.5**	**3.6**	**3.6**	1	2	1	**Serrate rarely Coarsely dentate**
Dolakha	300	30.1	2.3	1.9	1	1	2	Serrate
Panchthar	330	18.5	3.7	4	2	2	1	Serrate

It is remarkable to note that morpho-geographical characters of different populations of *G. diversifolia* from five different localities are variable and overlap with each other in terms of leaf length (cm); number of leaf lobes; petiole (cm); number of clumps; number of nodes; male flower bud; perianth lobes; stamens; female flower; and seeds. They do not give stable conclusive distinguishing characters to separate the populations. However, population from Kathmandu district shows some diagnostic characters (see Table 12). This demands to further study *G. diversifolia* at the genetic level.

4.1.1.9 *Girardinia diversifolia* (Link) Friis subsp. *suborbiculata* (C.J.Chen) C.J. Chen & Friis (Urticaceae): probably a new record for Nepal and Bhutan

Herb, monoecious. Stem and petioles pubescent, armed with stringent hairs. Leaf blade unlobed, suboricular or ovate-suborbicular, 6-7 x 5-6.5 cm, leaf base trancuate or rounded, apex shortly acuminate, margin dentate or double dentate, sharp stinging trichomes at abaxial surface, trichomes at veins at abaxial surface. Male inflorescene spicate. Female inflorescences axillary.

This taxon is morphologically distinct from *Girardinia diversifolia* subsp. *diversifolia* in having unlobed, suborbicular or ovate-suborbicular leaves with dentate margins and much shorter stipules.

Flowering: September to October

Habitat: Growing along the roadside at an altitude of 2,000- 2,500m

Distribution: Nepal, India, China, Korea

4.1.2 Genetic variation of *G. diversifolia* subsp. *diversifolia*

Genetic variation in the species ultimately affects the sustainability of natural resources (Firn & Jones, 2000). This study has assessed the genetic variation within and among the population of *G. diversifolia* subsp. *diversifolia* collected from eastern region, central region and western region of Nepal. Genetic uniformity within a species or population is important for the uniformity and quality products of fiber prepared from *G. diversifolia*.

4.1.2.1 Quantitative and qualitative analysis of extracted DNA

Rich in viscous, phenolics, secondary metabolites, and compounds such as tannin contained in Urticaceae family inhibit high-quality genomic DNA extraction (Sarrazola *et al*., 2019). During DNA extraction from *G. diversifolia* subsp. d*iversifolia* appeared dark and viscous substance that inhibited the production ofgood quality DNA and hindered PCR amplification. Therefore, modified Doyle and Doyle (1987) plant DNA extraction procedure was used. Before the extraction of genomic DNA, 5% Polyvinylpyrrolidone (PVP) and 5% β-mercaptoethanol were added to CTAB buffer. Mean ratio of absorbance (260/280) nm, mean ratio of absorbance

(260/230) nm, and mean concentration of DNA extracted from *G. diversifolia* subsp. *diversifolia* are presented in Table 13.

Table 13: Quantitative and qualitative analysis of the extracted DNA

S. No	Protocol	Mean ratio of absorbance (260/280) nm	Mean ratio of absorbance (260/230) nm	Mean concentration (ng/µL)	Colour/ Viscosity
1	Doyle and Doyle (1987) protocol	2.20	1.10	331.39	Dark and viscous
2	Modified protocol	1.83	2.20	445	Clear and nonviscous

The Doyle and Doyle (1987) method showed a dark and viscous DNA content indicating high polyphenol content in the leaf tissue, so modification was necessary. The modified procedure produced 445 ng/µL of genomic DNA. The purity was between 1.8-2.0 which indicates the minimum contamination of the secondary metabolites in the extracted DNA.

The standardized conditions for DNA extraction from *G. diversifolia* are shown in Table 14.

Table 14: The standardized condition for DNA extraction from *G. diversifolia*

S. No	Parameters	Tested range	Standardized condition	Inference
1	NaCl	1M, 2M, 3M, 4M, 5M, 6M	5 M	Removed polysaccharides
2	PVP	1%, 2%, 3%, 4%, 5%, 6%	5%	Absorbed polyphenols
3	β-mercaptoethanol	1%, 2%, 3%, 4%, 5%, 6%	5%	Extracted clear DNA pellet
4	Ammonium acetate	1M, 1.5M, 2M, 2.5M, 3M, 3.5M, 4M, 4.5M, 5M, 5.5M, 6M, 6.5M, 7M, 7.5M, 8M, 8.5M	7.5 M	Sugar phosphate backbone charges neutralized

4.1.2.2 Optimization of ISSR PCR and primers selection

PCR optimization is important to obtain DNA bands. This research attempted two options using ready to use PCR Master Mix (5X PCR Master Mix) of New England Biolab and separate *Taq* polymerase, dNTPs, from VIVANTIS by using standard protocol. Despite several attempts, PCR was inhibited due to the presence of phenolics, secondary metabolites, viscous compounds in extracted DNA (Sarrazola *et al*., 2019). Diverse ranges of manipulation in DNA template concentration (25-100)ng, Magnesium chloride concentration (1.5-4)mM, dNTPs concentrations (0.1-0.6) µM , *Taq* polymerase concentrations (0.5-3) units, and varied in annealing temperatures (45-52) °C were performed for the optimization of ISSR PCR (Table 15).

Table 15: PCR parameter testing and optimized conditions

S. No	PCR parameters	Tested ranges	Optimum conditions
1	Concentration of DNA (ng)	25, 37.5, 50, 62.5, 75, 87.5, 100	50
2	Deoxynucleotide triphosphate (dNTPs) (mM)	0.1, 0.2, 0.3, 0.4, 0.5, 0.6	0.6
3	Magnesium chloride (mM)	1.5, 2, 2.5, 3, 3.5, 4	4
4	Primer concentration (µM)	0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9	0.7
5	*Taq* DNA polymerase (Units)	0.5,1, 1.5, 2, 2.5, 3	2
6	Annealing temperature (°C)	45-52	45

The best conditions for ISSR-PCR reaction were found at 50 ng of DNA, 0.6 mM dNTPs, 4 mM $MgCl_2$, 2 units *Taq* polymerase, 0.7 µM primer concentrations, and 45°C annealing temperature in total 15 µL PCR reaction volumes. Agarose gel electrophoresis shows reproducible clear bands in (Figure 28).

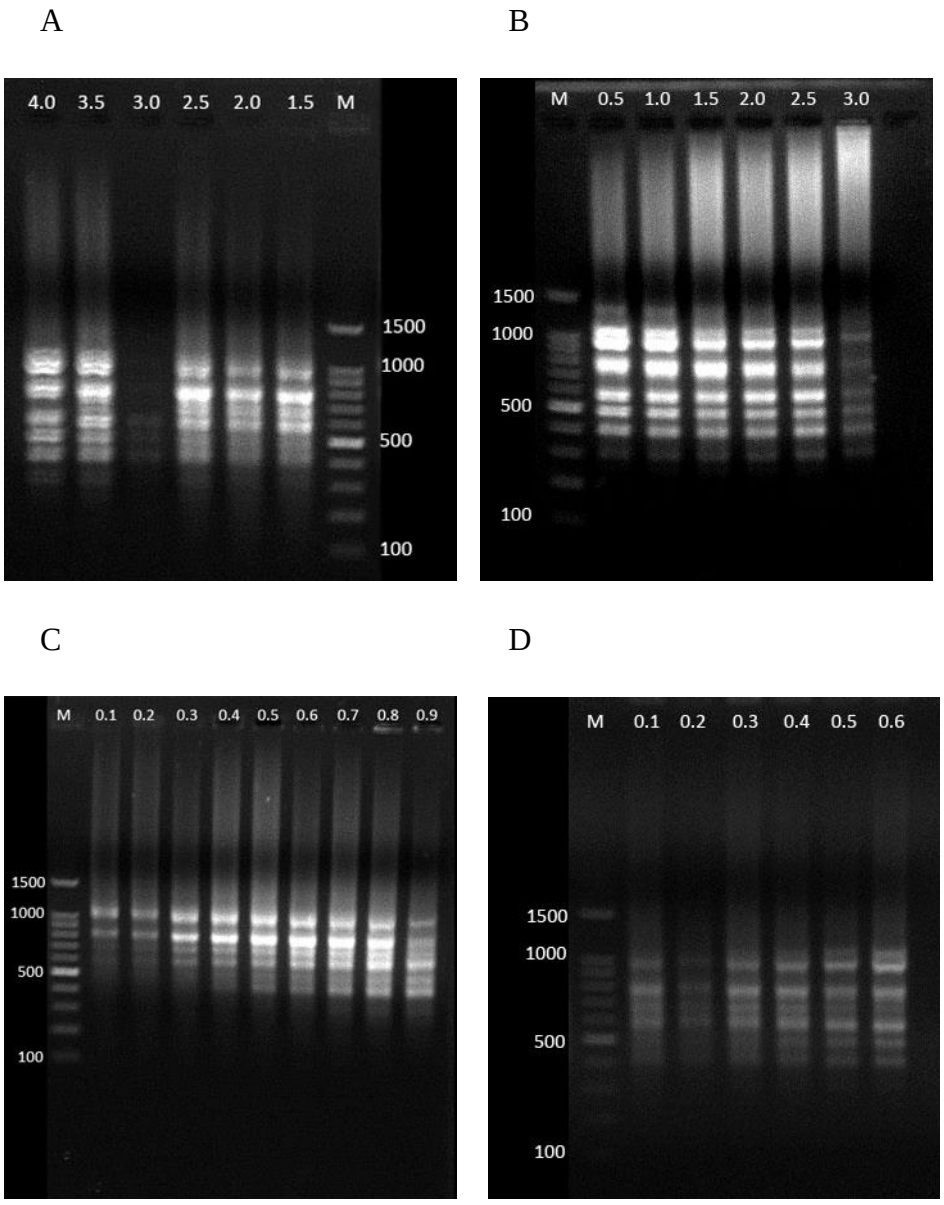


Figure 28: Optimization of DNA samples of *G. diversifolia* using the ISSR primer

Gel pictures of ISSR-PCR for the selection of $MgCl_2$, *Taq* polymerase, primer, dNTPs concentration using UBC 817 primer. Lane M- marker (100 bp); (A) Each lane is marked with the respective concentration of $MgCl_2$ (1.5mM to 4mM); (B) Each lane is marked with the respective concentration of *Taq* polymerase (0.5U to 3U); (C) Each lane is marked with the respective concentration of primer (0.1 µM to 0.9 µM); (D) Each lane is marked with the respective concentration of dNTPs (0.1mM to 0.6 mM).

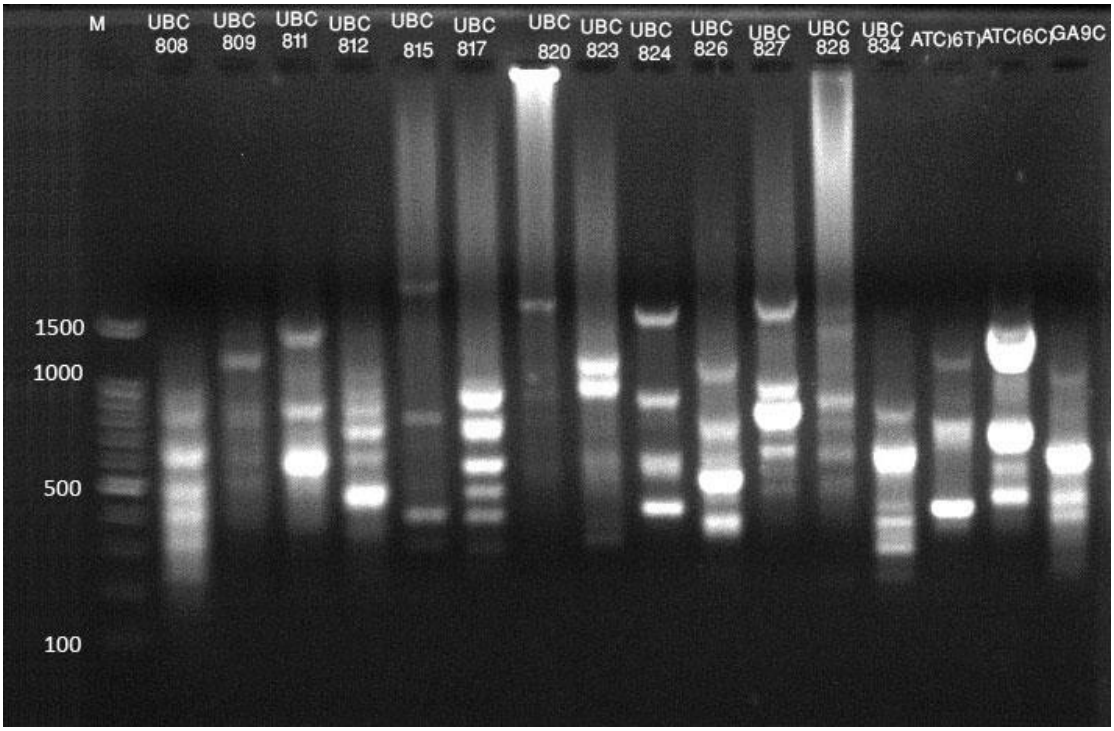


Figure 29: 16 ISSR primers test to optimize PCR condition for *G. diversifolia* subsp. *diversifolia*

M-marker (100 bp, Lane 1 UBC 808; Lane 2: UBC 809; Lane 3UBC 811; Lane 4 UBC 812; Lane 5 UBC 815; Lane 6 UBC 817; Lane 7 UBC 820; Lane 8- UBC 823; Lane 9-UBC 824; Lane 10-UBC 826; Lane 11 UBC 827; Lane 12 UBC 828; Lane 13 UBC 834; Lane 14 ATC(6T); Lane 15 ATC(6C); Lane 16 GA(9C)

Of the sixteen screened ISSR primers, ten ISSR primers showed clear reproducible bands (Table 16). These experiments were repeated twice (Figure 29). The best ISSR-PCR conditions were utilized to study genetic variation in *G. diversifolia*.

4.1.2.3 ISSR profiling and genetic diversity assessment

In this study, sixteen ISSR primers were taken based on PCR amplification as shown in *Urtica dioica* of Urticaceae family (Haghpanah *et al*., 2016). The gradient PCR program was performed to select the primer for best amplification (Table 15). Out of the sixteen primers ten UBC primers were selected to study the genetic variation of *G. diversifolia* that showed clear bands (Table 16). A total of 131 ISSR loci were amplified with an average amplification of thirteen bands per primer across 45 samples of *G. diversifolia,* 98.09% of amplified bands were found to be polymorphic (Table 16).

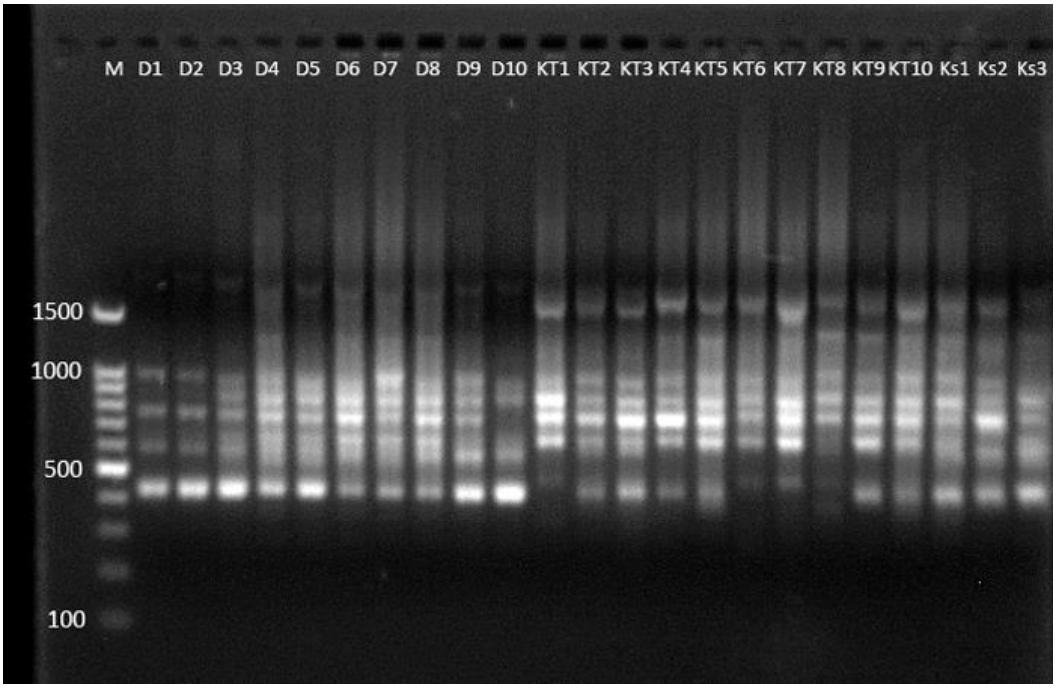
Table 16: Number of amplified DNA bands and percentage of polymorphism.

S.No	Primers	Primer sequence (3'-5')	Annealing temperature (°C)	TNB	NPB	P%
1	UBC 834	(AG)8YT	45	18	18	100
2	UBC 812	(GA)8A	52	18	18	100
3	UBC 808	(AG)8C	45	14	14	100
4	UBC 824	(TC)8G	45	12	12	100
5	UBC 811	(GA)8C	48	14	14	100
6	UBC 828	(TG)8A	45	15	15	100
7	UBC 826	(AC)8C	50	11	10	90.91
8	UBC 817	(CA)8A	52	11	11	100
9	ISSR-18	(ATC)6T	45	8	8	100
10	ISSR-19	(ATC)6C	45	10	9	90
	Total			131	129	98.09

NPB= number of polymorphic bands; TNB= total number of bands; P%= polymorphism percentage

The result of 45 samples of *G. diversifolia* using UBC 824 primer for ISSR amplification is shown in Figure 30.

A



B

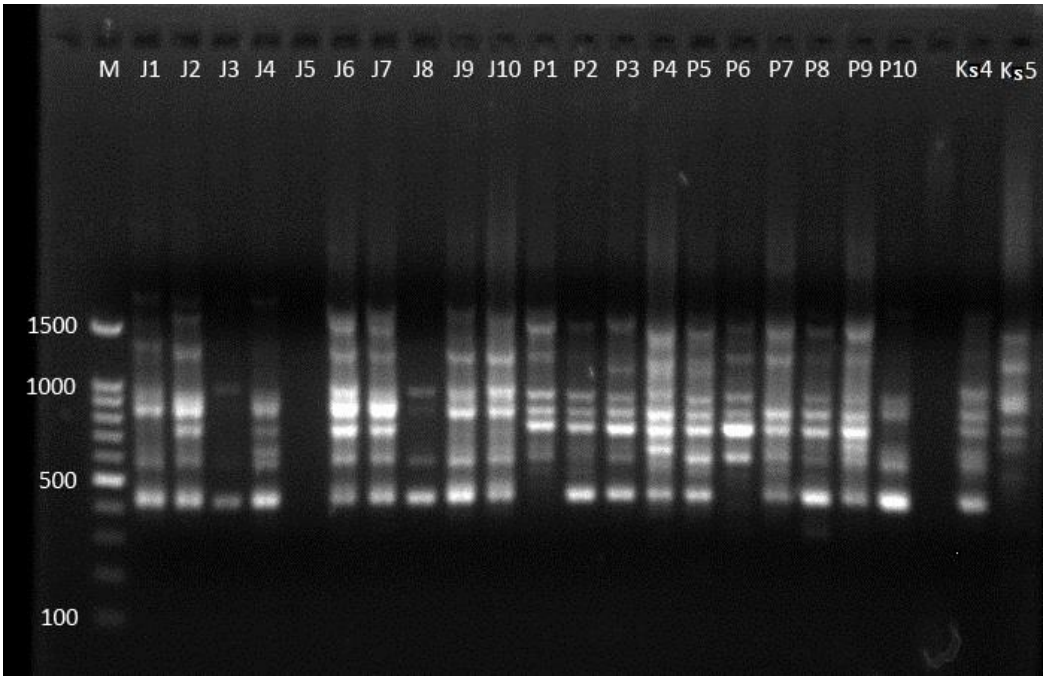


Figure 30: Primer UBC824 and ISSR amplification results (**A, B)** of 45 samples of *G. diversifolia*.

A, Lane M= DNA markers 100bp, the bands represent 100 bp to 1500 bp from top to bottom. Lane1-lane10 (D1 to D10: Samples from Darchula District); Lane 11- Lane 22 (KT1-KT10: Samples from Kathmandu District); Lane 23- Lane 24 (Ks1 to Ks3: Samples from Kaski District). B, Lane M= DNA markers 100bp, Lane1-lane10 (J1 to J10: Samples from Dolakha District); Lane 11- Lane 22 (P1 to P10: Samples from Panchthar District); Lane 23- Lane 24 (Ks4- Ks5: Samples from Kaski District).

Polymorphism information content (PIC) is measured based on the number of alleles and their distribution in the population. Ten primers selected in this study showed relatively reasonable distribution in the population of *G. diversifolia*. The average PIC value of the primer in this study was found to be 0.350.

Table 17: Assessment of PIC, $I_{B,}$ and Rp of selected ISSR primers

S.N.	Primers	PIC	I_B range	I_B average	Rp
1	UBC 812	0.266	0.08-1.8	0.454	8.177
2	UBC 834	0.252	0.04-1.7	0.429	7.733
3	UBC 808	0.381	0.2-1.5	0.663	9.288
4	UBC 824	0.414	0.3-1.4	0.770	9.244
5	UBC 811	0.396	0.3-1.7	0.742	10.400
6	UBC 828	0.428	0.2-1.6	0.794	11.911
7	UBC 826	0.272	0-2	0.892	9.822
8	UBC 817	0.388	0.4-1.9	0.913	10.044
9	ISSR-18	0.409	0.3-1.6	1.088	8.711
10	ISSR-19	0.295	0.04-1.7	0.951	9.511
	Mean	**0.350**	**-**	**0.770**	**9.484**

4.1.2.4 Similarity coefficient, cluster analysis and construction of UPGMA based dendrogram

Using binary matrix, obtained bands from gel electrophoresis, different similarity matrices were generated using binary data based on presence and absence of ISSR loci from SimQual computational algorithm of NTSYS-PC, version2.02i, Exeter software, Setauket, Newyork, USA (Rohlf, 1997). Jaccard's (J) similarity indices were obtained with range of 0.24-1.

The genetic similarity was calculated from 45 samples of *G. diversifolia* that were collected from Darchula to Panchthar Districts. Jaccard's coefficient produced five major clusters (i.e A, B, C, D, E) and had a similarity of 0.50. Cluster A comprised samples from Darchula District, Cluster B comprised samples from Kathmandu, Cluster C comprised samples from Dolakha, Cluster D comprised samples from Kaski and Cluster E comprised samples from Panchthar District. Cluster A (samples from Darchula District were further sub-clustered at the similarity coefficient level of 0.74 and 0.90); Cluster B (samples from Kathmandu District were further sub-clustered at 0.75 and 0.89); Cluster C (samples from Dolakha District were further sub-clustered

at 0.56 and 0.83), Cluster D (samples from Kaski District were further sub-clustered at 0.75 and 0.74); and Cluster E (samples from Panchthar District were further sub-clustered at 0.61 and 0.53) (Figure 31).

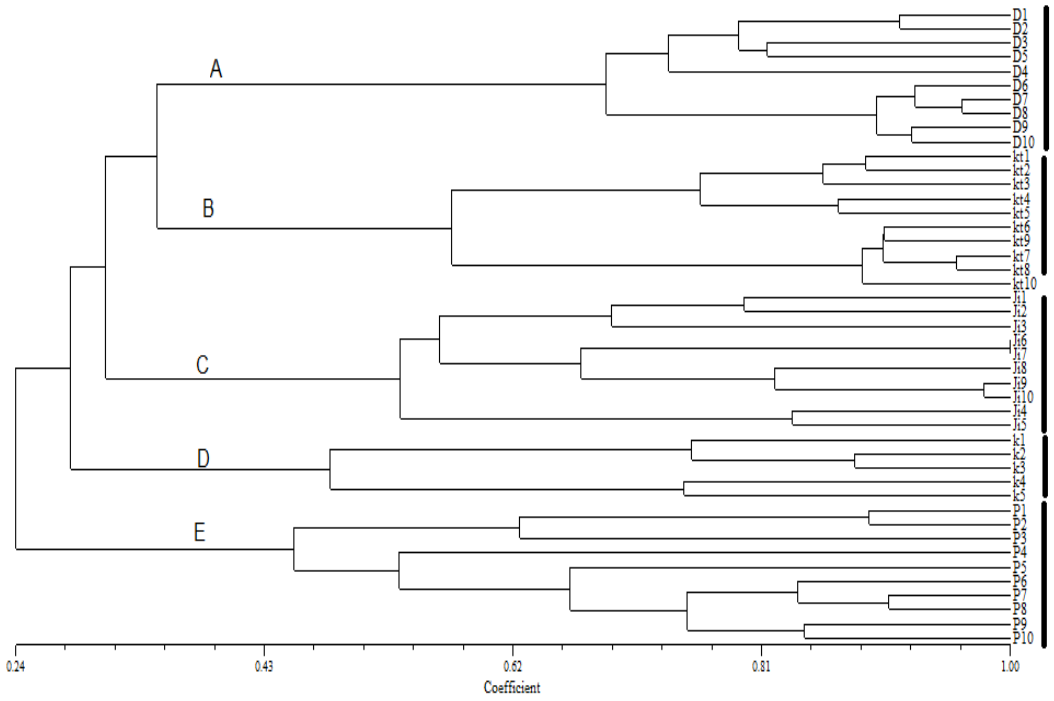


Figure 31: UPGMA cluster analysis using Jaccard's coefficient based dendrogram showing the genetic relationship among 45 samples of *G. diversifolia* using 10 ISSR primers. The clusters are labeled as A, B, C, D, E. (D1-D10) Darchula; (Kt1-Kt10) Kathmandu; (Ji1-Ji10) Dolakha; (K1-k5) Kaski; (P1-P10) Panchthar District.

4.1.2.5 Mantel test

The correlation values were obtained from the comparison of original matrix by applying Mantel test. The correlation between Dice (D) similarity and Jaccard (J) matrices has shown the highest significance (0.9909) when compared with Simple matching (SM) and J (0.96536), SM and D (0.96213), suggesting that Dice or Jaccard matrix are suitable for simple matching coefficient (Table 18).

Table 18: Correlation coefficient (2 way) from the Mantel test of original matrices.

	Simple matching	Jaccard	Dice
Simple matching	*****	-	-
Jaccard	0.96536	*****	
Dice	0.96213	0.99094	*****

4.1.2.6 3D plot

The construction of 3D plot was conducted with an analysis of Eigen vector using NTSYS-PC 2.02i for all the samples of *G. diversifolia* using Dice similarity matrix (Figure 32). Comparable dispersion and scatterness were shown from different regions of Nepal.

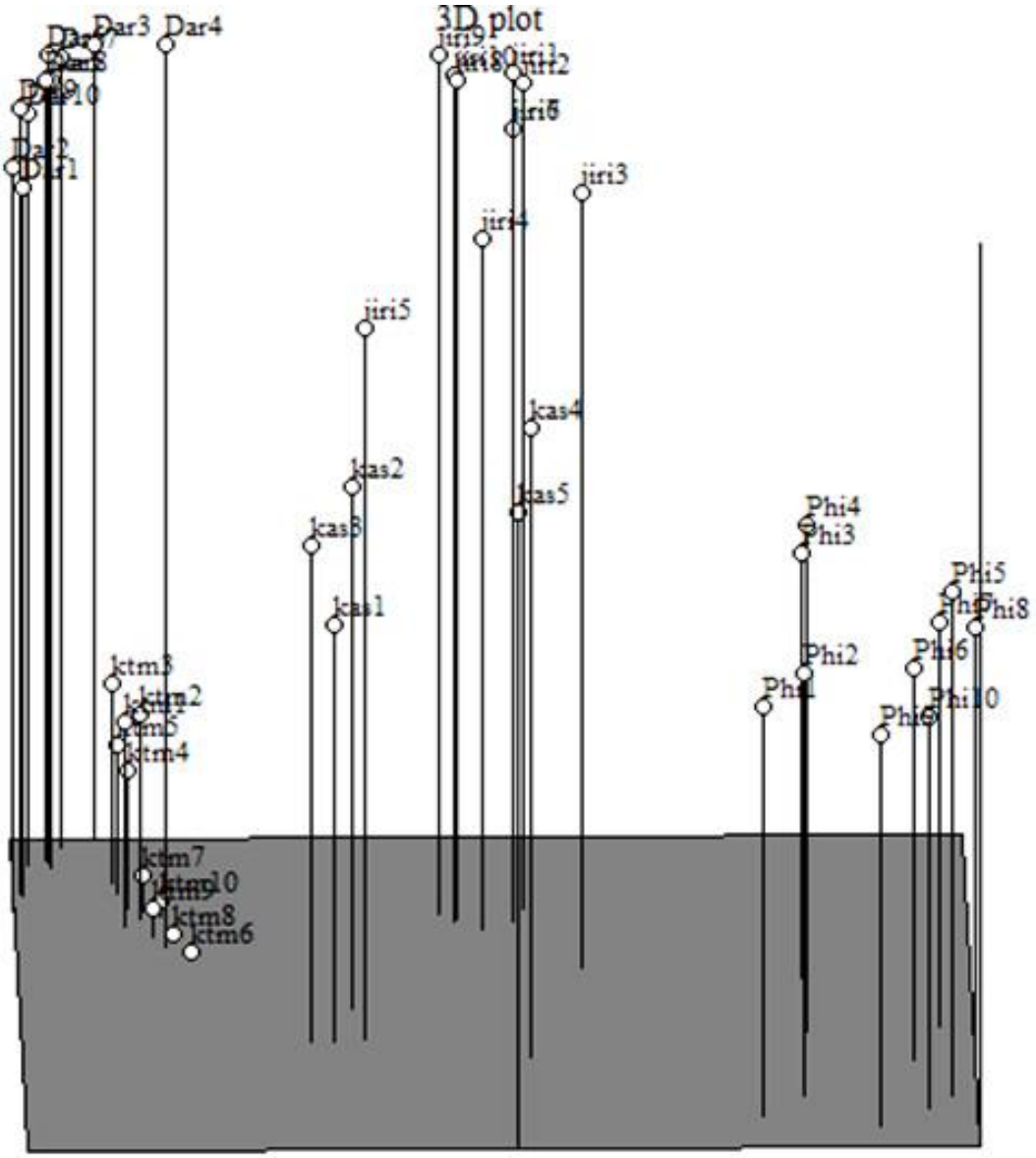


Figure 32: The 3D plot was made using NTSYS- PC (version 2.02i**)** to evaluate the dispersion of all collected samples representing Phi (Panchthar District), Kas (Kaski District); Jiri (Dolakha District); Ktm (Kathmandu District); Dar (Darchula District).

The plant samples from Dolakha District and Panchthar District have the highest genetic identity between the population (0.7489) and the shortest genetic distance (0.2891). The maximum genetic distance between the population from Darchula

District and Panchthar District was (0.4561) with minimum genetic identity was (0.6338).

4.1.2.7 Genetic variation within different populations of *G. diversifolia*

Out of selected populations, the samples from Dolakha District have the highest variation in population, with Nei's genetic diversity being 0.1928 and Shannon's information index being 0.2852. Samples of Darchula District had the lowest degree of variation in population with Nei's genetic diversity 0.110 and Shannon's information index 0.1629 (Table 19).

Table 19: Genetic variability within the population shown by POPGENE ver 1.32

Population	Sample size	Number of polymorphic bands	Percentage of polymorphic bands (PPB)	Nei's gene diversity (H)	Shannon's information index (I)
Panchthar	10	62	47.33	0.1536	0.2323
Dolakha	10	68	51.91	0.1928	0.2852
Kathmandu	10	44	33.59	0.1267	0.1872
Kaski	5	45	34.35	0.1255	0.1863
Darchula	10	38	29.01	0.1110	0.1629

Percentage of polymorphic bands (PPB); Nei's gene diversity (H); Shannon's information index (I).

4.1.2.8 Total genetic differentiation

Heterozygosity within the population (Hs) was in the range of 0.094 to 0.182 with the average of 0.141. Total heterozygosity (Ht) ranged from 0.265 to 0.408. The mean genetic differentiation (Gst) between populations over all loci was 0.594 and the average gene flow (Nm) was 0.355 (Table 20).

Table 20: Genetic variation within and between the *G. diversifolia* populations.

S.No	Primers	Ne	H	I	Ht	Hs	Gst	Nm
1	UBC 812	1.406	0.265	0.423	0.265	0.142	0.462	0.581
2	UBC 834	1.387	0.252	0.404	0.266	0.094	0.646	0.273
3	UBC 808	1.649	0.381	0.565	0.37	0.164	0.559	0.393
4	UBC 824	1.713	0.405	0.592	0.397	0.112	0.718	0.195
5	UBC 811	1.648	0.385	0.571	0.384	0.182	0.524	0.453
6	UBC 828	1.642	0.378	0.561	0.364	0.136	0.625	0.299
7	UBC 826	1.559	0.322	0.484	0.332	0.101	0.695	0.218
8	UBC 817	1.649	0.364	0.536	0.363	0.170	0.531	0.440
9	ISSR- 18	1.744	0.416	0.603	0.408	0.180	0.558	0.395
10	ISSR-19	1.581	0.327	0.489	0.347	0.130	0.623	0.302
	Mean	**1.598**	**0.349**	**0.523**	**0.350**	**0.141**	**0.594**	**0.355**
	SD	**0.118**	**0.056**	**0.069**	**0.049**	**0.032**	**0.080**	**0.119**

H= Nei's (1973) gene diversity; I=Shannon's information index; ne= effective number of alleles; Ht= Total heterozygosity/diversity; Hs=mean heterozygosity/gene diversity within population; Gst=genetic differentiation between population; Nm=gene flow.

4.1.2.9 Analysis of molecular variance of *G. diversifolia*

The partitioning of variations within and among populations of *G. diversifolia* was analyzed by AMOVA, which revealed that 60% of the total genetic variation was among the populations and 40% of the total genetic variation was within the populations. The P value <0.001 was statistically significant for the difference within and among the populations (Table 21).

Table 21: Analysis of molecular variance (AMOVA)

Variation	Degree of freedom (DF)	Sum of Square (SS)	Mean sum of square (MS)	Estimated variance	Total variance	P value
Among population	4	593.856	148.464	15.540	60%	< 0.001
Within population	40	413.300	10.333	10.333	40%	<0.001
Total	44	1007.156	-	25.872	100%	

Sum of Square (SS), Degree of freedom (DF), Mean sum of square (MS), Estimated variance, percentage of variation and significance based on permutation across the full data set.

4.1.2.10 Principal coordinate analysis (PCoA)

The data obtained using ten ISSR primers were used in principal coordinate analysis (PCoA) using Jaccard's coefficient of similarity. The first PC1, PC2, PC3 described a total variation of 52.73 with 22.07, 17.28, and 13.38 variations respectively. The group of individuals using two coordinates are shown in Figure 33. PCoA analysis classified genotypes into four different groups.

Principal coordinate analysis (PCoA) confirmed the clustering of 45 *G. diversifolia* accessions into clearly distinguishable five main populations using 10 ISSR primers Table 22. Five distinguishable populations are from: Dar (Darchula District), Jiri (Dolakha District), Kath (Kathmandu District), Phi (Panchthar District), Kas (Kaski District).

Table 22: Principal Coordinate Analysis (PCoA) using ISSR markers

Axis	**1**	**2**	**3**
Percentage	22.07	17.28	13.38
Cumulative percentage	22.07	39.36	52.73

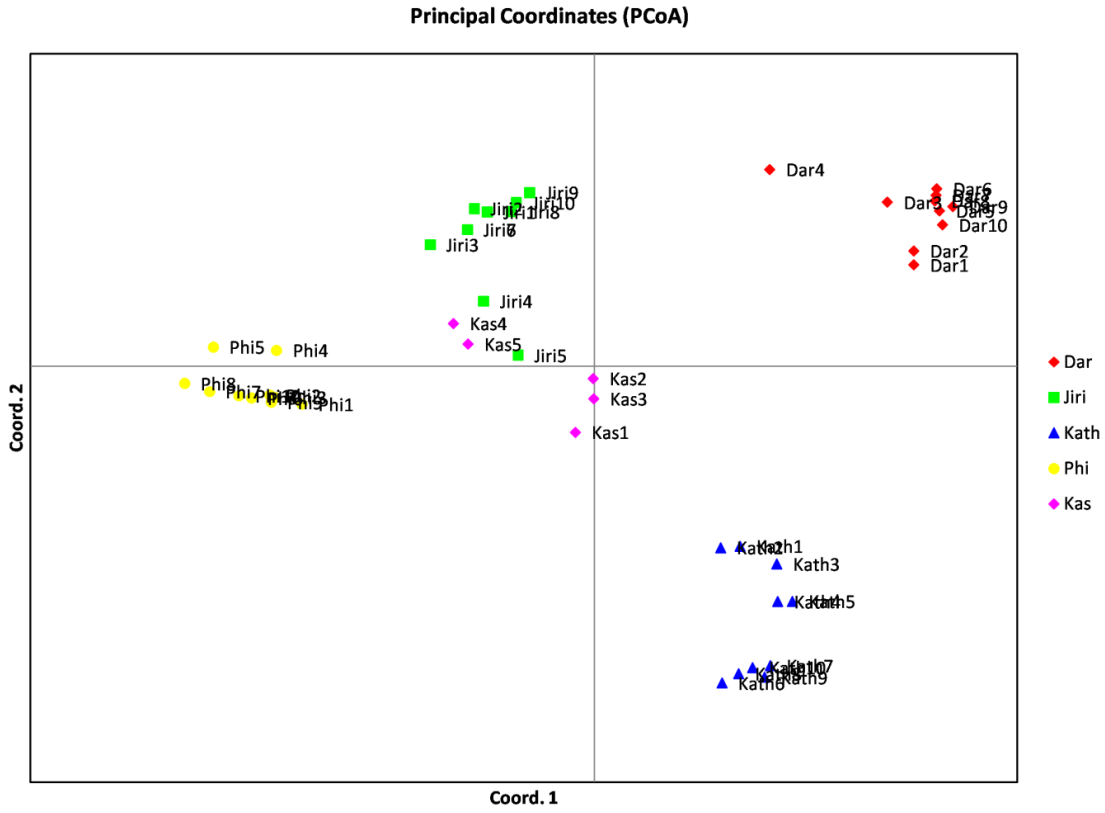
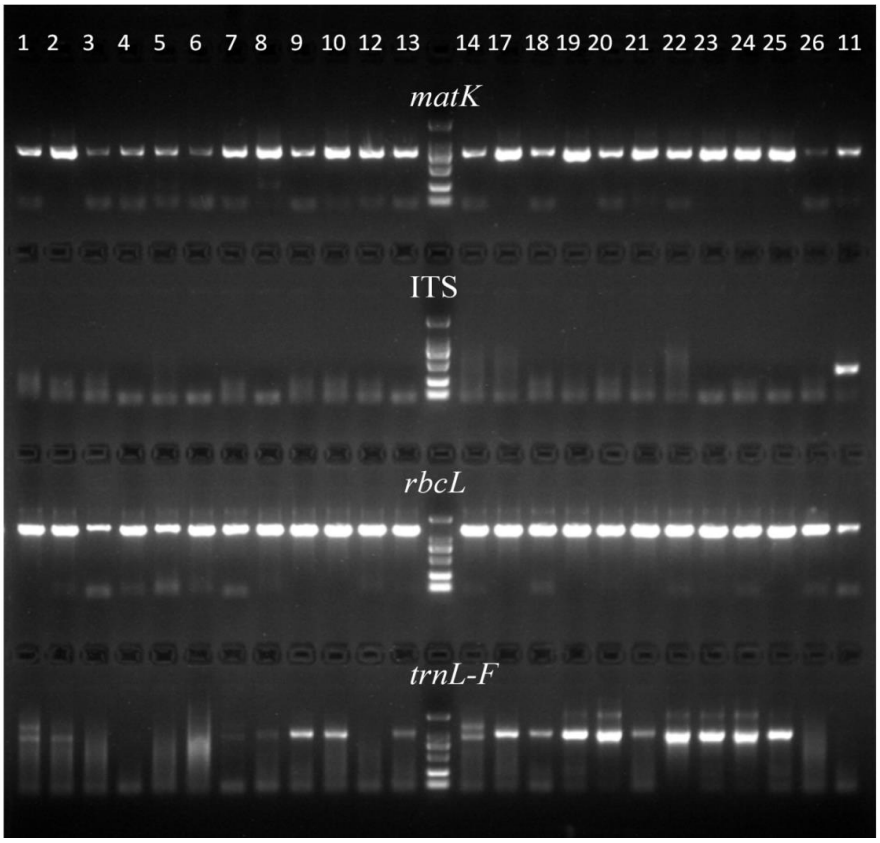


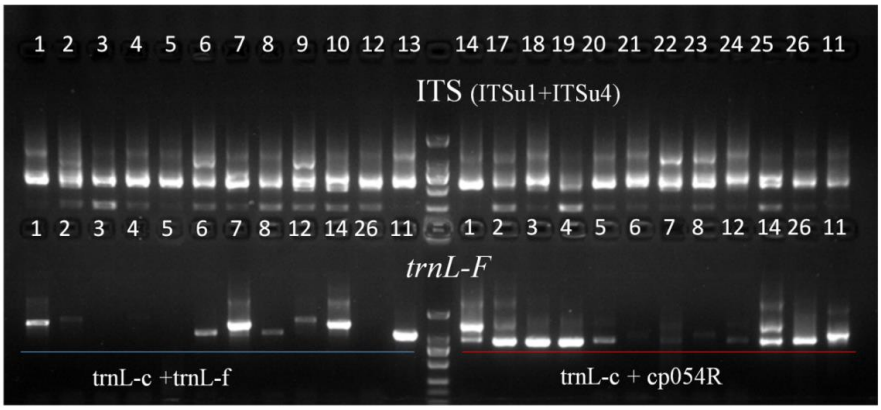
Figure 33: Principal coordinates analysis (PCoA) among 45 *G. diversifolia* samples using 10 ISSR primers: Dar (Darchula); Kas (Kaski); Kath (Kathmandu); Jiri (Dolakha); Phi (Panchthar)

4.1.3 DNA sequencing and phylogenetic study

The samples to be sequenced were selected based on the intensity of DNA band observed. The accessions after decoding from 1234...., (Pan1, Pan2, Pan3, Pan4, Pan5) were selected from Panchthar District, Ktm1, Ktm2, Ktm3, Ktm4, Ktm5 were selected from Kathmandu District, and Dar1, Dar2, Dar3, Dar4, Dar5 from Darchula District (Figure 34).



(A) Amplifying *matK*, ITS, *rbcL*, *trnL-F* markers for 24 samples



(B) ITS and *trnL-F* genes for samples of *G. diversifolia*.

Figure 34: (**A**) Amplifying *matK*, ITS, *rbcL*, *trnlF* markers for 24 DNA samples of *G. diversifolia*, (**B**) Amplifying ITS and *trnL-F* genes.

4.1.3.1 BLAST results

All sequenced samples were performed using Basic Local Alignment Search Tool (BLAST) to obtain sequences that produce the significant alignments. Sequences samples showed 98% to 99% identification with *G. diversifolia* subsp. *diversifolia* voucher GZ160713-6 with the total score value ranging from 1158 to 2290 and E value -0. Similarly, DNA sequences of *rbcL* region were BLAST. Since, there were not any verified sequence databases of *rbcL* gene of *G. diversifolia*.

DNA sequence of ITS showed 98% identification with *G. diversifolia* voucher GZ160713-6, accession no KY425766.1. Similarly, *matK* sequence showed 100% identification with *G. diversifolia* subsp. *diversifolia* isolate Dt_096 maturase K (*matK*) gene, accession no MK931205.1. DNA sequences of *trnL-F* region showed 100% identification with *G. diversifolia* subsp. *diversifolia* isolate G31 tRNA-Leu (trnL) gene with total score of 1559 and E-value O (Table 23).

Table 23: BLAST results obtained from consensus sequences for identification

DNA region	**Query samples**	**Identity with**	**Total score**	**Query Cover %**	**E-Value**	**Identify %**	**Accession no**
ITS	Dar1, Dar5	*G .diversifolia* voucher GZ160713-6	1166	100	0	98.07	KY425766.1
	Ktm1, Ktm2, Ktm 3, Ktm4, , Ktm5	*G. diversifolia* voucher GZ160713-6	1164	100	0	98.07	KY425766.**1**
	Pan1, Pan2, Pan3, Pan 4, Pan 5	*G. diversifolia* voucher GZ160713-6	1164	100	0	98.07	KY425766.**1**
matK	Dar1, Dar2, Dar3, Dar 4, Dar5, Ktm1, Ktm2, Ktm3, Ktm4, Pan1 , Pan2, Pan 3, Pan 4, Pan 5	*G. diversifolia* subsp. *diversifolia* isolate Dt_096 maturase K (*matK*) gene	1360	100	0	100.00	MK931**20**5.1
trnl F	Dar1, Dar2, Dar3, Dar 4, Dar5, Ktm1, Ktm2, Ktm3, Ktm4, Pan 1 , Pan 2, Pan2, Pan 3, Pan 4,Pan 5	*G. diversifolia* subsp. *diversifolia* isolate G31 tRNA-Leu (trnL) gene,	1559	99	0	100.00	KF138336.1

4.1.3.2 Phylogenetic analysis

4.1.3.2.1 Selection of the best fit model of nucleotide substitution

Before the construction of phylogenetic trees generated from sequences of *G. diversifolia* accessions of different populations from Nepal, the best fit model of nucleotide substitution was selected for ITS, *matK*, *rbcL*, *trnL-F* sequences using Jmodel test 2.1.4 (Darriba *et al*., 2012).

The lowest BIC score was found to be 6069.153 for T92+G (Tamura 3 parameter) for ITS sequences-based tree construction. Likewise, *matK* sequences-based tree construction showed the lowest BIC score i.e. 4051.263 for T92 parameter. For *rbcL* based tree construction BIC score was found to be 5479.45 for T92+G parameter. Similarly, *trnL-F* sequences-based tree construction showed the lowest BIC as 5389.145 T92 (Table 24). Therefore, Tamura 3 parameter was selected as the best model of nucleotide substitution.

Table 24: Best fit model of nucleotide substitution for sequences of four different DNA regions

	ITS	*matK*	*rbcL*	*trnL-F*
Best model	T92+G(6069.15)	T92(4051.26)	T92+G(5479.45)	T92(5389.14)

Where, T92 = Tamura 3parameter

+G = Gamma distribution

4.1.3.2.2 Phylogenetic tree construction

MatK gene segment was highly conserved, there was a single substitution mutation (A-T). Samples from Kathmandu had 'T' (Thymine) instead of 'A' (Adenine) from the rest of the samples (Figure 35).

Sequences from NCBI and sequences from our study showed the sequences under the Genus *Girardinia* formed three clades (Figure 36). The *G. diversifolia* sequences from Kathmandu and Panchthar of Nepal formed a clade with the subspecies *G. diversifolia* subsp. *diversifolia*. Another clade was formed from the sequences of subspecies *G. diversifolia* subsp *triloba*, whereas the third clade was formed from sequences of *G. diversifolia* subsp. *suborbiculata*.

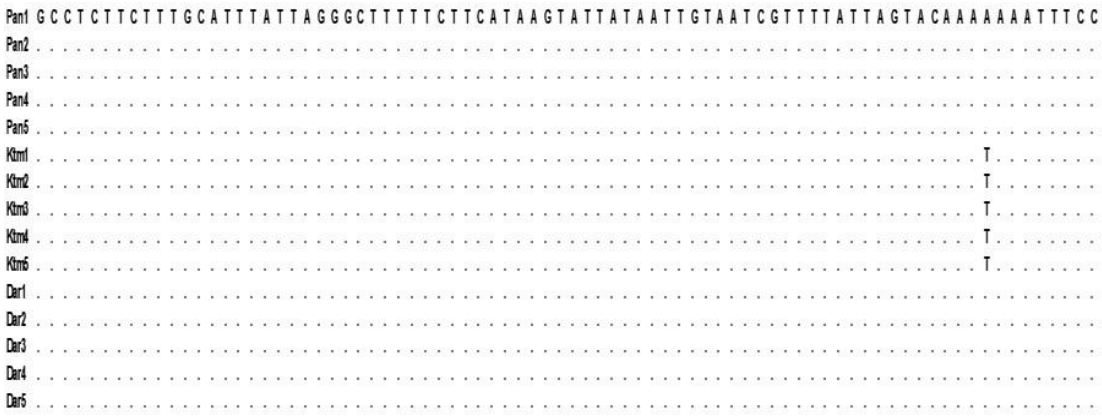


Figure 35: Variable sites of *MatK* region among the samples of *G. diversifolia* using multiple sequence alignment.

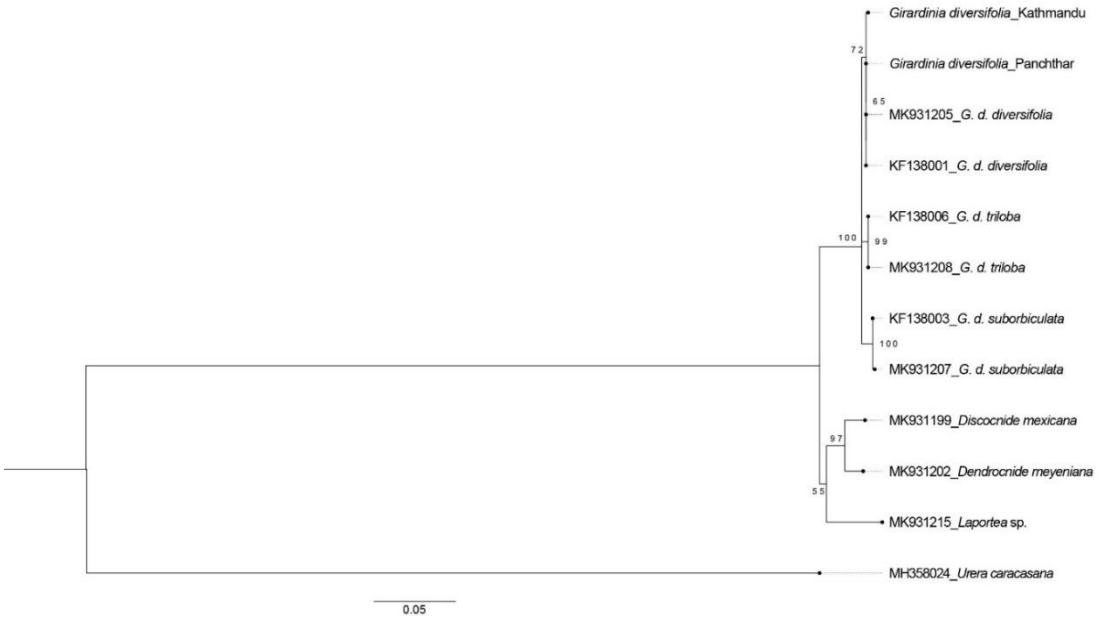


Figure 36: Maximum likelihood (ML) tree for *matK* gene (736 bp). Node values represent RAxML percentage bootstrap probability.

4.1.4 Fiber processing

Spray retting method (SRM) was used to extract fiber from the bark of *G. diversifolia*. There was no need for steam treatment and high temperature boiling and fiber cooking, which ultimately saved fuel. Foulk (2001) has suggested that normal atmospheric conditions in SRM are conducive to enzyme penetration and fiber retting.

Using the spray retting method (SRM), the effects of various chemical and enzymatic retting agents on the fiber were studied, the results of which are described below.

4.1.4.1 Spectroscopic characterization using fourier transform infrared spectroscopy (FTIR).

The FTIR spectra of treated and untreated fiber of *G. diversifolia*, position and probable assignments using different retting agents are given in Figure 37 and Table 25.

The peak at 3313 cm^{-1} shows hydroxy group, H- bonded, O-H stretch which is the characteristics of hydroxy compound group frequencies and alcohol (Coates 2000 & Kumar *et al*., 2017). The peak at 2927 cm^{-1} is due to aliphatic alkane or alkyl group frequencies showing methyl/methylene C-H stretching and bending vibration of cellulose and hemicellulose constituents of the fiber (Kale *et al*., 2018). The peak at 1647 cm^{-1} corresponds to alkenyl C=C stretch, primary, secondary amine NH bend. Peak at 1558 cm^{-1} corresponds to carboxylic acid groups of hemicellulose, aliphatic nitro compounds. The peak at 1319 cm^{-1} corresponds to aromatic primary, secondary, and tertiary amine C-N stretch. The peak at 1026 cm^{-1} corresponds to cyclohexane ring vibrations, C-F stretch, aliphatic phosphates (-P-O-C-) stretch, and primary secondary C-N stretch. The peak at 894 cm^{-1} corresponds aromatic C-H out-of-plane bend, 1,3-disubstitution (meta), aromatic phosphate (-P-O-C) stretch, β-glucosidic linkages in hemicelluloses, cellulose and =C–H bending alkenes (Kumar *et al*., 2017). The peak at 779 cm^{-1} corresponds aromatic C-H out-of-plane bend. The peak at 667 cm^{-1} shows aromatic C-H out-of-plane bend, aliphatic bromo compounds, C-Br stretch, thioethers, and alcohol. Infrared transmittance peaks (frequency number cm^{-1}) of fiber constituents obtained after treatment with given chemical are given in Figure 37 and Table 25.

The reduction of the peak intensity at 1650- 1640 cm-1 in NaOH and ENZ/CH treated fiber indicates that hemicellulose component is reduced by alkalization and enzyme/ chelator. The peak at 1647 cm-1 in untreated fiber showed hemicellulose constitutes the bonded water. Peak disappearance in ENZ/CH and NaOH treated fibers and shifting of peak at lower number in PENZ/CH indicates removal of bonded water from hemicellulose. The peak at 1319 cm-1 showed that in all the treated fibers except NaOH treated fiber, hemicellulose can be reduced using alkali in this fiber (Sim et al., 2012).

The wavenumber of the peak positions for different functional groups is presented in Figure 37 representing untreated natural fiber (NAT), NaOH treated (NaOH),

pectinase enzyme treated (ENZ), pectinase enzyme with chelator (ENZ/CH) and crude papaya enzyme with chelator (PENZ/CH) respectively.

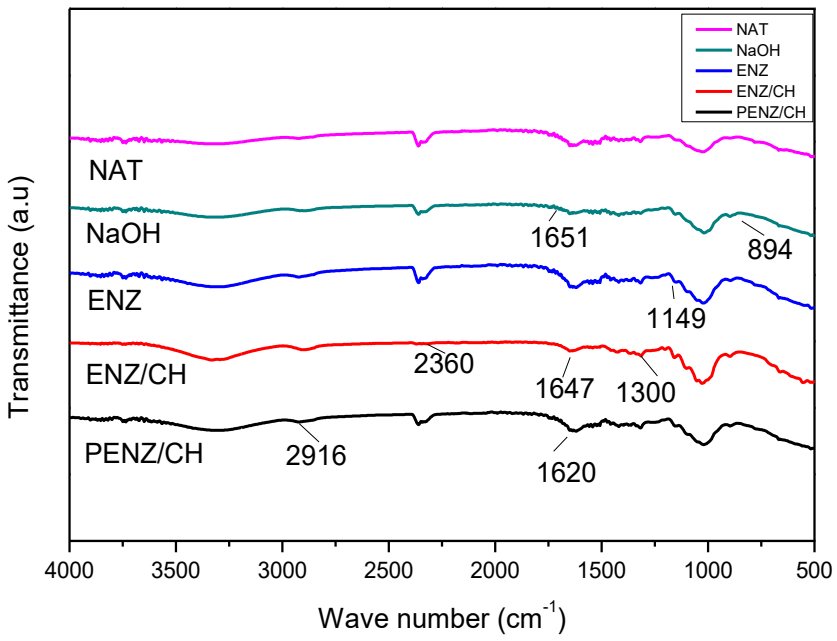


Figure 37: FTIR spectra of untreated (NAT); Sodium hydroxide (NaOH); Pectinase enzyme (ENZ); Pectinase/EDTA (ENZ/CH); and enzyme from papaya pericarp/EDTA (PENZ/CH) treated fiber.

Table 25: Infrared transmittance peaks of fiber constituents (frequency number cm^{-1})

Functional group	**NAT**	**ENZ/CH**	**NaOH**	**PENZ/CH**	**ENZ**
-OH	3313	3332	3329	3325	3332
C-H	2927	2897	2900	2916	2920
C=C	1647	-	-	1620	1620
C-C, N-0	1558	1423	1419	1419	1419
C-F, C-N	1026	1026	1018	1022	1022
=C-H		898	894	894	894
-OH		555	-	667	667

4.1.4.2 XRD analysis of untreated and treated nettle fiber

Structure of cellulose in fiber can be determined by observing crystalline and amorphous regions using XRD (Mwaikambo & Ansell 2002, Zhao *et al*., 2007). The use of XRD has mostly been used to evaluate fiber containing cellulose as a major component (Park *et al*., 2010). X -ray diffractograms of treated and untreated nettle fiber are shown in (Figure 38). All the samples showed characteristics peak of cellulose I ($22 \geq 2\theta \leq 23$) which corresponded to cryst Allographic plane 002 (Sawpan *et al*., 2011); another peak at 15.3 corresponded to cryst Allographic plane 101 (Cao & Tan, 2005). The measured crystallinity index (CI) showed the highest crystallinity in NAT (91%). Among the treatments, PENZ/CH (87%) treatment was observed to have highest CI followed by NaOH (84%), ENZ (84%), ENZ/CH (79%). The highest CI observed was that of NAT, followed by all the treatments. (Figure 38).

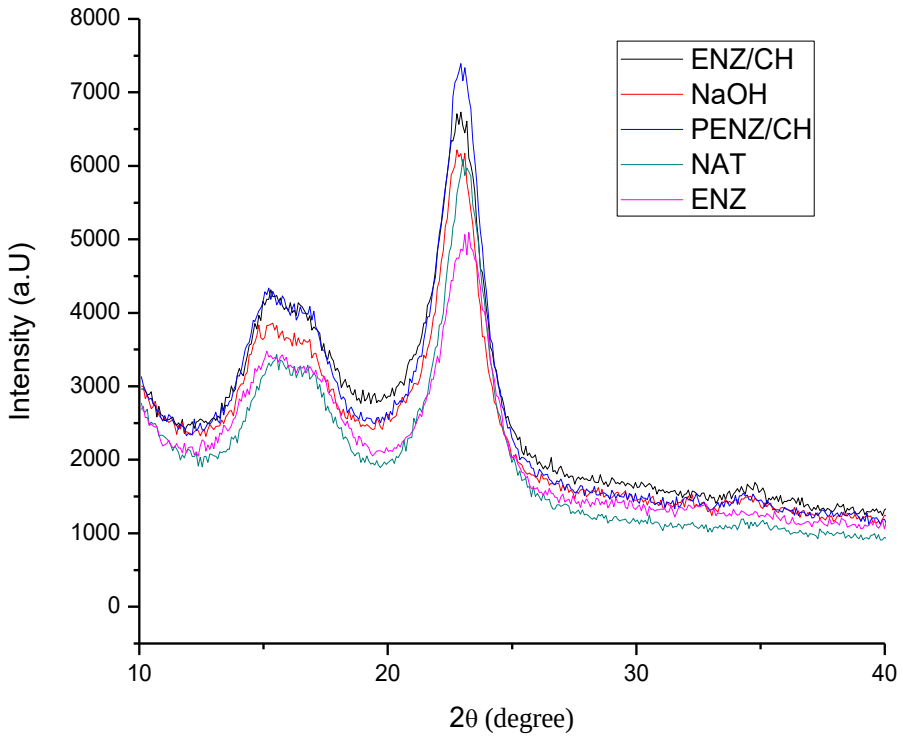


Figure 38: X-ray diffractogram of untreated and treated *G. diversifolia.*

4.1.4.3 Optical Microscopy

Photographic documentation of the fibers was summarized using macro-photographic images and micrographs by means of optical microscopy in the figures (Figure 39 and

Figure 40). The clear differences in shape (see Figure 39, A) untreated fiber, colour (Figure 39, B) and overall fineness between the differently treated fiber (see Figure 39, B,C,D,E). The fineness can be assessed in more detailed by optical micrographs in Figure 40. Few samples (sample B, D, E) were characterized by fine morphology where most fibers were separated (see Figure 40). The qualitative assessment of the morphology of samples are: A, very coarse; B, most fiber separated; C, fibers partly separated; D, most fiber separated; E, fibers partly separated. The fiber treated with NaOH and ENZ/CH are mostly separated than ENZ and PENZ/CH treated fiber.

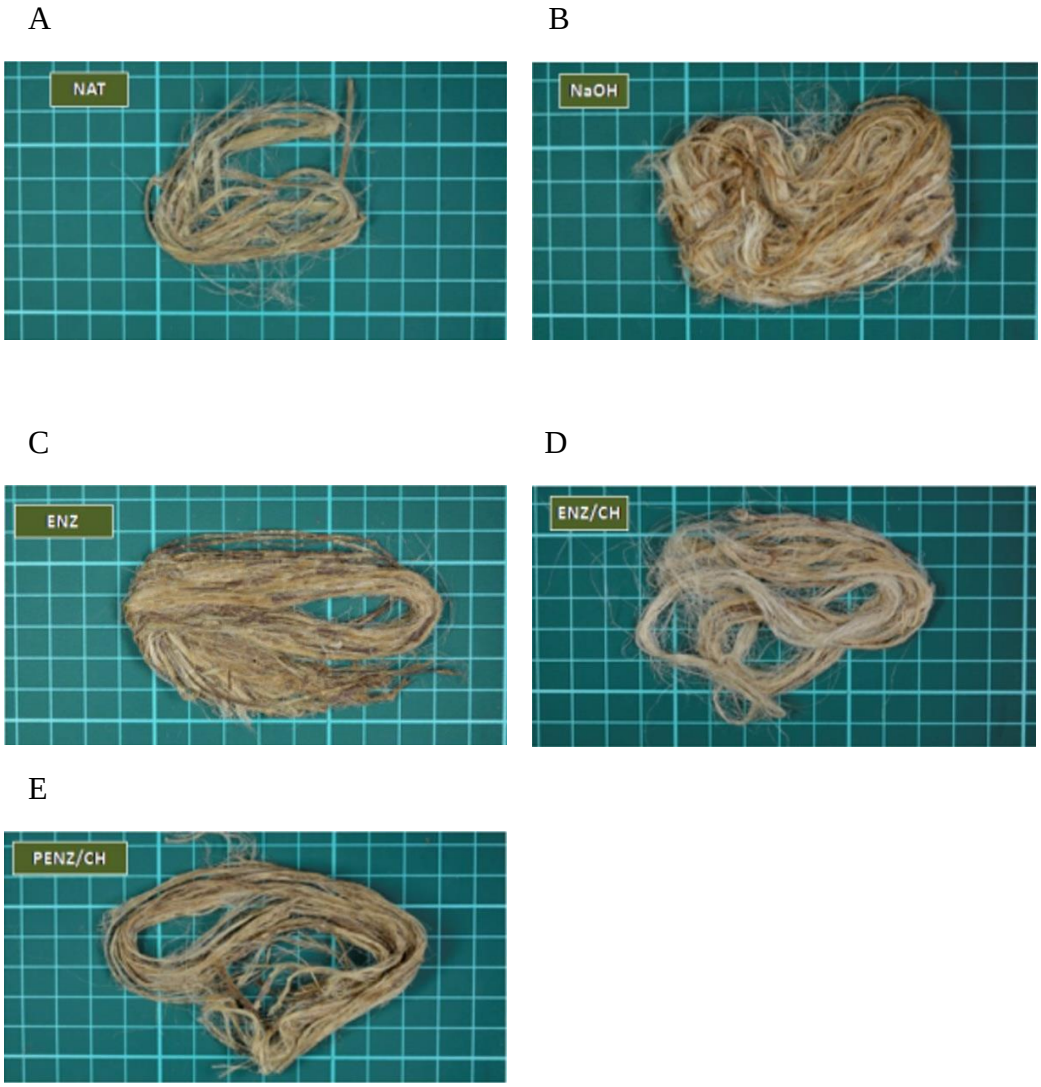


Figure 39: Macroscopic pictures of differently treated fiber; the scale of the raster is 1 cm × 1 cm A: NAT; B: NaOH, C: ENZ, D ENZ/CH, E: PENZ/CH)

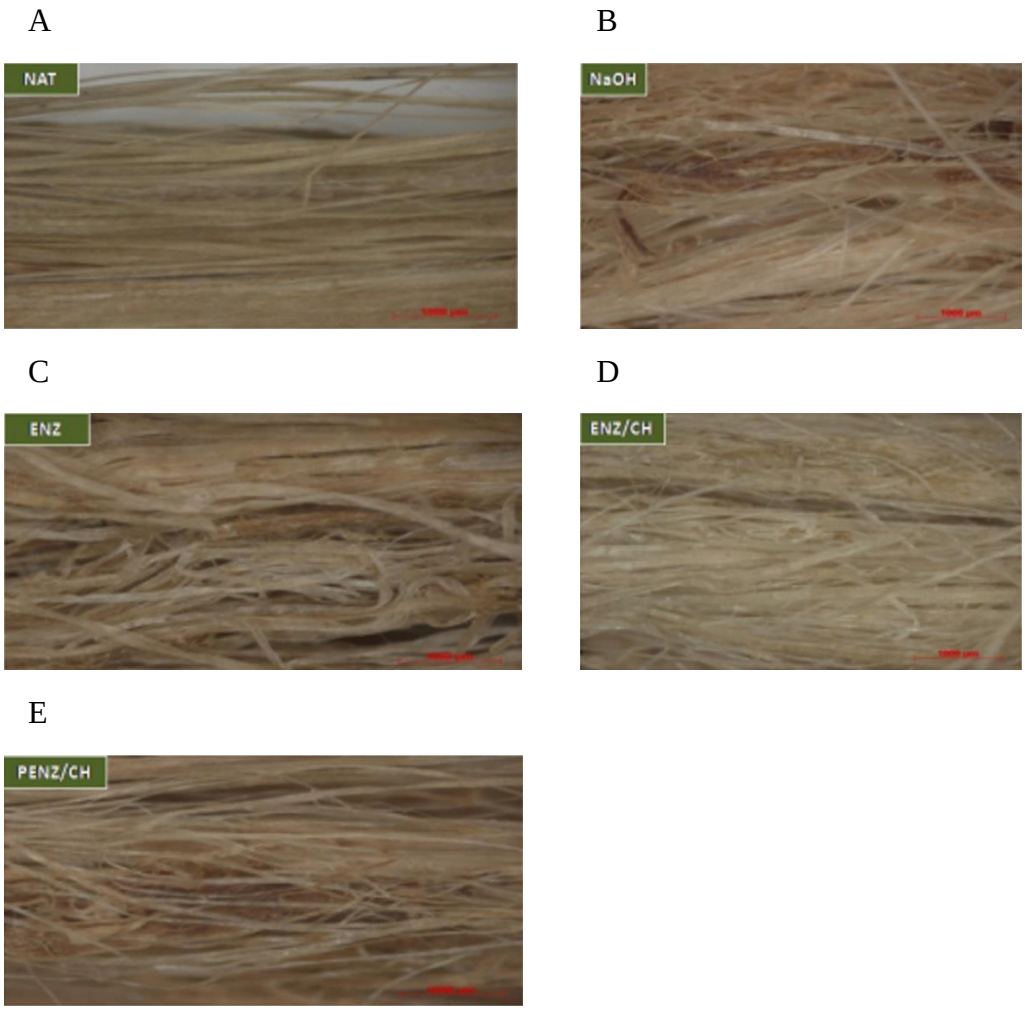


Figure 40: Micrographs of fibers retting in sodium hydroxide, pectinase only, pectinase/chelator, Papaya extract/chelator. A: NAT; B: NaOH, C: ENZ, D ENZ/CH, E: PENZ/CH)

4.1.4.4 Uniaxial tensile testing

The mass- related load vs. strain diagrams of the collected samples are summarized in (Figure 41). As typical for irregular natural fibers the shape and size of the diagrams scatter to a high degree which may mask possible relationships between the mechanical properties and the morphology of the fibers and fiber preparation (Figure 41). Mass-related load vs. strain diagrams from uniaxial tensile testing treated with NaOH (Figure 41B) showed less scattered graph. This means fiber treated with NaOH showed relatively regular tensile strength than other treated fibers.

Mass of fiber bundles is summarized below (Table 26). The average mass of ENZ/CH treated fiber was found highest 39.14 mg than fiber treated with ENZ (30.84 mg), NaOH (30.72 mg), PENZ/CH (23.8 mg) and NAT (19.64 mg).

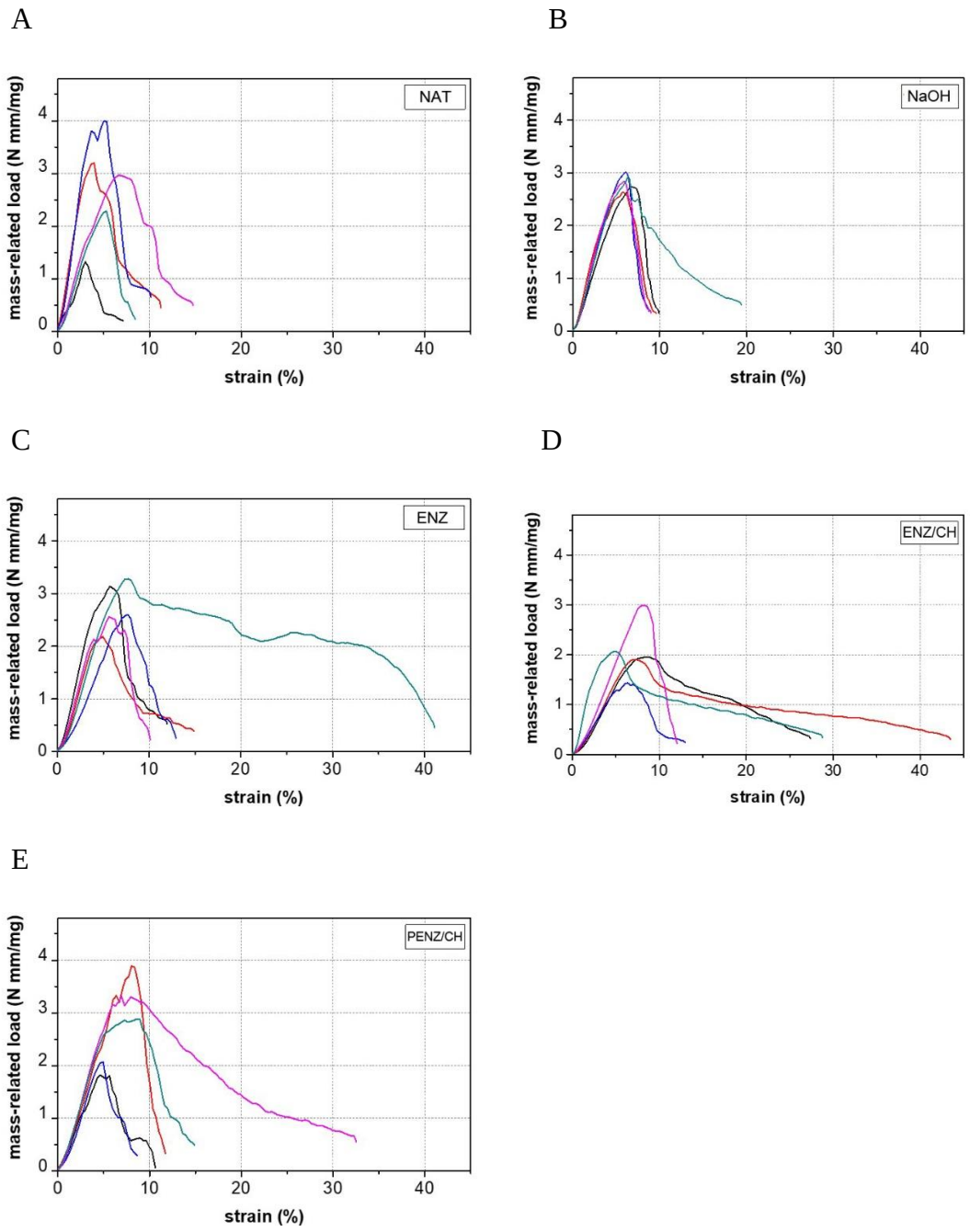


Figure 41: Mass-related load vs. strain diagrams from uniaxial tensile testing. A: NAT; B: NaOH, C: ENZ, D ENZ/CH, E: PENZ/CH)

Two parameters were collected from the mass-related load vs. strain diagrams as summarized in Figure 42. The mass-related tensile strength is related to the maximum load and the mass-related elastic modulus is calculated as the maximum slope of the mass related load vs. strain diagrams before passing through the maximum. The mean values are given in Table 27.

Table 26: Mass of the fiber bundles

No.	Mass (mg)				
	NAT	**NaOH**	**ENZ**	**ENZ/CH**	**PENZ/CH**
1	9.1	32.5	30.5	43.6	24.9
2	12.8	33.1	37.0	44.5	16.1
3	11.9	28.7	23.8	36.5	20.1
4	34.6	29.9	30.6	27.9	31.4
5	29.8	29.4	32.3	43.2	26.5
Average	19.64	30.72	30.84	39.14	23.8

From Figure 42 A, it is clear that most single tensile strength modulus data points are located in well-defined scatter band. Furthermore, as shown in Figure 42 A and Table 27, the mean data of most fibers are scattered in a narrow range close to 2.77 N mm/mg for the mass-related tensile strength and 62.8 N mm/mg for mass-related elastic modulus respectively. The average tensile strength 2.83±0.15 (N mm/mg) and elastic modulus 68.4±9.4 (N mm/mg) of NaOH treated fiber showed highest (Figure 42 B), compared with other treatments such as enzyme with chelator D, (ENZ/CH); A, (NAT); C, Enzyme only (ENZ); and crude papaya enzyme with chelator (PENZ/CH). This means NaOH treated fiber possesses highest tensile strength than other treated fiber.

A B

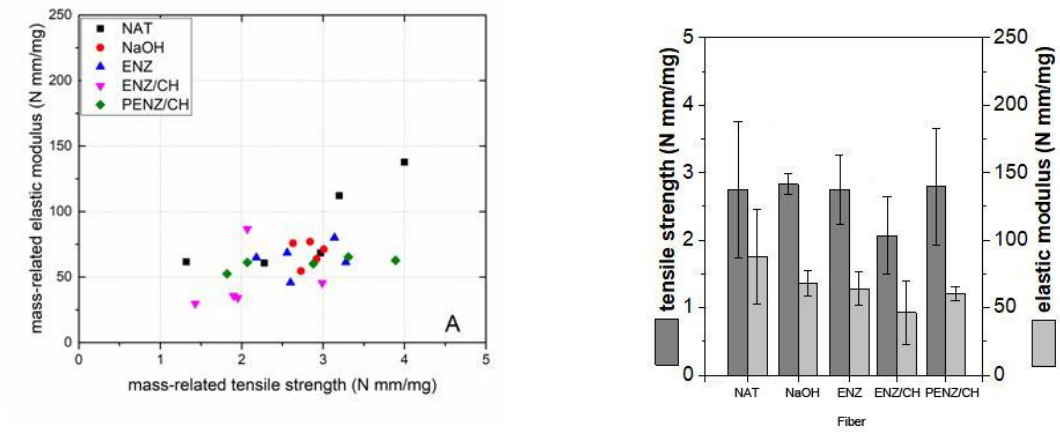


Figure 42: Uniaxial tensile testing (A) scatter band, (B) Mass-related tensile strength N mm/mg, and mass related-elastic modulus N mm/mg.

Table 27: Mean measurement of mass-related tensile strength and mass related elastic modulus.

Symbols	Mass-related tensile strength (N mm/mg)	Mass related elastic modulus (N mm/mg)
NAT	2.75±1.01	88.0±35.0
NaOH	2.83±0.15	68.4±9.4
ENZ	2.75±0.51	64.0±12.4
ENZ/CH	2.07±0.57	46.4±23.4
PENZ/CH	2.79±0.86	60.4±4.8

4.1.5 Use pattern of *G. diversifolia*

4.1.5.1 Cultural use of *G. diversifolia* by indigenous peoples of Nepal

Rai community particularly Kulung Rai firmly believes that *G. diversifolia* is associated with the creation of the world. They do have a distinct use of clothes prepared from *G. diversifolia* in different occasions. Like in the rite for god "Nagi" they offer the cloth made from *G. diversifolia* (Dougal, 1973). In the marriage ceremony, at the time of entering groom's house and in the funeral ceremony too *G. diversifolia* cloth is one of the essential items. The traditional green and red colour stripped *G. diversifolia* fabric called "lalachaar" or "lukspa" is used by Shaman's during rituals (Figure 43). During funeral rites of Kulung Rai, dyed *G. diversifolia* cloth is the important requirement.

The materials required for dyeing *G. diversifolia* cloth are known only to a few older people, who maintain purity during funerals. Even if the corpse is wrapped after dyeing the cloth, the cloth should be taken out before cremation. Natural dye materials for textiles can also provide market opportunities for natural fibers. Rai communities use *G. diversifolia* as fodder for livestock.

Figure 43: Fabric of naturally dyed *G. diversifolia*

Fiber of *G. diversifolia* has also been found to be used in weaving in other parts of Nepal e.g., Gurung community living in the Annapurna Conservation Area (ACA) prepares "Bhangra" using fiber of *G. diversifolia*. There are two types of bhangra locally made by local community in this area, such as "masino kuldu" which is soft in texture and "gardu kuldu" which is coarse in texture (Gurung *et al*., 2012). Festivals like "Tamu Losar" of Gurung community, men wear soft bhangras and white shirts, which are also used to carry luggage. They use coarse bhangras as sacks for harvesting crops, collecting firewood and gathering grass (Gurung *et al*., 2012). Chhantyal communities living in Baglung and Myagdi Districts of Nepal, exchange *G. diversifolia* cloth with surrounding villagers to develop social relations called Gharpati (house owner) and Sangina (guest). Therefore, *G. diversifolia* cloth is used as gift and is believed to bring good luck to the family (Pun, 2011).

4.1.5.2 Traditional use and processing of *G. diversifolia*

Various parts of *G. diversifolia* like roots, stems, leaves, and inflorescences are used by local healers (Vaidhya). Local ethnic communities such as Thagunna, Dhami and Bohora living in Darchula District use *G. diversifolia* to treat joint pain, gastritis, headache, asthma, and tuberculosis.

Table 28: Use of *G. diversifolia* by local communities in Nepal.

Parts used	Traditional use
Root	Root juice is used in the treatment of gastritis. The root paste is used as a medicine to treat dog bites and to reduce joint pain.
Bark	Bark of the plant is used to make various items such as "Namlo" (porter strap), bags, fishing nets, jackets, sacks and carpets. Cloth made from *G. diversifolia* is believed to have an anti-allergic effect on the skin.
Leaf	The leaf juice is used in the treatment of headaches, tuberculosis, and joint pain. It is also used as a vegetable.
Stem	To heal a broken bone, the stalks are heated and wrapped around the legs and arms.
Inflorescence	Used as vegetable.

Figure 44: Traditional technique used to extract fiber from the bark of *G. diversifolia*, and knitting equipment used in Nepal. (A) Harvest from the forest. (B) Cooking of *G. diversifolia* fiber in iron drum. (C) Drying of fiber. (D) Spinning of fiber. (E) Weaving technique in wooden loom. (F) Finished products (Source: Subedee *et al*., 2020)

People of Nepal use locally available material to extract fiber from the stem of *G. diversifolia*. The processing practices and equipment used are listed in Table 29.

Table 29: Traditional processing equipment, materials, and technologies for *G. diversifolia* fiber used by the local communities of Nepal.

Harvesting	*G. diversifolia* is harvested from community forests and national forests using a variety of iron sickles (Hasiya, Khurpa, Khukuri). Porter straps (namlo) made from *G. diversifolia* are used by the locals to carry heavy loads.
Wrapping cloth	Inoperative clothes are used to remove the bark from *G. diversifolia* due to the presence of stinging hairs.
Cooking drum	Iron drums are used to boil the bark of *G. diversifolia*. Aluminum containers should not be used because caustic soda or sodium hydroxide (NaOH) and wood ash may react with aluminum containers.
Cooking stove	The traditional three-stone stove is used to boil the bark of *G.diversifolia*, which consumes a large amount of firewood.
Wood ash	Bark soaked in water for 24 hours is mixed with wood ash and boiled for 3 to 4 hours.
Caustic soda	The bark of *G. diversifolia* is strongly bound to the pectin molecule. This non-fibrous part is difficult to remove. Caustic soda is used to remove the non-fibrous substances of the bark, about two grams of caustic soda is used in1 kg of bark.
Wooden hammer	"Mungro" the traditional wooden hammer is used to remove the non-fibrous substances of the bark.
White clay, talc (Kamero)	White clay called "kamero" is often used to soften fibers. This white clay helps in removing the non-fibrous part.
Hand spindle (Katuwa)	The hand spindle "Katuwa" is used for spinning fiber because it is portable and can be used while walking.
Handloom	Locally available species of *Prunus cerasoides, Pinus wallichiana, Dendrocalamus* species and *Quercus lanata,* are used to make handlooms.

4.1.5.3 Harvesting

Every year from October to December the local communities harvest *G. diversifolia* from the forest. Knife and hand gloves are used to remove bark (Figure 44). In some parts of far-western Nepal, people take stem from the forest to their homes, but in eastern Nepal, most of the people in Sankhuwasabha District take only bark of *G. diversifolia* from the forest.

4.1.5.4 Income generation from *G. diversifolia*

G. diversifolia is an alternative source of income for people living in Shankhuwashbha District and Darchula District. Primarily, women are involved in harvesting, processing and product design. The women sell bark, spinned fiber, bags, jackets and garments made from *G. diversifolia* in national markets (Adhikari *et al*., 2018; Subedee *et al*., 2017). Study conducted by ICIMOD (2015), Adhikari *et al*., (2018) and Subedee et al., (2017) showed that the annual income of working women in Allo sector of Darchula District was about NRs 35,000 which helped women to meet their household expenses.

4.1.5.5 Cost and benefit analysis during harvesting and processing of *G. diversifolia*

Costs and benefits were analyzed in 2017 in consultation with entrepreneurs and Allo workers of Khar VDC in Darchula District. Detailed cost analysis from harvesting to making goods from *G. diversifolia* is given in Table 30, the study showed less profit in selling dry Allo bark, about 20.45 percent loss in the selling of spinned yarn, and 64 percent more profit in selling woven Allo cloth.

Table 30: Cost analysis of *G. diversifolia* during processing, and weaving in Darchula District.

Steps	Activities	Weight (kg)	Day/man	Cost NRs	Selling price per kg)	Selling price (NRs)	Profit %	Loss%
1.	Green, fresh bark collection from forest	25	1	400				
2	Drying Allo Bark	An average of 5 kg dry bark remains after drying.			Dried bark NRs 100/kg	500	25	
3	Firewood for cooking “Allo	50 kg		400				
4	Cooking, beating, washing, and mixing with Kamero (soil)	4 kg of wool produced (1 kg gets wasted while beating)	2	800				
5	Spinning yarn	Average of 3.5 kg remains (0.5 gm get wasted while spinning)	7	2800 1600 4400	+ = 1000/kg	3500		20.45
6	Preparation of Loom		1	400				
7	Weaving of Allo Cloth	3.5 kg yarn (From 1 kg yarn 3m cloth can be produced)	4	1600 4800 6400	+ = 1000/m	10,500	64	
	Total		15	12,800		14,500	13	

4.1.5.6 *G. diversifolia* in natural habitat of Nepal

Focus group discussions, key informant interviews, and semi-structured questionnaires were used to collect the information regarding the status of *G.*

diversifolia in its natural habitat. *G. diversifolia* fiber processers, fiber collectors, and traders were invited to focus group discussions and informal meetings. Out of total of 110 respondents, 89% responded that the natural habitat of *G. diversifolia* was disturbed. Due to natural habitat destruction, the growth of plant species was found to be declining. Detailed analysis is given in Figure 45.

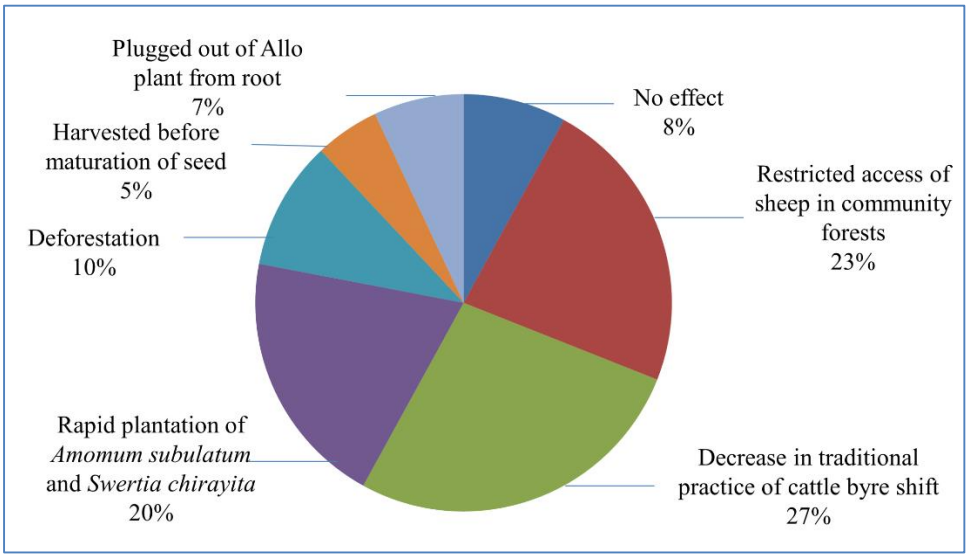


Figure 45: Causes of declining natural habitat of *G. diversifolia.*

Sheep farming is one of the alternative sources of income for the people of Darchula District. It is also the source of meat (food), wool, and manure. Sheep excrement is a good source of manure for *G. diversifolia*. *G. diversifolia* seed stuck to the sheep's wool during grazing helps in spreading seeds in forest and thus enhances plant regeneration. Sheep are still used for transportation of goods in some of the VDCs of Darchula District. Many traditional routes pass through the forests. Nowadays CFUGs do not allow sheep grazing in community forests; Thus, the lack of access to traditional grazing areas in the forest has affected sheep farming, and it had a major impact on the natural resurgence of *G. diversifolia*. Approximately 23% of respondents reported that the loss of *G. diversifolia* in natural habitat is due to the decrease in sheep numbers. Approximately 27% of the respondents mentioned that the loss of natural habitat of *G. diversifolia* is due to the decrease in traditional practice of cattle shifting. Approximately 20% of the respondents said that *G. diversifolia* habitat damage was caused by the increase in cultivation of large cardamom (*Amomum subulatum*) and Chirayito (*Swertia chirayita*) in previously *G. diversifolia* growing lands in eastern region of Nepal. About 10% of respondents reported that deforestation was the cause of the *G. diversifolia* population decline. *G. diversifolia*

grows in wet, damp and shady places; therefore, disruption of natural habitat affects the regeneration of the plant. Approximately 7% responded that the removal of the entire *G. diversifolia* plant to support the regeneration of fodder grass for domestic animals contributes to the loss of *G. diversifolia*, while about 8% responded no effect on the natural habitat. Only 5% realized the destruction is caused by the harvest of plants before seed maturation.

4.1.5.7 Community practice of resources distribution

Kulung community of Sankuwasabha District is following the calendar while harvesting *G. diversifolia*. They harvest *G. diversifolia* on special days in special months of a year. The Kulung community has formed a collection and processing group of *G. diversifolia*. Every year on the 1st day of Mangsir (mid-November) the Kulung community collects *G. diversifolia* from forest. They collect *G. diversifolia* from the forest for a week. According to collectors, the main reason for harvesting in mid-November is seed maturation, which helps in regeneration after mature seeds spread around the soil. They distribute the collected bark of *G. diversifolia* evenly to their members. However, no such community management practices have been found for *G. diversifolia* in the far west and central regions of Nepal. Disorganized collection of *G. diversifolia* has led to the degradation of natural habitat.

4.1.5.8 SWOT analysis

As the products made from Allo are likely to become popular in the national and international markets and create employment opportunities for rural people, it is important to know the strengths, weakness, opportunities, and threats of Allo value chain of Nepal, this research has done SWOT analysis. SWOT analysis showed that traditional knowledge on use of locally available material for Allo processing is a strength and immature collection, use of old equipments, use of caustic soda are some weakness and threats. The overall strength, weaknesses, opportunities, and threats for *G. diversifolia* in Nepal are listed below (Table 31).

Table 31: Strength, weakness, opportunity, and threat of Allo value chain present in Nepal.

	Strength	**Weakness**	**Opportunity**	**Threat**
Input/Equipment Supply	• Locally available resources (water, fire wood, and Ash) for Allo processing. • Handmade *Charkhas* (spinning wheels) and weaving machines made of strong wood can be manufactured locally.	• Immature collection from Allo's natural habitat. • Lack of awareness in farming practices and irregular collection from natural habitats.	• Locally available natural dyeing materials for Allo's products.	• Extreme use of caustic soda. Immature collection.
Production	• Sufficient availability of raw material in both national and community forests. • Women find opportunities to work on Allo related activities	• Allo is being harvested directly from the forest and its availability has also declined due to lack of cultivation practices.	• Can be cultivated on barren and private land. It is also possible to generate income.	• Immature and over-exploited. • There is a possibility that the plant itself will be affected when it is collected indiscriminately.
Processing	• Knowledge of processing is traditionally available. • Easy availability of water and fuel wood.	• Old equipments, lack of maintenance. • Lack of knowledge on proper use of caustic soda.	• Job creation and potential to develop entrepreneurship.	• Deforestation, forest fire, grass were saved for livestock but Allo plants were uprooted
Trade	• Demand for Allo's products has grown significantly	• Lack of quality and quantity of products to meet local and export markets.	• Allo products are getting popular in national and international markets.	• Processing techniques that are difficult to operate and time consuming. • The market price of Allo's products is very expensive.

4.2 Discussion

In the first part, the morpho-geographical analysis of *G. diversifolia* from Nepal has been discussed. In Nepal, two subspecies of *G. diversifolia* occur, these are *G. diversifolia* subsp. *diversifolia* and *G. diversifolia* subsp. *suborbiculata* (C.J. Chen) C.J. Chen & Friis; the later taxon is probably a new record for Nepal. The taxon is also probably a new record for Bhutan. Further, in the second part, genetic variation

of *G. diversifolia* subsp. *diversifolia* collected from the five populations representing eastern, central, and western regions of Nepal has been assessed. The five populations of *G. diversifolia* show a moderate degree of morphological and geographical divergence that are discussed below.

4.2.1 Morpho-geographical study of *G. diversifolia* in Nepal

During the course of scrutinizing the herbarium specimens of *Girardinia diversifolia* collected from Nepal and deposited at KATH, most of the specimens are identified as *G. diversifolia* subsp. *diversifolia* which has wide range of distribution (Chapter 4:4.1). However, one of the specimens of *G. diversifolia* was found interesting. The detailed critical study of the specimen after matching the specimen with published literatures Ambrish & Srivastava (2016); Shu *et al*. (2003), and matching with the protologue revealed that the specimen named as *G. heterophylla* belonged to an allied subspecies, i.e. *G. diversifolia* (Link.) Friis subsp. *suborbiculata* (C.J. Chen) C.J. Chen & Friis. Further, during a transboundary Kangchenjunga Landscape collaborative programme, I visited some parts of Bhutan and collected herbarium specimens of *Girardinia diversifolia*. The specimens were also identified as *G. diversifolia* (Link.) Friis subsp. *suborbiculata* (C.J. Chen) C.J. Chen & Friis.

In the previous studies from Nepal (Rajbhandari *et al.,* 2019; and Shrestha *et al.,* 2022); and Bhutan (Grierson & Long, 1983), only *G. diversifolia* subsp. *diversifolia* has been recorded. Therefore, *G. diversifolia* (Link.) Friis subsp. *suborbiculata* (C.J. Chen) C.J. Chen & Friis is probably a new record for Nepal and Bhutan, and publication based on new record of *G. diversifolia* subsp. *suborbiculata* is on pipeline. The study also confirms that characters of leaves, i.e. leaf shape, leaf lobes, leaf base, and leaf margin are good taxonomic characters to delimit the taxa into subspecies level.

It is remarkable to note that morpho-geographical characters of different populations of *G. diversifolia* subsp. *diversifolia* from five different localities of Nepal are variable and overlap with each other in terms of height and diameter of stems, leaf length (cm); number of leaf lobes; male flower bud; stamens; female flower; and seeds. They do not give stable conclusive distinguishing characters to separate the populations. However, population from Kathmandu District shows some diagnostic

characters, such as height of plant, diameter of stem and coarsely dentate leaf margin (see Table 12). This demands to further study *G. diversifolia* at the genetic level.

The average height of *G. diversifolia* is 150 cm to 300 cm (Lanzilao *et al.,* 2016; Singh & Shrestha, 1988). In the present study, the *G. diversifolia* collected from the Panchthar District has the tallest height i.e., 330 cm, and the population of the Kathmandu District having shortest height i.e., 240 cm.

Stems are herbaceous, erect, and cylindrical, woody at base, straight, branched, 5-angled, and range from 100 cm to 300 cm tall. Stem surfaces are covered with stinging hairs (Friis, 1989). There are very few studies on the stem morphology of *G. diversifolia.* Sethmann, (2004) cultivated seeds from Sankhuwasabha District of Nepal in Hamburg, Germany, and studied the stem anatomy where stems are divided into five equal segments in length. A comparative study of the stem diameter from different places in Nepal is lacking. This study compared the basal diameter of the mature stem. The average stem basal diameter of *G. diversifolia* subsp. *diversifolia* from the Kathmandu District was the highest, 37.5 mm, and the stem basal diameter of *G. diversifolia* subsp. *diversifolia* from the Panchthar District had the lowest diameter, i.e. 18.5 mm. Local communities involved in the processing and manufacturing of textile products from *G. diversifolia* prefer as well as consider that smaller stem diameters and longer lengths of stem yield for best quality of fibers. However, from our study, basal diameter and length of stem seem to be a poor predictors for population/ecotypes delimitation of *G. diversifolia* subsp. *diversifolia.*

The *G. diversifolia* has tremendous variation in leaf morphology. This has led to distinguish *G. diversifolia* into different subspecies (Friis, 1989; Shu *et al.,* 2003). The plant has various degrees of division in the leaf (Friis, 1981). *G. diversifolia* subsp. *suborbiculata* has leaves suborbicular, base rounded or truncate, leaf blades not lobed, or rarely 3-lobed, margin dentate or doubly dentate; *G. diversifolia* subsp. *diversifolia* has leaves ovate, leaf base subtruncate, 3, 5, or 7-lobed, margin regularly serrate or doubly serrate at leaf base; and *G. diversifolia* subsp. *triloba* has leaves broadly ovate to obovate, often 3-lobed, lobes triangular, terminal one 3-7 cm, lateral lobes 1.5-3 cm, base truncate or cordate, margin regularly dentate or doubly dentate margin (Shu *et al*., 2003). These results confirm previous findings by Friis (1981), Shu *et al*., (2003), Ambrish & Srivastava (2016).

Among the three subspecies of *G. diversifolia*, *G. diversifolia* subsp. *diversifolia* seems a polymorphic species with variations in the morphology of leaves. These morphological variations in leaves, in particular leaf margin, have led taxonomists to describe different taxa, and more than a dozen of synonyms have been established. Some of the common synonyms include *Urtica diversifolia* Link, Enum. Hort. Berol. 2: 385. 1822; *Girardinia chingiana* Chien; *G. condensata* (Steudel) Weddell; *G. cuspidata* Weddell subsp. *grammata* C. J. Chen; *G. diversifolia* subsp. *ciliata* (C. J. Chen) H. W. Li; *G. formosana* Hayata; *G. heterophylla* (Vahl) Decaisne; *G. leschenaultiana* Decaisne; *G. longispica* Handel-Mazzetti; *G. longispica* subsp. *conferta* C. J. Chen; *G. palmata* Blume; *G. palmata* subsp. *ciliata* C. J. Chen; *G. suborbiculata* subsp. *grammata* (C. J. Chen) C. J. Chen; *G. vitifolia* Franchet; *U. buraei* H. Léveillé; *U. condensata* Steudel; *U. heterophylla* D. Don; *U. lobotifolia* S. S. Ying; *U. palmata* Forsskål (Shu *et al.,* 2003).

The population study of *G. diversifolia* subsp. *diversifolia* shows leaf blades ovate, to oblate in outline, base cordate, or subtruncate. But, leaf lobes vary from 1 to 7 lobes; in general, young leaves are undivided, and the mature ones are 3 to 7 lobed, 10 to 14 cm long, and often are as broad as the length or slightly smaller leaf base obtuse in populations from Darchula, Kaski, Dolakha and Panchthar Districts; whereas, obtuse to truncate from Kathmandu District; margin serrate from the population of Darchula, Dolakha and Panchthar Districts or coarsely serrate, rarely coarsely dentate from the population of Kaski and Kathmandu Districts. The average length of stipules of *G. diversifolia* from Dolakha District is 2 cm, which is the longest as compared to the samples from Darchula, Kaski, Kathmandu, and Panchthar Districts which were 1 cm long. Despite of the above distinguishing characters, the leaves of *G. diversifolia* subsp. *diversifolia* are very variably divided, with various degrees of divisions of the leaves on the same plant, and are always with three major nerves from the base of the leaf blade. The variations in leaf characters collected from different populations do not allow to keep them as distinct varieties. However, some diagnostic characters among the populations from different localities have been observed where intermediary forms (such as leaf base obtuse from Darchula, Kaski, Dolakha, and Panchthar District or obtuse to truncate from Kathmandu District; leaf margin serrate from Darchula, Dolakha and Panchthar District or coarsely serrate from Kaski District or coarsely dentate from Kathmandu District) have also been observed. So, we

consider *G. diversifolia* subsp. *diversifolia* as one polymorphic taxon with no further intraspecific taxa.

Some species of the Urticaceae family only contain the pustulate trichomes in veins at the abaxial surface of the leaf such as *Urtica thunbergiana (*Fu *et al*. 2003). The trichomes of *G. diversifolia* are found in both the adaxial and veins of the abaxial surface. Fu *et al*. (2003) recorded the longest trichomes of *G. diversifolia* 5.62 mm compared with the trichomes of *Dendrocnide meyeniana, Urtica thunbergiana,* 0.37 mm, and 2.65 mm respectively. From the present study, the longest length of trichomes of *G. diversifolia* is from Panchthar District i.e., 3.7 mm and 4 mm at the adaxial and abaxial surface respectively among the collected samples from Nepal. A number of trichomes per cm^2, however, varies. For example, number of trichomes at adaxial surface is 3 per cm^2 from Kaski District. Despite, some differences in the number of trichomes, this does not provide good indicator to delineate the populations into further infraspecific taxa.

This study also resembles with the male flower having perianth 4, stamens 4, and rudimentary ovary conspicuous; female flowers 3 lobes, tubular. The achenes are broad, compressed, 2 to 3.05 mm long, and 1.55 to 2.75 mm broad; cotyledons broad, persistent stigma usually reflexed (Friis, 1981; Friis, 1993; Kim *et al*., 2015, Shu *et al*., 2003).

G. diversifolia is found in 55 districts of Nepal (Bhandari, 2019), especially in hilly areas having high humidity and cold weather. The result of the distribution pattern of *G. diversifolia* of Nepal agrees with previous results, for example, the taxon is widely distributed in tropical Africa to Madagascar (Brink, 2009), subtropical and temperate Asia (Polunin & Stainton, 1984).

Various studies mention that *G. diversifolia* subsp. *diversifolia* is found at an altitude of 1,000 m to 3, 000m above sea level (Lanzilao *et al.,* 2016; Hara *et al*. 1982; Hooker, 1992; Polunin & Stainton, 1984) and 1,200 m to 3,000 m (Barakoti & Shrestha, 2008; Shrestha *et al.* 2020). Geographical distribution seems fairly a good predictor of the infraspecific relationship within *G. diversifolia*, and this result is in contrast to *Urtica thunbergiana* (Große-Veldmann, 2017). In China, three subspecies of *G. diversifolia* have somewhat distinct as well as superimposed distribution

patterns. For example, *G. diversifolia* subsp. *diversifolia* occurs in between 1,500-2,800 m; *G. diversifolia* subsp. *suborbiculata* between (?100)400-800 m; and *G. diversifolia* subsp. *triloba* between 300-1800 m (Shu *et al.*, 2003). Two subspecies of *Girardinia diversifolia* have been reported from India; the first one *G. diversifolia* subsp. *diversifolia* is widely distributed; and the second one is *G. diversifolia* subsp. *suborbiculata* which is reported a new record of subspecies for India from Western Himalaya (Garhwal), Uttrakhanda collected at an altitude of 1,800-2,500m (Ambrish & Srivastava, 2016). In Bhutan, only *G. diversifolia* subsp. *diversifolia* occurs (Grierson & Long, 1983). However, during the course of study, we have collected herbarium specimens from Bhutan which belongs to *G. diversifolia* subsp. *suborbiculata*; this subspecies is probably a new record for Bhutan, and the publication of the new record is on pipeline.

In the present study, the samples collected from Nepal ranged from 900 m to 2,700 m above sea level. This shows that *G. diversifolia* subsp. *diversifolia* in Nepal has wide geographical range compared to China and India where the taxon is distributed from 1,500 to 2,800 m (Shu *et al*., 2003) This may be attributed to adaptation in local environmental conditions (Große-Veldmann, 2017); however, populations of *G. diversifolia* subsp. *diversifolia* fail to diverge from the common gene pool nor do they develop any isolation mechanisms. These findings are in consistent with the findings of *Urtica dioica*, another species from Urticaceae where morphologically similar populations do not develop isolation mechanism (Große-Veldmann, 2017). Further study requires study of trait variation of *G. diversifoilia* subsp. *diversifolia* along elevation gradients or variation of phenotypic plasticity among different populations.

4.2.2 Genetic Variation in *G. diversifolia*

4.2.2.1 DNA extraction and method modified

Using the standard protocol of Doyle and Doyle (1987), several attempts were made to isolate genomic DNA from *G. diversifolia*. Many barriers were faced from the very first stage of DNA extraction, including cell lysis and DNA elution. Due to the presence of various polyphenols and secondary metabolites in *G. diversifolia*, the Doyle and Doyle (1987) protocol could not produce good quality DNA, and ultimately inhibited the PCR amplification. According to Khanuja *et al*. (1999), the

biochemical components in the plant tissue of different species differ, thus standard protocol may not provide optimal DNA yield, and closely related species also may require different methods. According to Porebski *et al*. (1997) increased concentration of PVP and β-mercaptoethanol in the extraction buffer plays an important role in neutralizing the oxidation of tannins, polyphenols, and secondary metabolites. Thus, procedure described by Doyle and Doyle (1987) was modified by increasing the concentration of β-mercaptoethanol (2% to 5%), increasing the concentration of NaCl from 2 M to 5 M, addition of Phenol:Chloroform:Isoamyl Alcohol for deproteinization process and increasing the concentration of PVP (1% to 5%). Similarly, by modifying the CTAB protocol of Doyle & Doyle in *Tridax procumbens*, *Aloe barbadensis*, *Cissus quadrangularis*, *Catharanthus roseus*, *Tinospora cordifolia* and *Dracaena cambodiana*, quality genomic DNA was extracted from the plants (Aboul-Maaty *et al*., 2019, Tiwari *et al*., 2012).

According to Arruda *et al*. (2017) the ratio of absorbance at A260 / A280 nm range are recommended from 1.8-2.0, and the ratio of absorbance at A260 / 230 nm are recommended from 2.0-2.22, for impure free DNA. Absorbance above 2.0 indicates the contamination of phenol in extracted DNA, while low absorbance indicates the presence of protein. The modified protocol showed average value of 1.83 which confirmed the extraction of pure DNA in the A260 / A280 and A260 / A230 ratios. The optimized method showed an average value of 400 ng / µl DNA extracted from the leaves of *G. diversifolia*.

Polysaccharide in the isolated DNA of *G. diversifolia* was effectively removed by using 5 M NaCl (Table 14). Studies conducted in other plant species such as *Capsicum* sp., *Mangifera indica*, *Eclipta alba*, and *Aegle marmelos* have shown that this modification effectively removed polysaccharides (Chandran, 2010; Devi *et al*., 2018; Kit & Chandra *et al*., 2010; Kumar *et al*., 2018; Shukla *et al*., 2018). Similarly, 5 M NaCl concentration removed polysaccharide contamination and reduced polyphenols from the leaves of *Grewia asiatica* (Shukla *et al*., 2018).

During genomic DNA extraction process, high concentration of PVP in the extraction buffer improves DNA quality by removing secondary metabolites (Osena *et al*., 2017). Similarly, Some studies suggested using 5% PVP in CTAB buffer such as in *Vigna* sp. (Choudhary *et al*., 2008); *Mimosa tenuiflora* (Arruda *et al*., 2017) to obtain

good quality DNA pellet. Therefore 5% PVP was mixed in 2% CTAB buffer which removed polyphenols.

Proteins are reduced by β-mercaptoethanol (Tiwari *et al*., 2012). According to Arruda *et al*. (2017), high concentrations of β-mercaptoethanol is essential for the reduction of polyphenols during the extraction of genomic DNA from plants. The modified protocol contains 5% β-mercaptoethanol instead of the 2% used by Doyle and Doyle, (1987), which was useful to reduce gray DNA pellet and clear DNA pellet was obtained. Similarly, a study in *Litchi chinensis* (Arruda *et al*., 2017; Puchooa, 2004) showed that increased concentration of β-mercaptoethanol helped produce clear DNA pellet. Phenol:Chloroform:Isoamyl Alcohol is used to remove polyphenols (Anerao *et al*., 2016). Polyphenols often damage genomic DNA, therefore, Phenol: Chloroform:Isoamyl Alcohol was used which removed polyphenols, and thus pure genomic DNA was extracted.

4.2.2.2 ISSR- PCR optimization and primers selection

ISSR is a molecular technique that involves the use of microsatellite sequences as a primer and is used to study genetic variations in plant species. It depends on the quantity and quality of extracted DNA. The selection of primer is another important factor because the same primer may perform different PCR amplification results in different species (Khanuja *et al*., 1999).

Optimization of ISSR-PCR is important to obtain the desirable reproducible DNA band pattern for genetic variation analysis (Amiteye, 2021). The sensitive reaction parameters which need to be optimized are purity and concentration of template DNA, $Mgcl_2$ concentration, primer concentration, dNTPs concentration, *Taq* polymerase concentration, and PCR cycling conditions (Mohamad *et al*., 2017). After genomic DNA was extracted from *G. diversifolia*, PCR was performed using Master Mix (New England Biolab). PCR master mix failed to show the result therefore, separate *Taq* polymerase, dNTPs, from VIVANTIS (Malaysia) were used ISSR-PCR was optimized using genomic DNA extracted from *G. diversifolia.* Five main factors; dNTPs, $MgCl_2$, *Taq* DNA polymerase, template DNA, primers (University of British Columbia, Canada), and their concentrations were optimized. The universal ISSR primer of University of British Columbia (UBC) was used to optimize PCR

amplification. Thermocycler was programmed by testing various conditions using gradient PCR.

The genomic DNA concentration of 50 ng, 4 mM $Mgcl_2$, 0.6 mM dNTPs, 0.7 µM primer, 2 units *Taq* DNA polymerase, and annealing temperature of 45°C were found optimum for ISSR-PCR amplification. The clear reproducible bands have been obtained using the optimized condition. Finally, optimized PCR program was as follows: 93°C for 3 min, followed by 45 cycles of denaturing at 93°C for 30 s, annealing of primers at (45-52°C) for 45 s, extension at 72°C for 2 min, final extension at 72°C for 10 min and holding temperature at 4°C. Based on the literature review this is the first study focused on *G. diversifolia* using ISSR PCR reaction, but similar optimization process was also performed on *Oryza sativa*, *Nothapodytes nimmoniana* (Mohamad *et al*., 2017; Sane *et al*., 2012).

Sixteen screened ISSR primers were selected based on previous research on *Urtica dioica* of Urticaceae Family (Haghpanah *et al*., 2016). Out of sixteen ISSR primers the ten ISSR primers were selected in this study (Table 16) indicated the existence of microsatellite regions of (GA)n, (TC)n, (AT)n, (AC)n in *G. diversifolia* which were also found in *Urtica dioica* (Haghpanah *et al*., 2016) and *Boehmeria nivea* (Liu *et al*., 2009). Genetic polymorphism observed from 10 ISSR markers for *G. diversifolia* is higher (98.09 %) in comparison to the results observed for *Urtica dioica* (68%) (Haghpanah *et al*., 2016), and *Boehmeria* species (96.3%) (Zhang *et al*., 2014).

4.2.2.3 Genetic variation in the population of *G. diversifolia* from Nepal using ISSR markers.

ISSR markers are reproducible and sensitive markers (Zietkiewicz *et al*., 1994). Very limited studies have been performed to investigate the variation in *G. diversifolia*.

Polymorphism information content (PIC) is a widely used metric of the use of molecular markers calculated based on the number of alleles and their distribution in population (Anderson *et al*., 1993; Botstein *et al*., 1980; Nagy *et al*., 2012). The maximum value of PIC in dominant marker is 0.5 (Chesnokov & Artemyeva, 2015). In this study, the average value of PIC of the primer was found to be 0.350. Sixteen ISSR primers were selected based on previous research on *Urtica dioica* of Urticaceae

family (Haghpanah *et al*., 2016). Out of the sixteen ISSR primers, ten ISSR primers showed clear, reproducible bands in *G. diversifolia*. The ten primers selected in this study had relatively proper distribution in population of *G. diversifolia*. UBC primer 828 gave relatively high 0.428 PIC value in the study. A study conducted on the highly used natural fiber producing plant *Linum usitatissimum* showed the PIC value of 0.367 (Kumari *et al*., 2017).

ISSR markers system assessed the genetic variation and similarities among and within the five populations of *G. diversifolia* from eastern, central and western Nepal. The results of the similarity coefficient show that the genetic variation ranged from 0.16. to 0.98. High level of genetic variations were recorded from the population of Dolakha (PPB 51.91%, H (0.1928), I (0.2852) and lower level was recorded from the population of Darchula PPB (29.01%), H (0.1110), I (0.1629) (Table 20).

The study obtained the effective number of alleles (1.38-1.74). Nei (1978) classified genetic differentiation between population (Gst) into three classes: low (Gst<0.05), moderate (0.05< Gst<0.15) and high (Gst>0.15). The genetic structure obtained in the study suggested that the genetic differentiation (Gst 0.594) is higher than the average (Table 20). The Shannon's index varies from 0 to 1 and the values closer to zero represent lower genetic diversity (Monfared *et al*., 2018). The Shannon's diversity index obtained in the study was in an average of 0.523. The Shannon's diversity index from Dolakha (0.2852) was the most diverse population compared to Panchthar (0.2323), Kathmandu (0.1872), Kaski (0.1863), and Darchula (0.1629). High genetic differentiation in this species suggested that the individual populations are fairly isolated to reproduce and there is little current gene flow between them. The value of Nm was found 0.355. The value of gene flow (Nm) < 1 which denotes less than one migrant per generation into a population is the threshold value at which the differentiation occurs in population in a significant amount (Slatkin, 1985). Less than one Nm showed that the diversity maintained in the population is prone to genetic drift (Wright, 1949).

The highest genetic identity was 0.756, which was found in the populations of Dolakha and Panchthar, with least genetic distance (0.278). The population of Darchula and Panchthar, which have a lowest genetic identity of 0.638, have a highest genetic distance of 0.4481 (Table 32).

Table 32: Nei's unbiased measure of genetic identity (above diagonal) and genetic distance (below diagonal)

Population	Darchula	Dolakha	Kathmandu	Panchthar	Kaski
Darchula	****	0.728	0.721	**0.638**	0.697
Dolakha	0.316	****	0.714	**0.756**	0.746
Kathmandu	0.326	0.335	****	0.682	0.714
Panchthar	0.448	**0.278**	0.382	****	0.731
Kaski	0.360	0.292	0.336	0.312	****

In General, it is agreed that plant genetic variation changes with time and distance (Loveless, 1984). The extent and distribution of genetic diversity in a plant species depend on time and frequency with various factors such as its ecological and geographical factors, evolution and breeding system, and human factors (Rao *et al*., 2002).

Analysis of molecular variance (AMOVA) of *G. diversifolia* showed that higher distribution of genetic variation is present among the populations (60%) compared to within the populations (40%) with a significance value of $p < 0.001$. The genetic diversity is closely correlated with different factors like effective population size, breeding system, natural selection, and life history traits (including life form, ecological tolerance, seed dispersal, and gene flow) (Slatkin, 1985). For *G. diversifolia*, the plant harbors shady moist habitat, commonly found in high altitudes above 1,000 m to 2,700 m in the mountain gorges. The similar analysis was observed in *Stylosanthes scabra* (Costa *et al*., 2018), *Zingiber officinale* (Das *et al*., 2017), *Oryza granulata* (Qian & Hong *et al*., 2001) and *Cypripedium japonicum* (Tian *et al*., 2018). The study showed that there is clear association between geographical origin and genetic similarity among populations distributed in different regions.

The present study also revealed that high level of genetic differentiation is present among populations from Darchula District and Panchthar District and genetic variation increased with an increase in geographical distance. The Gst value of 0.594 also strengthened the result of the study. The Nm value less than 1, Gst value more than 0.15, and clear cluster formation depending upon geographic distance suggested that high level of genetic variance is present among the population of *G. diversifolia*.

4.2.3 Phylogenetic analysis

Girardina was found to be a sister to *Dendrocnide* (Kim *et al*., 2015). The phylogenetic relationship between *Girardina* and *Dendrocnide-Discocnide* was indicated using DNA sequence data (Wu *et al*., 2013). Where, within *G. diversifolia,* three subspecies were recognized globally based on the number of lobes of leaf blades (Chen *et al*., 2003): subsp. *diversifolia,* subsp. *triloba* (C. J. Chen) C. J. Chen & Friis, and subsp. *suborbiculata* (C. J. Chen) C. J. Chen & Friis. However, from the morphological and phylogenetic study conducted from the collected samples of Nepal, the results indicated the presence of subspecies i.e *G. diversifolia* subsp. *diversifolia.* Study of microsatellite region by ISSR showed genetic variation in *G. diversifolia*. Since ISSR is a dominant marker, it appears that specific co-dominant markers may be required for further detail studies.

4.2.3.1 Characteristics of ITS, *rbcL, matK, trnH-psbA, trnl-F*

The study of morphological characteristics was not sufficient to identify the homologous variations in *G. diversifolia*, thus molecular study was selected. This study has selected several universal molecular markers such as *matK*, ITS, *rbcL*, *trnL-F*. These markers are successfully used to study other genera from Urticaceae Family (Runqing *et al*., 2015). Among the selected markers, *matK* gene only divided *G. diversifolia* into two clades (Figure 36). Molecular marker *matK* has placed the population of Darchula District and Panchthar District in one clade and the population of Kathmandu in another clade. From this result it can be referred that *G. diversifolia* from Kathmandu District has genetic variation on the basis of maturase gene (Figure 35). This type of variation is new for *G. diversifolia*. It is known that the variability in the ITS, *rbcL, trnL-F* is relatively lower compared to the *matK* region (Yang *et al*., 2012). Our data also agreed with the claim that there are no variable sites found in the ITS, *rbcL,* and *trnL-F* gene. ISSR study showed high diversity among the populations and low diversity within the populations from Panchthar, Kathmandu, and Darchula Districts. ISSR study indicated variation in the microsatellite region of *G. diversifolia* and DNA sequences mostly *matK* gene indicated variation in maturase gene.

4.2.4 Fiber processing

Matrix degrading chemicals and enzymes with chelators in the spray enzyme retting (SER) method make the retting process extremely easy (Akin *et al*., 2000; Akin *et al*., 2002). Ethylenediaminetetraacetic Acid (EDTA) is an effective chelator at pH 5.0 which sequesters calcium ions from the fiber matrix solution (Adamsen *et al*., 2002). Enzymatic retting method extracts quality fiber in less time than other methods, but it is also a very expensive technology (Paridah *et al*., 2011). Enzymatic treatment facilitates the swelling of natural fiber shown in the study of jute yarn, hydrophilicity can be improved by treatment of pectinase by removal of hydrophobic pectin (Dhiman & Battan, 2008). According to Martial *et al*. (2017), papaya pericarp extracted pectinase could be a potential source for degrading pectin due to their broad substrate specificity with high stability. This study also showed that pectinase enzyme from papaya pericarp has the potential to be used for retting of natural fiber. Alkaline and enzymatic retting process have saved the fuel than the traditional dew retting, steam explosion and high temperature boiling technique (Dreyer *et al*., 2002).

4.2.4.1 Spectroscopic characterization

The reduction of the peak intensity found at around 1650- 1640 cm^{-1} in NaOH and ENZ/CH treated fiber indicates the reaction of the C=O bonds of hemicelluloses (Figure 37). This reaction indicates that hemicellulose component of this fiber can be reduced by alkalization and enzyme with a chelator. The peak observed in 1647 cm^{-1} in untreated fiber showed the bonded water in hemicellulose. Disapperance of this peak in ENZ/CH and NaOH treated fibers, shifted of peak at lower number in PENZ/CH indicates removal of bonded water from hemicellulose (Kabir *et al*., 2013). The characteristic peak at 1319 cm^{-1} was abundantly present in all the treated fibers except NaOH treated fiber indicating that hemicellulose can be reduced using alkali in this fiber (Sim *et al*., 2012).

The peak at 2920 cm^{-1} of untreated fiber shifted to 2897 cm^{-1} in ENZ/CH, 2900 cm^{-1} in NaOH and 2926 cm^{-1} in PENZ/CH which indicates the removal of lignin constituents (Kabir *et al*., 2013). There was lowering of peak intensity at 1419 cm^{-1} in ENZ/CH, PENZ/CH, NaOH, and ENZ treated fiber, C-C stretch in aromatic and CH_2 bending in lignin are considered (Figure 37). It shows that treated fiber helps to remove some amount of lignin from fiber (Kumar *et al*., 2017). The characteristic

peak at 1026 cm^{-1} is almost similar to NAT and ENZ/CH but shifts to 1018 cm^{-1} in NaOH treated fiber. The shifts observed indicate that few amounts of lignin is removed from the NaOH treated fiber but an insignificant effect was seen in PENZ/CH, ENZ treated fibers.

Reduction in the intensity of peak at 894 cm^{-1} in ENZ/CH treated fibers was observed in C-H bonding of amorphous and crystalline cellulose (Kabir *et al*., 2013).

The above analysis showed that NaOH, ENZ/CH, and PENZ/CH treatments have a degree of reactivity in removing lignin as well as hemicellulose components of the fiber. However, ENZ showed much less reactivity on the fiber component because there are very few changes in FTIR spectra compared to untreated NAT fiber.

4.2.4.2 XRD analysis of treated and untreated fiber

The XRD analysis of treated and untreated fiber samples showed characteristics peak of cellulose I ($22 \geq 2\theta \leq 23$) which corresponded to cryst Allographic plane 002 (Sawpan *et al*., 2011); and another peak at 15.3 corresponded to cryst Allographic plane 101 (Cao and Tan, 2005). The measured crystallinity index (CI) showed following order: NAT (91%)> PENZ/CH (87%)> NaOH (84%)> ENZ (84%)> ENZ/CH (79%). The Highest CI observed was that of NAT, followed by all the treatments. Among the treatments, PENZ/CH treatment was observed to have highest CI followed by NaOH treatment (Figure 38). The increased CI after PENZ/CH and NaOH treatments indicated improvements in the structure of cellulose.

4.2.4.3 Optical Microscopy and Uniaxial tensile testing

In Figure 39 and 40 the photographical documentation of the fiber is summarized using macro-photographic images and micrographs by means of optical microscopy. Already by naked eyes clear differences in shape (see fiber NaOH), colour (see fiber ENZ/CH) (Figure 39) and overall fineness between the different fibers types have been recognized. Especially the overall fineness can be assessed in more detail by the optical micrographs (Figure 40).

Natural fibers often show a high degree of variability in their fiber properties (Hearle & Morton, 2008). Fiber properties of *G. diversifolia* depend on various parameters such as stem section, growth conditions (location, fertilizer), air humidity and temperature (Sethman, 2004). According to Shrestha (1998) *G. diversifolia* stem

harvesting time showed differences in fiber quality. The tensile strength of *G. diversifolia* fiber has been shown to range from 178 MPA to 550 MPA while the Young's modulus has been shown to range from 5.8 to 22.5 GPA (Sethmann, 2004). *G. diversifolia* is the longest fiber among any bast fiber (Lanzilao *et al*., 2016). Several studies have concluded that alkali treatment conditions are conducive to an increase in the tensile properties of bast fiber. However, some studies have indicated a decrease in tensile properties, thus uniformity between the tensile properties of bast fibers and the alkali treatment conditions is not known. Kumar *et al*., (2017) used 5% to 10% NaOH concentrations in the fiber of *G. diversifolia* and showed increase in the tensile strength. The present work has also shown that tensile strength is 2.83±0.15 (N mm/mg) which is higher than that of natural untreated fiber 2.75±1.01 (N mm/mg), while 5% NaOH has been taken as the base. Similarly, fiber tensile strength increased by 81% in ramie, and 56% in coir after alkali treatment (Suriaman *et al*., 2021). Likewise, the application of pectinase enzyme has been found to increase fiber tensile strength in some studies and decrease fiber tensile strength in others. Saleem et al., (2008) in a study on reinforced thermoplastic composites using hemp fiber showed a decrease in tensile strength when treated with pectinase enzyme. Li & Pickering (2008) demonstrated an improvement of 19% in the tensile strength of hemp fiber composite materials when using pectinase enzyme with chelating agents. Akin et al., (2000) observed weaker strength of flax fiber due to enzyme treatment, similarly, our experiment also showed a weaker tensile strength of *G. diversifolia* fiber, but the crystallinity index of the fiber was found to be better.

Mass-related elastic modulus with NaOH-treated *G. diversifolia* fiber showed 68.4 ± 9.4 (N mm/mg) which is lower than natural untreated fiber 88.0±35.0 (N mm/mg) but showed much higher mass-related elastic modulus than other tested fibers. Sequentially, the mass-related tensile test showed a pattern of NaOH (2.83 ± 0.15)> PENZ / CH (2.79 ± 0.86)> ENZ (2.75 ± 0.51)> ENZ / CH (2.07 ± 0.57) while mass-related elastic modulus showed the pattern of NaOH (68.4 ± 9.4)> ENZ (64.0 ± 12.4)> PENZ / CH (60.4 ± 4.8)> ENZ / CH (46.4 ± 23.4) (Table 27). From this observation, it can be said that NaOH can retain fiber using the Spray Retting Method. The use of plant-based enzymes (crude papaya pericarp extract) has shown good results in enzymatic rettings, thus highlighting the importance of future studies for extraction of natural fiber using enzymatic method.

4.2.5 Traditional use and processing of *G. diversifolia*

G. diversifolia is used in traditional medicine to reduce disorders such as constipation (Thapa, 2012; Gurung *et al*., 2012), for smooth delivery of babies (Pande *et al*., 2007), to treat headache, joint pain (Manandhar, 2002), and fever. Root powder is taken with hot water to treat headache, joint pain, tuberculosis (Gurung *et al*., 2012; Subedee *et al*., 2020), internal injuries and blood purification (Rokaya *et al*., 2010). Due to the many uses of *G. diversifolia*, it is considered to be an important valuable plant.

Previously, *G. diversifolia* was traditionally processed only using white wood ash. But recently, caustic soda has been used instead of traditional processing method. Here, caustic soda can replace waxy material of fiber faster than wood ash, but it pollutes the water (Gurung 2012; Deokota & Chhetri 2009; Adhikari *et al*., 2018; Subedee *et al*., 2020). Due to which the use of caustic soda cannot be considered sustainable.

Indigenous peoples and local communities of Nepal mostly use traditional three-stone stove for boiling the bark of *G. diversifolia* (Subedee *et al*., 2017). The traditional three-stone stove is less effective for boiling purposes because it consumes more firewood and emits more smoke (Specht *et al*., 2015). Therefore, rocket stove technology may be an alternative option to reduce these problems (Subedee *et al*., 2017). Likewise, white clay (*Kamero*) is important for softening the fiber. Since, *Kamero* is a locally available material and cheaper, it is requisite to study the effect of different concentrations and properties of *Kamero* in the future.

Katuwa (hand spindle) is a portable yarn spinning tool but produces very little spinned fiber. Therefore, modern spinning machines (motorized spinning wheels) can be an alternative device that allows fiber spinners to produce large amount of spinned fiber. Wooden handlooms are still used in weaving, it is difficult to operate and only three meters of fabric can be produced per day. Wooden handlooms can be replaced by metal handlooms.

According to the analysis of cost and benefits of *G. diversifolia* during processing, and weaving (Table 30), it was found that selling woven cloth is more profitable than selling yarn. Therefore, people working in *G. diversifolia* are advised to sell woven clothees. Sweaters woven from the fibers of *G. diversifolia* by local women are in

high demand in national and international markets (MEDEP, 2010). In this way, off farm employment and extra income for women is increased. However, regular training is important to enhance their skills and techniques for processing and knitting the fibers.

Indigenous peoples and local communities are harvesting *G. diversifolia* from the forest areas. They have to travel long distances to collect *G. diversifolia* and have to spend at least a week in the forest. Another issue is massive plantation of large cardamom (*Amomum subulatum*) and Chirayito (*Swertia chirayita*) at the natural habitat of *G. diversifolia* in eastern Nepal. Large cardamom and Chirayito may be a source of high income but massive cultivation of these species replaces the natural habitat of *G. diversifolia* which may affect the ecosysterm in the long run. Therefore, it seems necessary to conserve the natural habitat of *G. diversifolia*.

Many stakeholders (farmers, collectors, traders and entrepreneurs) involved in activities related to *G. diversifolia* reported that various tax policies were not clear when raw bark and its finished marketable products were brought from village to national and international markets.

CHAPTER 5

5. CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The morpho-geographical and molecular studies conducted on the samples collected from Nepal have shown significant variations in *G. diversifolia*. In Nepal, two subspecies of *G. diversifolia* occur these are *G. diversifolia* subsp. *diversifolia* and *G. diversifolia* subsp. *suborbiculata* (C.J. Chen) C.J. Chen & Friis; the later taxon is probably a new record for Nepal. The taxon is also probably a new record for Bhutan. Morpho-geographical studies of the five populations studied have shown that *G. diversifolia* subsp. *diversifolia* is a polymorphic species with variations in the morphology of leaves. The study also confirms that characters of leaves, i.e. leaf shape, leaf lobes, leaf base, and leaf margin are good taxonomic characters to delimit the taxa into subspecies level.

G. diversifolia is rich in polyphenols, secondary metabolities and viscous compounds which inhibited high quality genomic DNA extraction. Therefore, the Doyle and Doyle (1987) protocol was modified. The modified protocol includes 5% β-mercaptoethanol, 5% PVP, and 5M NaCl in DNA extraction buffer. The modified Doyle and Doyle (1987) protocol successfully isolated high quality genomic DNA from *G. diversifolia* Clear and amenable DNA bands were obtained from the extracted DNA after optimizing ISSR-PCR. The results confirmed that the modified protocol is suitable for extracting genomic DNA from *G. diversifolia* and can be applied to other plant species having high concentrations of secondary metabolites. DNA sequencing have been done from the universal molecular markers such as *matK*, ITS, *rbcL*, *trnL-F*. Among the selected markers, *matK* only divided *G. diversifolia* in two clades. Molecular marker *matK* has placed the population of Darchula District and Panchthar District in one clade and the population of Kathmandu in another clade. The research may be useful in the future for the analysis of molecular characterization, genetic diversity analysis of allied taxa.

The ISSR markers allowed determination of the genetic variability in *G. diversifolia* by grouping them in terms of their geographical locations. Comprehensive molecular

analysis revealed that *G. diversifolia* has low variation within population and high genetic differentiation among different populations. Because of this variability, sustainable harvesting and cultivation of this plant can be a viable option for its conservation.

Lack of technological know-how is a big limiting factor for the effective extration of fiber from *G. diversifolia.* The study inferred that sodium hydroxide and plant- based enzyme can retain strong fiber from *G. diversifolia* using the Spray Retting Method. Retting process with NaOH and plant-based enzymes have shown some increase in tensile strength compared to untreated fibers of *G. diversifolia*. The research also showed the possibilites of plant based enzymatic retting would be one of the ecofriendly retting methods and further study of the plant-based enzyme is needed.

G. diversifolia has medicinal, economic, and cultural importance for the indigenous peoples and local communities of Nepal. Traditional and cultural beliefs are the main factors to conserve the populations in its natural habitat. In terms of economic opportunity and medicinal values, *G. diversifolia* is one of the alternative livelihood options to the people of Nepal.

5.2 Recommendations

1. Future studies should be directed at high-resolution molecular markers to identify infraspecific relationships in *G. diversifolia* subsp. *diversifolia* and *G. diversifolia* subsp. *suborbiculata*.
2. Research and development in cultivation, and sustainable harvesting is important for the stakeholders involved in *G. diversifola* related activities as a source of income in Nepal.
3. Allo is the identity of various indigenous peoples and local communities of Nepal and has the potential to create off farm employment for the rural people of Nepal. Therefore, further long term plans for sustainable harvesting and conservation of the species are needed.
4. Resource inventory of Allo must be carried out periodically in community managed and government managed forests, because it provides the baseline data on the total available resources of Allo in Nepal.

5. There is a need to create a database to document the communities, stakeholders, individuals involved in Allo related works, which make easier to solve their existing problems.
6. For sustainable income generation it is nececssary to focus on the long- term results related with presence of Allo in the wild rather than being allured by short term monetary value generated with the cultivation of large cardamom.
7. Enzyme retting process may be an alternative to chemical retting method because use of NaOH effects environment as well as the Allo workers.

CHAPTER 6

6. SUMMARY

This thesis explored the morpho-geographical, genetic variation, the effects of chemicals and enzymes on fiber, and social-ecological perspectives of *G. diversifolia* in Nepal. The thesis consists of six different chapters and a reference section.

The first chapter provides a general introduction to the Urticaceae family, tribe, species, fiber, and the uses of this plant. Indigenous peoples and local communities of Nepal have used this plant for traditional uses and economic purposes but showed lack of research from social-ecological perspectives. Although there are many morphological variations in *G. diversifolia* found in Nepal, there is no comprehensive study undertaken that represents the whole of Nepal and the use of molecular tools and techniques has been used to study its genetic variation. The fiber produced from this plant has been processed in Nepal in the inefficient way; therefore the use of chemical and enzymatic retting process and its effects have been shown.

The second chapter describes about the relevant literature reviewed. This chapter described about the nomenclature complexity of *G. diversifolia* and its subspecies: *G. diversifolia* subsp. *diversifolia*; *G. diversifolia* subsp. *suborbiculata*; *G. diversifolia* subsp. *triloba*. Brief global distribution of this plant has been provided where this plant is present in South Africa, Ethiopia, southward to Zimbabwe, Angola, and Madagascar, in Asia: from Kashmir to Sikkim, Burma, Nepal, Bhutan, Srilanka, Indonesia and China. Medicinal, cultural and economic values have been reviewed from the published articles about this plant. Morphological variation, cytological studies, molecular phylogenetic relationships have also been reviewed. The importance of ISSR, ITS, *matK, trnlF, rbcl* markers have been reviewed. Various fiber properties including physical properties, morphological characteristics, fiber processing using alkali and enzymatic treatments and the tensile strength and elastic modulus have been reviewed.

The third chapter describes the materials and methods. This chapter shows the method of collecting samples collected from different altitudes of 1,000-3,000 meter above sea level from the far western, central and eastern regions of Nepal. Rapid Rural

Appraisal (RRA)/ Participatory rural appraisal (PRA), key informant survey, herbarium specimen preparation, and morpho-geographical study have been described for documenting traditional knowledge, morphological variation and fiber processing practices. The protocol of Doyle and Doyle (1984) has been modified for DNA isolation. ISSR markers have been used to study genetic variation. Molecular markers such as *rbcL*, *matK*, *trnL-F* have been used for phylogenetic studies. FTIR, Xray diffraction, microscopy and tensile testing methods have been used to test the fiber properties of this plant using commercial enzymes, plant-based enzymes and NaOH.

The fourth chapter described about the results and discussion. Based on the study, two subspecies of *G. diversifolia* occur in Nepal, these are *G. diversifolia* subsp. *diversifolia* and *G. diversifolia* subsp. *suborbiculate* (C.J. Chen) C.J. Chen & Friis; the later taxon is probalby a new record for the Flora of Nepal. The taxon is also probably a new record for the Flora of Bhutan. The new record of the taxon is in process of publication. *G. diversifolia* is found in 55 districts of Nepal, especially in hilly areas having high humidity and cold weather. Out of these 55 districts of Nepal, Solukhumbu and Sankhuwasabha Districts of eastern Nepal are the main districts in terms of availability of *G. diversifolia.* Similarly, Nuwakot, Ramechhap, Sindhupalchowk and Dolakha Districts are from the central Nepal, while Parbat, Myagdi Districts from western Nepal and Rukum, Rolpa, Dolpa, Humla, Jumla, Dailekh and Pyuthan are from the midwestern Nepal. Similarly, Dadeldhura, Bajhang and Darchula Districts from the far west are ahead in terms of availability of *G. diversifolia.* The morpho-geographical study showed that *G. diversifolia* found in Dolakha District has lower number of trichomes on the abaxial surface than plants found in Darchula, Kaski, Kathmandu and Panchthar Districts. However, *G. diversifolia* found in Panchthar District are tall having cylindrical stem and small diameter with long internodes and are considered in terms of excellent fiber quality.

Molecular studies were done on the plant samples collected from different parts of Nepal. The main task for molecular studies is DNA extraction from the collected samples. Due to the presence of various polyphenols and secondary metabolites in *G. diversifolia*, the Doyle and Doyle (1987) protocol is modified to produce good quality DNA Pure and clear DNA. The modified Doyle and Doyle method produced 445 ng/μL of DNA, and the purity ranged from 1.8-2.0 indicating the minimum

contamination of metabolites. The genomic DNA concentration 50 ng, 4 mM $Mgcl_{2,}$ 0.6 mM dNTPS, 0.7 µM primer, 2 units *Taq* DNA polymerase and annealing temperature 45°C were found optimum for ISSR-PCR amplification. The clear reproducible bands have been obtained using the optimized condition. Finally, optimized PCR program was as follows: 93°C for 3 min, followed by 45 cycles of denaturing at 93°C for 30 s, annealing of primers at (45-52°C) for 45 s, extension at 72°C for 2 min, final extension at 72°C for 10 min and holding temperature at 4°C. The modified technique was found to be suitable for isolation of genomic DNA of *G. diversifolia*.

The ISSR markers system evaluated genetic diversity and similarities among and within the five populations of *G. diversifolia* from western, central and eastern Nepal. Obtained results from the similarity coefficient indicated that genetic variation ranged from 0.98 to 0.16. High level of genetic diversity was recorded from the population of Dolakha PPB (51.91%, H (0.1928), I (0.2852) and lower level was recorded from the population of Darchula PPB (29.01%), H (0.1110), I (0.1629). The effective number of alleles was (ne) 1.38 to1.74. The genetic structure obtained in the study suggested that the differentiation coefficients (Gst 0.594) is higher than the average coefficients. The Shannon's diversity index obtained in the study was in the average of 0.523. The Shannon's diversity index from Dolakha (0.285) showed the most diverse population compared to Panchthar (0.232), Kathmandu (0.1872), Kaski (0.186) and Darchula (0.162) Districts. High genetic differentiation in this species suggested that the individual populations have been reproductively isolated and has little current gene flow between them. The value of gene flow (Nm) was 0.355. The Nm less than one suggested that the diversity maintained in the population is prone to genetic drift. Analysis of molecular variance (AMOVA) of *G. diversifolia* showed that higher distribution of genetic variation is present among the populations (60%) compared to within the populations (40%) with the significance value of $p < 0.001$.

This study has selected several universal molecular markers such as *matK*, ITS, *rbcL*, *trnL-F*. These markers are successfully used to study other genera from Urticaceae Family (Runqing *et al.*, 2015). Among the selected markers, *matK* gene only divided *G. diversifolia* in two clades. Molecular marker *matK* has placed the population of Darchula District and Panchthar District in one clade and the population of

Kathmandu in another clade. From this result it can be referred that *G. diversifolia* from Kathmandu District has genetic variation on the basis of maturase gene. This type of variation is new for *G. diversifolia*. It is known that the variability in the ITS, *rbcL*, *trnL-F* is relatively lower compared to the *matK* region. Our own data has agreed with the claim that there is no variable sites were found in ITS, *rbcL* and *trnL-F* gene.

The fiber of this plant was extracted in traditional way which was cumbersome and time consuming. But chemical and enzymatic methods have been used in this research. A study of the effects of this enzyme and NaOH on fiber yielded the following results: tensile strength has been found 2.83±0.15 which is higher than natural untreated fiber 2.75±1.01, while 5% NaOH has been taken as the base. Mass-related elastic modulus with NaOH-treated *G. diversifolia* fiber showed 68.4 ± 9.4 which is lower than natural untreated fiber but with much higher mass-related elastic modulus than other tested fibers. Sequentially, the mass-related tensile test showed a pattern of NaOH (2.83 ± 0.15)> PENZ / CH (2.79 ± 0.86)> ENZ (2.75 ± 0.51)> ENZ / CH (2.07 ± 0.57) while mass-related elastic modulus showed the pattern of NaOH (68.4 ± 9.4)> ENZ (64.0 ± 12.4)> PENZ / CH (60.4 ± 4.8)> ENZ / CH (46.4 ± 23.4). From this observation, it can be said that NaOH can retain strong fiber using the Spray Retting Method. The plant based enzyme (crude papaya pericarp extract) has also shown good results in the enzymatic retting used, so the research highlighted the importance to conduct in-depth study of the papaya based enzymatic retting in the future.

Fifth chapter shows the conclusion of the study. The result of morpho-geographical study revealed the presence of two subspecies of *G. diversifolia* in Nepal and Bhutan. Molecular study indicated that variations in *G. diversifolia* subsp. *diversifolia* are not uniform and is not sufficient to distinguish different subspecies thus it can be concluded that the samples collected during the study from different places of Nepal is *G. diversifolia* subsp. *diversifolia.* The ISSR markers allowed determination of the genetic variability in *G. diversifolia* by grouping them in terms of their geographical locations. Morpho-geographical study showed that in *G. diversifolia* there are many differences in trichomes and leaf length but these differences are not homologous and their structure also differs within the same species. Comprehensive molecular analysis

revealed that *G. diversifolia* has low genetic diversity within population and high genetic differentiation among the population. *G. diversifolia* has medicinal, economic, and cultural importance for the indigenous peoples and local communities of Nepal. Traditional and cultural beliefs are the important factors to conserve natural population of *G. diversifolia*. In terms of economic opportunity and medicinal value, *G. diversifolia* is an alternative source of income for people living in rural Nepal.

Chapter six summarizes the thesis writing

REFERENCES

Aboul-Maaty, N. A. F., & Oraby, H. A. S. (2019). Extraction of high-quality genomic DNA from different plant orders applying a modified CTAB-based method. *Bulletin of the National Research Centre*, **43**(1):1-10.

Adamsen, A. P., Akin, D. E., & Rigsby, L. L. (2002). Chelating Agents and Enzyme Retting of Flax, *Textile Research Journal*, **72**(4):296-302.

Adhikari, L., Shrestha, A. J., Dorji, T., Lemke, E., & Subedee, B. R. (2018). Transforming the lives of mountain women through the Himalayan nettle value chain: a case study from Darchula, far west Nepal. *Mountain Research and Development*, **38**(1):4-13.

Aggarwal, A., & Mali, R. R. (2015). *Ocimum tenuiflorum*–A Medicinal Plants with its versatile uses. *International Journal of Recent Advances in Science and Technology*, **2**(2): 1-10.

Akbay, P., Basaran, A. A., Undeger, U. & Basaran, N. (2003). In vitro immunomodulatory activity of flavonoid glycosides from *Urtica dioica* L. *Phytotherapy Research*, **17**(1):34–37.

Akin, D. E., Dodd, R. B., Perkins, W., Henriksson, G., & Eriksson, K. E. L. (2000). Spray Enzymatic Retting: A New Method for Processing Flax Fibers. *Textile Research Journal*, **70**(6):486-494.

Akin, D. E., Foulk J. A & Dodd, R. B. (2002). Influence on Flax Fibers of Components in Enzyme Retting Formulations. *Textile Research Journal*, **72(**6):510-514

Álvarez, I. & Wendel, J. F. (2003). Ribosomal ITS sequences and plant phylogenetic inference. *Molecular Phylogenetics and Evolution*, **29**(3):417-434.

Amaducci, S., & Gusovius, H. J. (2010). *Hemp–cultivation, extraction and processing. Industrial Applications of Natural Fibers Structure, Properties*

and Technical Applications. United Kingdom: John Wiley & Sons, Ltd, Chichester, West Sussex.

Ambrish, K., & Srivastava, S. K. (2016). *Girardinia diversifolia* (Link) Friis subsp. *suborbiculata* (CJ Chen) CJ Chen & Friis (Urticaceae): A New Record for India. *Indian Journal of Forestry*, **39**(2):151-153.

Amiteye, S. (2021). Basic concepts and methodologies of DNA marker systems in plant molecular breeding, *Heliyon*, **7**(10): e08093

Ana-Cruz, M. C., Helena, M. E., & Yacenia, M. C. (2017). Molecular characterization of *Chenopodium quinoa* Willd. using inter-simple sequence repeat (ISSR) markers. *African Journal of Biotechnology*, **16**(10): 483-489.

Anderson, J.A., Churchill, G. A., Autrique, J. E., Tanksley, S. D., & Sorrells, M. E. (1993). Optimizing parental selection for genetic linkage maps. *Genome*, **36**(1):181-186.

Anerao, J., Jha, V., & Desai, N. (2016). Optimization of DNA extraction methods from *Garcinia* species for ISSR-PCR, RAPD-PCR and DNA barcoding. *Asian Journal of Biotechnology*, ***9*:**35-42.

Arruda, S. R., Pereira, D. G., Silva-Castro, M. M., Brito, M. G., & Waldschmidt, A. M. (2017). An optimized protocol for DNA extraction in plants with a high content of secondary metabolites, based on leaves of *Mimosa tenuiflora* (Willd.) Poir. (Leguminosae). *Genetics and Molecular Research*, **16**(3):1-9.

Aslan, M., Carrasco, G. C., Sørensen, B. F., & Madsen, B. (2011). Strength variability of single flax fibers. *Journal of Materials Science*, **46**(19):6344-6354.

Avise, J. C., & Hamrick, J. L. (eds.) (1996). *Conservation genetics: case histories from nature*. Chapman & Hall , New York,

Bajpai, P. K., Meena, D., Vatsa, S. & Singh, I. (2013). Tensile behavior of nettle fiber composites exposed to various environments. *Journal of Natural Fibers*, **10**(3):244-256.

Barakoti, T. & Shrestha, K. (2008). Commercial utilization of 'Allo' (*Girardinia diversifolia*) by the Rais of Sankhuwasabha for income generation. *Banko Janakari*, **18**(1):18–24.

Bhandari, P., Gurung, M. B., Subedi, C. K., Chaudhary, R. P., Basnet, K. B., Gurung, J., Uprety, Y., Neupane, A., & Shrestha, K. K. (2021). Traditional use of medicinal plants in the Chyangthapu-Phalaicha biological sub-corridor, Panchthar District, Kangchenjunga Landscape, Nepal. *Ethnobotany Research and Applications,* **22**(25):1-43

Bhandari, P. L. (2019).Value chain analysis of Allo (*Girardinia diversifolia*) in Solukhumbhu district of Nepal. *Archives of Agriculture and Environmental Science*, **4**(4): 410-423.

Bharati, S, (2018). An improved protocol for genomic DNA extraction and purification from the leaves of *Terminalia Arjuna* (Roxb.) Wigh and Arnis. *International Research Journal of Biological Sciences,* **7**(10): 1-6

Bharmauria, V., Narang, N., Verma, V., & Sharma, S. (2009). Genetic variation and polymorphism in the Himalayan nettle plant *Urtica dioica* based on RAPD marker. *Journal of Medicinal Plants Research*, **3**(3):166-170.

Bhatt, J., Kumar, S., Patel, S., & Solanki, R. (2017). Sequence-related amplified polymorphism (SRAP) markers based genetic diversity analysis of cumin genotypes. *Annals of Agrarian Science*, 15(4): 434-438.

Bhattarai, S., Chaudhary, R. P., Quave, C. L., & Taylor, R. S. (2010). The use of medicinal plants in the trans-himalayan arid zone of Mustang district, Nepal. *Journal of Ethnobiology and Ethnomedicine*, **6**(1):1-11.

Binneck, E., Nedel, J. L., & Dellagostin, O. A. (2002). RAPD analysis on cultivar identification: a useful methodology. *Revista Brasileira de Farmacognosia-Brazilian Journal of Pharmacognosy*, **24**(1)**:** 183-196.

Bismarck, A., Aranberri., Askargorta, I. A., Springer, J., Lampke, T., Wielage, B., Stamboulis, A., Shenderovich, I & Limbach, H. H. (2002). Surface

characterization of flax, hemp and cellulose fibers; surface properties and the water uptake behavior. *Polymer composites*, **23**(5):872-894.

Bledzki, A. K., Reihmane, S., & Gassan, J. (1996). Properties and modification methods for vegetable fibers for natural fiber composites. *Journal of Applied Polymer Science*, **59**(8):1329-1336.

Botstein, D., White, R. L., Skolnick, M., & Davis, R. W. (1980). Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *American Journal of Human Genetics*, **32**(3):314-331.

Brink, M., (2009). *Girardinia diversifolia* (Link) Friis. Record from PROTA4U. Brink, M. & Achigan-Dako, E.G. (Editors). PROTA (Plant Resources of Tropical Africa / Ressources végétales de l'Afrique tropicale), Wageningen, Netherlands. Accessed on (21/09/2021)

Bruhlmann, F., Kim, K. S., Zimmerman, W. and Fiechter, A. (1994), 'Pectinolytic enzymes from actinomycetes for the degumming of ramie bast fibers', *Applied and Environmental Microbiology*, **60**(6):2107–12.

Burkhardt L. (2018). Verzeichnis eponymischer Pflanzen-namen – Erweiterte Edition. Index of Eponymic Plant Names – Extended Edition. Index des Noms éponymiques des Plantes. – Édition augmentée. – Berlin: Botanic Garden and Botanical Museum Berlin, Freie Universität Berlin. – Published at https://doi.org/10.3372/epolist2018 Accessed on (21/09/2021)

Cao, Y., & Tan, H. (2005). Study on crystal structures of enzyme-hydrolyzed cellulosic materials by X-ray diffraction. *Enzyme and Microbial Technology*, **36**(2-3):314-317.

Célino, A., Fréour, S., Jacquemin, F., & Casari, P. (2014). The hygroscopic behavior of plant fibers: a review. *Frontiers in Chemistry*, **1**:43.

Chanysi, H. G. (2011). *A resource manual on 'Allo' cultivation, conservation, processing, weaving and market management.* Kathmandu: Shree Manjushree press.

Chase, M. W., Cowan, R. S., Hollingsworth, P. M., Van Den Berg, C., Madriñán, S., Petersen, G., Seberg, O., Jorgsensen, T., Cameron. K. M., Carine, M., Pederson N., Hedderson T. A. J., Conrad, F., Salazar G.A., Richardson J.E., Holligsworth M.L., Barraclough T.G., Kelly. L. & Wilkinson, M. (2007). A proposal for a standardised protocol to barcode all land plants. *Taxon*, **56**(2), 295-299.

Chase, M. W., Soltis, D. E., Olmstead, R. G., Morgan, D., Les, D. H., Mishler, B. D., & Albert, V. A. (1993). Phylogenetics of seed plants: an analysis of nucleotide sequences from the plastid gene *rbcL*. *Annals of the Missouri Botanical Garden*, **80**(3):528-580.

Chen, C. J., Lin, Q. Friis, I., Wilmot-Dear, C. M., & Monro. A. K. (2003). Urticaceae.in: Z.Y. Wu, P.H. Raven, D.Y. Hong (Eds.), *Flora of China: Ulmaceae through Basellaceae.* Science Press, Beijing and Missouri Botanical Garden Press, St. Louis Flora of China **5**:76–189.

Cheng, T., Xu, C., Lei, L., Li, C., Zhang, Y., & Zhou, S. (2016). Barcoding the kingdom Plantae: new PCR primers for *ITS* regions of plants with improved universality and specificity. *Molecular Ecology Resources*, **16**(1):138-149.

Chesnokov Y.V & Artemyeva A.M., (2015).Evaluation of the Measure of Polymophism Informationof Genetic Diversity, *Agriculture Biology*. **50**:571-578

Choudhary, K., Mathur, N., Choudhary, O. P., & Pillai, U. (2008). Protocol for isolation of genomic DNA from dry and fresh leaves of *Vigna* species suitable for RAPD and restriction digestion. *Advances in Biological Research*, **2**:83-89.

Coates, J. (2000). Interpretation of infrared spectra, a practical approach. *Encyclopedia of analytical chemistry: applications, theory and instrumentation*. https://onlinelibrary.wiley.com/doi/epdf/10.1002/9780470027318.a5606 Accessed on 10 May 2019

Collard, B. C., Jahufer, M. Z. Z., Brouwer, J. B., & Pang, E. C. K. (2005). An introduction to markers, quantitative trait loci (QTL) mapping and marker-

assisted selection for crop improvement: the basic concepts. *Euphytica*, **142**(1):169-196.

Costa, J. C., Fracetto, G. G. M., Fracetto, F. J. C., Souza, T. C., Santos, M.V.F & Junior, M. A. L. (2018). Genetic diversity in natural populations of *Stylosanthes scabra* using ISSR markers. *Genetics and Molecular Research*, **18**(2):1-9

Cullings, K. W. (1992). Design and testing of a plant-specific PCR primer for ecological and evolutionary studies. *Molecular Ecology*, **1**(4): 233-240.

Darriba, D., Taboada, G. L., Doallo, R., & Posada, D. (2012). jModelTest 2: more models, new heuristics and parallel computing. *Nature methods*, **9**(8): 772-772.

Das, A., Gaur, M., Barik, D. P., & Subudhi, E. (2017). Genetic diversity analysis of 60 ginger germplasm core accessions using ISSR and SSR markers. *Plant Biosystems-An International Journal Dealing with all Aspects of Plant Biology*, **151**(5):822-832.

De Vicente, M.C., López, C. & Fulton, T. (eds.). (2004). *Genetic Diversity Analysis with Molecular Marker Data: Learning Module.* International Plant Genetic Resources Institute (IPGRI), Rome, Italy.

Deng, T., Kim, C., Zhang, D. G., Zhang, J. W., Li, Z. M., Nie, Z. L., & Sun, H. (2013). *Zhengyia shennongensis*: A new bulbiliferous genus and species of the nettle family (Urticaceae) from central China exhibiting parallel evolution of the bulbil trait. *Taxon*, **62**(1), 89-99.

Deokota, R., & Chhetri, R. B. (2009). Traditional knowledge on wild fiber processing of *Allo* in Bhedetar of Sunsari District, Nepal. *Journal of Science, Engineering and Technology*, **5** (1):136–42.

Devi, A. A., Brajendra, N., & Dinachandra, M. (2018). Genetic Diversity Analysis in Chilli (*Capsicum annuum* L.) Found in Manipur Using RAPD Markers. *International Journal of Current Microbiology and Applied Sciences*, **7**(10): 257-262.

Dhiman, S. S., Sharma, J., & Battan, B. (2008). Industrial applications and future prospects of microbial xylanases: a review. *BioResources*, **3**(4):1377-1402.

Dice, L. R. (1945). Measures of the amount of ecologic association between species. *Ecology*, **26**(3):297-302.

Dong, W., Xu, C., Cheng, T., Lin, K., & Zhou, S.(2013). Sequencing angiosperm plastid genomes made easy: a complete set of universal primers and a case study on the phylogeny of Saxifragales. *Genome Biology and Evolution*, **5**(5): 989-997.

Dougal, M. C. (1973). Structure and division in Kulung Rai society. *Kailash,* **1**:205 – 224.

Doyle, J. J., & Dickson, E. E. (1987). Preservation of plant samples for DNA restriction endonuclease analysis. *Taxon*, **36**(4), 715-722.

Doyle, J. J., & Doyle, J.L. (1987). A Rapid DNA Isolation Procedure for Small Quantities of Fresh Leaf Tissue. *Phytochemical Bulletin*, **19**:11-15

Dreyer, J., Müssig, J., Koschke, N., Ibenthal, W. D., & Harig, H. (2002). Comparison of enzymatically separated hemp and nettle fiber to chemically separated and steam exploded hemp fiber. *Journal of Industrial Hemp*, **7(**1): 43-59.

Dunsmore, J. (1998). Microenterprise development: Traditional skills and the reduction of poverty in highland Nepal. Himalaya, *Journal of the Association for Nepal and Himalayan Studies*, **18** (2):22–27.

Dutta, A., Singh, K., Singh, B., Shrma, Y. P., & Bussmann, R. W. (2021) veterinary practices from Gujjar and Bakarwal tribes of District Poonch, Jammu & Kashmir: A boon for animals from our ancestors, *Ethnobotany Research & Applications,* **21**(45): 1-18

Erzurumlu, G. S., Sultana, N., Vural, M., & Serce, S. (2018). Genetic and phenotypic variation among Turkish terrestrial orchid species as revealed by RAPD and

morphological characteristics. *Turkish Journal of Agriculture and Forestry*, **42(**4): 227-236.

Excoffier, L., Smouse, P. E., & Quattro, J. M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **131**(2): 479-491.

Fernie, A., & Klee, H. (2011). The use of natural genetic diversity in the understanding of metabolic organization and regulation. *Frontiers in Plant Science*, **2**:59.

Fidelis, M. E. A., Pereira, T. V. C., Gomes, O. D. F. M., Silva, F. A., & Toledo Filho, R. D. T. (2013). The effect of fiber morphology on the tensile strength of natural fibers. *Journal of Materials Research and Technology*, **2**(2):149-157

Fiore, V., Scalici, T., Bella, G. D., & Valenza, A. (2015). A review on basalt fiber and its composites. *Composites Part B: Engineering*, **74**:74-94.

Firn, R. D., & Jones, C. G. (2000). The evolution of secondary metabolism–a unifying model. *Molecular Microbiology*, **37**(5): 989–994.

Flood, P. J., Harbinson, J., & Aarts, M. G. (2011). Natural genetic variation in plant photosynthesis. *Trends in Plant Science*, **16**(6):327-335.

Forman, L & Bridson, D. M.(1989). *The herbarium handbook.* Royal Botanic Gardens, Edinburg

Foulk, J. A., Akin, D. E., & Dodd, R. B. (2001). Processing techniques for improving enzyme-retting of flax. *Industrial Crops and Products*, **13(**3):239-248.

Friis I. (1989). The Urticaceae:a systematic review. In: Crane, P. R,. Blackmore. S. (Eds.), *Evolution, Systematics and Fossil History of the Hamamelidae*, Oxford: Clarendon Press

Friis, I. (1993). *Urticaceae.* In *Flowering Plants· Dicotyledons* Springer, Berlin, Heidelberg.

Friis, I. (1981). A synopsis of Girardinia (Urticaceae). *Kew Bulletin,* **36** (1):143–57.

Fu Han-Yi, Chen S-J, & Huang, L. L. K (2003). Comparative study on the stinging trichomes and some related epidermal structures in the leaves of *Dendrocnide meyeniana*, *Girardinia diversifolia*, and *Urtica thunbergiana*. *Taiwania,* **48**(4):213-223.

Gorshkova, T. A., Salnikov, V. V., Pogodina, N. M., Chemikosova, S. B., Yablokova, E. V., Ulanov, A. V., Ageeva, M.V., Dam. J. E. G & Lozovaya, V. V. (2000). Composition and distribution of cell wall phenolic compounds in flax (*Linum usitatissimum* L.) stem tissues. *Annals of Botany*, **85**(4): 477-486.

Graham, G. C., Mayers, P., & Henry, R. J. (1994). A simplified method for the preparation of fungal genomic DNA for PCR and RAPD analysis. *Biotechniques*, **16**(1): 48-50.

Grierson, A. J., & Long, D. G. (1983). *Flora of Bhutan: including a record of plants from Sikkim*. Edinburgh: Royal Botanic Garden Edinburgh, 1(1):111

Grosse-Veldmann, B., Nürk, N. M., Smissen, R., Breitwieser, I., Quandt, D., & Weigend, M. (2016). Pulling the sting out of nettle systematics–a comprehensive phylogeny of the genus *Urtica* L. (Urticaceae). *Molecular Phylogenetics and Evolution*, **102**:9-19.

Große-Veldmann, B. (2017). *Systematics, taxonomy, and evolution of Urtica L. (Urticaceae)* (Doctoral dissertation, Universitäts-und Landesbibliothek Bonn, Germany).

Gurung, A., Flanigan, H., Ghimeray, A.K., Karki, R., Bista, R., & Gurung, O.P. (2012). Traditional knowledge of processing and use of the himalayan giant nettle (*Girardinia diversifolia* (Link) Friis) among the Gurungs of Sikles, Nepal. *Ethnobotany Research and Applications,* **10**:167–74.

Hadiah, J. T., Conn, B. J., & Quinn, C. J. (2008). Infra-familial phylogeny of Urticaceae, using chloroplast sequence data. *Australian Systematic Botany*, **21**(5):375-385.

Haghpanah, M., Kazemitabar, S.-K., Hashemi, S.-H., & Alavi, S.-M. (2016). Comparison of ISSR and AFLP markers in assessing genetic diversity among Nettle (*Uritica dioica* L.) populations. *Journal of Plant Molecular Breeding*, **4**(1):10–16.

Hamrick, J. L., Godt, M. J. W., & Sherman-Broyles, S. L. (1992). Factors influencing levels of genetic diversity in woody plant species. *Population Genetics of Forest Trees*, **6:**95-124.

Hara, H., Chater, A. O & Williams, L.H.J. (1982). *An Enumeration of the Flowering Plants of Nepal*, British Museum (Natural History): Trustees of British Museum, London, 3:203-204.

Hargitai, H., Rácz, I., & Anandjiwala, R. D. (2008). Development of hemp fiber reinforced polypropylene composites. *Journal of Thermoplastic Composite Materials*, **21**(2): 165-174.

Hearle, J. W., & Morton, W. E. (2008). *Physical properties of textile fibres*. Manchester: Textile Institute. Woodhead Publishing.

Hebert, P. D. N, Penton, E. H., Burns, J. M., Janzen, D. H., & Hallwachs, W. (2004). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences*, **101**(41):14812-14817.

Hooker, J. D. (1992). *The flora of British India*. (reprint) Bishen Singh Mahendra Pal Singh, Dehra Dun India 5:550-551

ICIMOD (International Centre for Integrated Mountain Development). (2015). Promotion of the Allo (Himalayan Nettle) Value Chain in Nepal: Sustainable Livelihoods in the Kailash Sacred Landscape. Kathmandu, Nepal: ICIMOD.

http://lib.icimod.org/record/31164/files/Allo_15.pdf; accessed on 15 September 2019.

Jaccard, P. (1908). Nouvelles recherches sur la distribution florale. *Bulletin de la Société Sciences Nat* ., **44**: 223-270.

Jankauskienė, Z., Butkutė, B., Gruzdevienė, E., Cesevičienė, J., & Fernando, A. L. (2015). Chemical composition and physical properties of dew-and water-retted hemp fibers. *Industrial Crops and Products*, **75**:206-211.

Kabir, M. M., Wang, H., Lau, K. T., & Cardona, F. (2013). Effects of chemical treatments on hemp fiber structure. *Applied Surface Science*, ***276***:13-23.

Keller, A., Förster, F., Müller, T., Dandekar, T., Schultz, J., & Wolf, M. (2010). Including RNA secondary structures improves accuracy and robustness in reconstruction of phylogenetic trees. *Biology Direct*, **5**(1):1-12.

Khalil, H. A., Hossain, M. S., Rosamah, E., Azli, N. A., Saddon, N., Davoudpoura, Davoudpora,Y., Islam, N., & Dungani, R. (2015). The role of soil properties and it's interaction towards quality plant fiber: A review. *Renewable and Sustainable Energy Reviews*, **43**:1006-1015.

Khanuja, S. P., Shasany, A. K., Darokar, M. P., & Kumar, S. (1999). Rapid isolation of DNA from dry and fresh samples of plants producing large amounts of secondary metabolites and essential oils. *Plant molecular biology Reporter*, **17**(1): 74-74.

Kim, C., Deng, T., Chase, M., Zhang, D. G., Nie, Z. L., & Sun, H. (2015). Generic phylogeny and character evolution in Urticeae (Urticaceae) inferred from nuclear and plastid DNA regions. *Taxon*, **64**(1):65-78.

Kit, Y. S., & Chandran, S. (2010). A simple, rapid and efficient method of isolating DNA from Chokanan mango (*Mangifera indica* L.). *African Journal of Biotechnology*, **9**(36):5805-5808

Kollipara K. P., Singh R. J., & Hymowitz. T. (1997). Phylogenetic and genomic relationships in the genus *Glycine* Willd. based on sequences from the ITS region of nuclear rDNA. *Genome*, **40**(1): 57-68.

Koohestani, B. Darban, A. K., Mokhtari, P., Yilmaz, E., & Darezereshki, E. (2019). Comparison of different natural fiber treatments: A literature review. *International Journal of Environmental Science and Technology*, **16**(1): 1-14.

Kozlowski, R. M. (2012). *Handbook of natural fibers: Processing and applications*. Woodhead Publishing Limited.

Kozlowski, R., Baraniecki, P., & Barriga-Bedoya, J. (2005). *Bast fibers (flax, hemp, jute, ramie, kenaf, abaca). Biodegradable and sustainable fibers.* England:Woodhead Publishing, Cambridge.

Kozlowski, R., Batog, J., Konczewicz, W., Mackiewicz-Talarczyk, M., Muzyczek, M., Sedelnik, N., & Tanska, B. (2006). Enzymes in bast fibrous plant processing. *Biotechnology letters*, **28**(10):761-765.

Kress W. J., Wurdack. K. J., Zimme. E. A. R., Weigt. L. A., & Janzen D. H. (2005). Use of DNA barcodes to identify flowering plants, *Proceedings of the National Academy of Sciences,* **102**(23):8369–8374.

Kress, W. J., & Erickson, D. L. (2007). A two-locus global DNA barcode for land plants: the coding *rbcL* gene complements the non-coding *trnH-psbA* spacer region. *PLoS one*, **2***(*6):e508.

Kumar, N., & Das, D. (2017). Alkali treatment on nettle fibers: Part I: investigation of chemical, structural, physical, and mechanical characteristics of alkali-treated nettle fibers. *The Journal of The Textile Institute*, **108**(8):1461-1467.

Kumar, N., & Das, D. (2017). Fibrous biocomposites from nettle (*Girardinia diversifolia*) and poly (lactic acid) fibers for automotive dashboard panel application. *Composites Part B: Engineering*, **130**:54-63.

Kumar, R., Singh, R. P., & Kataria, R. (2019). Study on flexural performance of fabricated natural fiber hybrid polypropylene composite: an experimental investigation through designed experiments. *World Journal of Engineering*. **16**(3):1-13

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, **35**(6):1547-1549.

Kumari, A., Paul, S., & Sharma, V. (2017). Genetic diversity analysis using RAPD and ISSR markers revealed discrete genetic makeup in relation to fiber and oil content in *Linum usitatissimum* L. genotypes. *The Nucleus*, **61**(1):45-53

Kunwar, R. M., Shrestha, K., Malla, S., Acharya, T., Sementelli, A. J., Kutal, D., & Bussmann, R. W. (2019). Relation of medicinal plants, their use patterns and availability in the lower Kailash Sacred Landscape, Nepal. *Ethnobotany Research and Applications*, **18**:1-14.

Lanfear, R., Calcott, B., Ho, S. Y., & Guindon, S. (2012). PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution*, **29**(6): 1695-1701.

Lanzilao, G., Goswami, P., & Blackburn, R. S. (2016). Study of the morphological characteristics and physical properties of Himalayan giant nettle (*Girardinia diversifolia* L.) fiber in comparison with European nettle (*Urtica dioica* L.) fiber. *Materials Letters*, **181**:200-203.

Li, Y., & Pickering, K. L. (2008). Hemp fibre reinforced composites using chelator and enzyme treatments. *Composites science and technology*, **68**(15): 3293-3298.

Liu, J., Shi, L., Han, J., Li, G., Lu, H., Hou, J., Zhou, X., Meng, F., & Downie, S. R. (2014). Identification of species in the angiosperm family Apiaceae using DNA barcodes. *Molecular Ecology Resources*, **14**(6): 1231-1238.

Liu, L., Meng, Z.-Q., Wang, B., Wang, X., Yang, J.-Y., & Peng, D. (2009). Genetic Diversity among Wild Resources of the Genus *Boehmeria* Jacq. From West China Determined Using Inter-simple Sequence Repeat and Rapid Amplification of Polymorphic DNA Markers. *Plant Production Science*, **12**(1):88–96.

Loveless, M. D., & Hamrick, J. L. (1984). Ecological determinants of genetic structure in plant populations. *Annual Review of Ecology and Systematics*, **15**(1):65-95.

Malla, S. B., Rajbhandari, S. B., Shrestha, T. B., Adhikari, P. M., Adhikari, S. R., and Shakya, P. R. (eds) (1986), *Flora of Kathmandu Valley*, Bulletin of the Department of Medicinal Plants, Number 11, Kathmandu: Department of Medicinal Plants, HMG.

Malla, B., Gauchan, D. P, & Chhetri, R. B. (2014). Medico-ethnobotanical investigation in Parbat district of Western Nepal. *Journal of Medicinal Plants Research*, **8** (2):95–108.

Manandhar, N. P. (2002). *Plants and people of Nepal*. Portland: Timber press.

Martial–Didier, A. K., Hubert. K. K., Parfait K. E. J., Constant Y. J. & Kablan T. (2017). Partial purification and characterization of two pectinases (polygalacturonase and pectin lyase) from Papaya Pericarp (*Carica papaya* cv.solo8). *Interanational. Journal of Current. Microbiology. Applied. Sciences*, **6**(6):2729-2739.

Marwal, A., & Gaur, R. K. (2020). *Molecular markers: Tool for genetic analysis*. *Animal Biotechnology* . India: Academic press.

MEDEP (Micro-Enterprise Development Programme). (2010). Value chain based approach to micro-enterprise development:Value chain analysis-'Allo'. Value chain analysis series. Bakhundole, Lalitpur. http://medep.org.np/epanel/publication/docs/Value%20Chain%20Analysis.pdf

Minh, B. Q., Nguyen, M. A. T., & Haeseler, A.V (2013). Ultrafast approximation for phylogenetic bootstrap. *Molecular Biology and Evolution*, **30**(5):1188-1195.

Mohamad, A., Alhasnawi, A. N., Kadhimi, A. A., Isahak, A., Yusoff, W. M. W., & Radziah, C. M. Z. C. (2017). DNA isolation and optimization of ISSR-PCR reaction system in *Oryza sativa* L. *International Journal on Advanced Science, Engineering and Information Technology*, **7**(6):2264.

Mohammadi, S. A., & Prasanna, B. M. (2003). Analysis of genetic diversity in crop plants—salient statistical tools and considerations. *Crop science*, **43**(4):1235-1248.

Mohanty, A. K., Misra, M., & Drzal, L. T. (2001). Surface modifications of natural fibers and performance of the resulting biocomposites: an overview. *Composite Interfaces*, **8**(5): 313-343.

Mondini, L., Noorani, A., & Pagnotta, M. A. (2009). Assessing plant genetic diversity by molecular tools. *Diversity*, **1**(1): 19-35

Monfared, M. A., Samsampour, D., Sharifi-Sirchi, G. R., & Sadeghi, F. (2018). Assessment of genetic diversity in *Salvadora persica* L. based on inter simple sequence repeat (ISSR) genetic marker. *Journal of Genetic Engineering and Biotechnology*, **16(**2): 661–667.

Mujeeb, F., Bajpai, P., Pathak, N., & Verma, S. R. (2017). *Genetic diversity analysis of medicinally important horticultural crop Aegle marmelos by ISSR Markers*. New York: Springer

Mwaikambo, L. Y., & Ansell, M. P. (2002). Chemical modification of hemp, sisal, jute, and kapok fibers by alkalization. *Journal of Applied Polymer Science*, **84**(12):2222-2234.

Nagy, S., Poczai, P., Cernák, I., Gorji, A. M., Hegedűs, G., & Taller, J. (2012). PICcalc: an online program to calculate polymorphic information content for molecular genetic studies. *Biochemical Genetics*, **50**(9-10):670-672.

Negawo, A. T., Jorge, A., Hanson, J., Teshome, A., Muktar, M. S., Azevedo, A. L. S.,Ledo, F. J., Machado, J. C., & Jones, C. S. (2018). Molecular markers as a tool for germplasm acquisition to enhance the genetic diversity of a Napier grass (*Cenchruspurpureus* syn. Pennisetum purpureum) collection. *Tropical Grasslands-Forrajes Tropicales*, **6**(2):58-69.

Nei, M., & Li, W. H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences*, **76**(10): 5269-5273.

Nguyen, L. T., Schmidt, H. A., Von Haeseler, A., & Minh, B. Q. (2015). IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Molecular Biology and Evolution*, **32**(1):268-274.

Njogu, P. M., Thoithi, G. N., Mwangi, J. W., Kamau, F. N., Kibwage, I. O., Kariuki, S. T., Yenesew, A., Mugo, H., & Mwalukumbi, J. M. (2011). Phytochemical and antimicrobial investigation of *Girardinia diversifolia* (Link) Friis (Urticaceae). *East and Central African Journal of Pharmaceutical Sciences*, **14**(3):89-94.

Osena, G., Nyaboga, E. N., & Amugune, N. O. (2017). Rapid and efficient isolation of high quality DNA from cassava (Manihot esculenta Crantz) suitable for PCR based downstream applications. *Annual Research & Review in Biology*, **12**(2):1-10.

Ostrom, E. (2009). A general framework for analyzing sustainability of social-ecological systems. *Science*, **325**(5939): 419-422.

Pandey, P. C., Tiwari, L., & Pande, H. C. (2007). Ethnoveterinary plants of Uttaranchal—A review. *Indian Journal of Traditional Knowledge*, **6**(3):444-458

Pandey, A., Lepcha, U. P., Gaira, K. S., Joshi, R., & Chettri, N. (2020). *Nettle plant (Girardinia diversifolia) fiber extraction and yarn making*. G. B. Pant National Institute of Himalayan Environment, India.

Pangeni, B., Bhattarai, S., Paudyal, H., & Chaudhary, R. P. (2020). Ethnobotanical Study of Magar Ethnic Community of Palpa District of Nepal. *Ethnobotany Research and Applications*, **20**(44):1-17.

Papadopoulou, E., Bikiaris, D., Chrysafis, K., Wladyka-Przybylak, M., Wesolek, D., Mankowski, J. Kolodziej, J., Baraniecki, P., Bujnowicz, K., & Gronberg, V. (2015). Value-added industrial products from bast fiber crops. *Industrial Crops and Products*, **68**:116-125.

Pargai, D., & Gahlot, M. (2020). The utilisation of Himalayan Nettle (*Girardinia diversifolia*) plant for development of UV protective textiles: A new perspective of a traditionally used plant, *Indian Journal of Traditional Knowledge,* **19**(4):910-915

Paridah, M. T., Basher, A. B., SaifulAzry, S., & Ahmed, Z. (2011). Retting process of some bast plant fibers and its effect on fiber quality: a review. *BioResources*, **6**(4): 5260-5281.

Park, Sunkyu, Baker, J. O., Himmel, M. E, Parilla, P. A, & Johnson, D. K. (2010). Cellulose crystallinity index: measurement techniques and their impact on interpreting cellulase performance. *Biotechnology for Biofuels* **3**(1): 10.

Peakall, R., & Smouse, P. E. (2006). GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes*, **6**(1):288-295.

Polunin, O., & Stainton, A. (1984). *Flowers of the Himalaya*. Oxford University Press.

Porebski, S., Bailey, L. G., & Baum, B. R. (1997). Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. *Plant Molecular Biology Reporter*, **15**(1): 8-15.

Prevost, A., & Wilkinson, M. J. (1999). A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. *Theoretical and Applied Genetics*, **98**(1):107-112.

Puchooa, D. (2004). A simple, rapid and efficient method for the extraction of genomic DNA from lychee (*Litchi chinensis* Sonn.). *African Journal of Biotechnology*, **3**(4):253-255.

Pun, Y. B. (2011). *Indigenous knowledge on 'Allo' management, a study on Chhantyal community of Mayadhi District*. Lalitpur: Social Inclusion Research Fund (SIRF) SNV.

Purohit, A. R., Verma, P. U., & Patel, N. J. (2012). Rapid and efficient procedure for isolation of high yielding DNA from castor (*Ricinus communis* L.). *International Journal of Scientific and Research Publications*, **2**(7): 1-4.

Qian, W., Ge, S., & Hong, D. Y. (2001). Genetic variation within and among populations of a wild rice *Oryza granulata* from China detected by RAPD and ISSR markers. *Theoretical and Applied Genetics*, **102**(2-3):440-449

Rajbhandari, K. R., & Rai, S. K. (2017). *A handbook of the flowering plants of Nepal*, Volume I, Kathmandu, Nepal: Department of Plant Resources.

Rambaut A, Suchard M, Xie D, Drummond A. (2014). Tracer v1.6. – Computer program and documentation. Accessed on 10 May 2019. https://www.scirp.org/reference/referencespapers.aspx?referenceid=2565011.

Rao, V. R & Hodgkin, T. (2002). Genetic diversity and conservation and utilization of plant genetic resources. *Plant Cell, Tissue and Organ Culture*, **68**: 1–19

Ratnaparkhe, M. B., Santra, D. K., Tullu, A., & Muehlbauer, F. J. (1998). Inheritance of inter-simple-sequence-repeat polymorphisms and linkage with a fusarium wilt resistance gene in chickpea. *Theoretical and Applied Genetics*, **96**:348-353.

Reddy, M. P., Sarla, N., & Siddiq, E. A. (2002). Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. *Euphytica*, **128 (**1):9-17.

Rejlová, L., Böhmová, A., Chumová, Z., Hořčicová, Š., Josefiová, J., Schmidt, P. A., Trávníček,P., Urfus,T., Vít, Pert & Chrtek, J. (2021). Disparity between

morphology and genetics in *Urtica dioica* (Urticaceae). *Botanical Journal of the Linnean Society*, **195**(4): 606-621.

Rokaya, M. B., Münzbergová, Z., & Timsina, B. (2010). Ethnobotanical study of medicinal plants from the Humla district of western Nepal. *Journal of Ethnopharmacology*, **130**(3):485-504.

Rohlf, F. J. (1997).*NTSYS-pc version. 2.02 i Numerical Taxonomy and Multivariate Analysis System*. NewYork, Applied Biostatistics Inc., Exeter Software: Setauket.

Roldan-Ruiz, I., Dendauw, J., Van Bockstaele, E., Depicker, A., & De Loose, M. A. F. L. P. (2000). AFLP markers reveal high polymorphic rates in ryegrasses (Lolium spp.). *Molecular breeding*, **6**(2): 125-134.

Ronquist, F., Huelsenbeck, J., & Teslenko, M. (2011). Draft MrBayes version 3.2 manual: tutorials and model summaries. *Distributed with the software from http://brahms. biology. rochester. edu/software. html.* Accessed on (03/07/ 2021)

Rowell, R. M. (2014). *The use of biomass to produce bio-based composites and building materials. In Advances in Biorefineries*. Woodhead Publishing.

Runqing, Y., Baloch, S. U., Lijun, L., Qihui, P., Songlin, G., Xinyue, Z., Bo, W., & Dingxiang, P. (2015). The phylogenetic relationships among germplasm resources of wild ramie (*Boehmeria nivea* l. gaud) in China based on *trn*l-f and its sequences. *Pakistan Journal of Botany*, **47**(4): 1451-1457.

Sá, O., Pereira, J. A., & Baptista, P. (2011). Optimization of DNA extraction for RAPD and ISSR analysis of *Arbutus unedo* L. leaves. *International Journal of Molecular Sciences,* **12**(6): 4156-4164.

Saleem, Z., Rennebaum, H., Pudel, F., & Grimm, E. (2008). Treating bast fibres with pectinase improves mechanical characteristics of reinforced thermoplastic composites. *Composites Science and Technology*, **68**(2): 471-476.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: a laboratory manual* (No. Ed. 2). Cold spring harbor laboratory press.

Sane.A., Kareem, V.K.A., Rajasekharan, P.E., & Kumar, T.V. (2012). Optimization of DNA Isolation and PCR Protocol for ISSR Analysis of *Nothapodytes nimmoniana*: A Threatened Anti-cancerous Medicinal Plant. *Asian Journal of Biotechnology*, **4**(2): 100-107.

Sarrazola, J. H., & Alzate, F. A. (2019). Obtaining DNA from Urticaceae: Overcoming the challenges associated with chemical compounds and herbarium specimens. *International Journal of Molecular Biology*, **4**(5):158-165.

Sarwat, M., Nabi, G., Das, S., & Srivastava, P. S. (2012). Molecular markers in medicinal plant biotechnology: past and present. *Critical Reviews in Biotechnology*, **32**(1):74-92.

Sawpan, M. A., Pickering, K. L., & Fernyhough, A. (2011). Effect of various chemical treatments on the fiber structure and tensile properties of industrial hemp fibers. *Composites Part A: Applied Science and Manufacturing*, **42**(8): 888-895.

Segal, L., Creely, J. J., Martin, A. E., & Conrad, C. M. (1959). An empirical method for estimating the degree of crystallinity of native cellulose using the X-ray diffractometer. *Textile Research Journal*, **29**(10): 786-794.

Sethmann, A., & Dreyling, G. (2001). *Girardinia diversifolia* (LINK) FRIIS-morphological and anatomical characteristics of a fiber plant. *Journal of Applied Botany* **75**: 112-117.

Sethmann, A., (2004). *Girardinia diversifolia* (L INK) F RIIS (Urticaceae)- a new fiber plant -Studies on the morphological and mechanical fiber characteristics (Doctoral dissertation). Department of Biology the University of Hamburg, Germany

Sett, S. K., Ray, S. C., & Mukherjee, A. (2016). Processing of Himalayan Giant Nettle (*Girardinia diversifolia*) and its Potential Uses in Textile Applications. https://www.academia.edu/10362418/Processing_of_Himalayan_Giant_Nettle_*Girardinia_Diversifolia*_and_its_Potential_uses_in_Textile_Applications. Accessed on 03/07/2021

Shannon, C. E. (1948). A mathematical theory of communication. *The Bell system Technical Journal*, **27**(3): 379-423.

Shrestha, A. J., Adhikari, L., Amatya, R., Subedee, B. R. & Dorji, T. (2018). Allo value chain in Darchula, Nepal: Process documentation. ICIMOD Working Paper 2018/9. Kathmandu: ICIMOD.

Shrestha, K. K., Bhandari, P., & Bhattarai, S. (2022). *Plants of Nepal (Gymnosperms and Angiosperms)*, Heritage publisher and distributors Pvt Ltd, Kathmandu, Nepal.

Shrestha, B. (1994). *Dye-Yielding Plants of Nepal*. Kathmandu: Research Centre for Applied Science and Technology, Tribhuvan University.

Shrestha, R., & T. Hoshion. (1998). Karyomorphological studies in *Girardinia diversifolia* (Link) Friis (Urticaceae) collected from Nepal. *Journal of Japanese Botany*,**73** (3):125–27.

Shrestha, S. S., Sut, S., Ferrarese, I., Barbon Di Marco, S., Zengin, G., Franco, M. D., Pant, D.R., Mahomoodaily, M. F., Ferri, N., Biancorosso, N., Maggi, F., Acqua, S. D., & Rajbhandary, S. (2020). Himalayan Nettle *Girardinia diversifolia* as a Candidate Ingredient for Pharmaceutical and Nutraceutical Applications—Phytochemical Analysis and In Vitro Bioassays. *Molecules*, **25**(7): 1563.

Shrestha, S., Shrestha, J., & Shah, K. K. (2020). Non-timber Forest products and their role in the livelihoods of people of Nepal: a critical review. *Grassroots Journal of Natural Resources*, **3**(2): 42-56.

Shu, X. Z. C., Jiarui, C., Friis, Ib., & Wilmot-Dear, M (2003) *Girardinia Gaudichaud-Beaupre. In: Flora of China* (eds. Z.Wu, P. H. Raven, & D. Hong, Volume 5: *Ulmaceae through Basellaceae*. Science Press, 5: 90-91.

Shukla, R., Sharma, D. C., Pathak, N., & Bajpai, P. (2018). Genomic DNA Isolation from High Polyphenolic Content *Grewia asiatica* L. Leaf Without Using Liquid Nitrogen. *Iranian Journal of Science and Technology, Transactions A: Science*, **42**(2): 347-351.

Sigdel, S. R., Rokaya, M. B., & Timsina, B. (2013). Plant inventory and ethnobotanical study of Khimti hydropower project, central Nepal. *Scientific World*, **11**(11): 105-112.

Silva, F. A., Chawla, N., & Filho. R. D. T. (2008). Tensile behavior of high performance natural (sisal) fibers. *Composites Science and Technology* **68**(15):3438-3443.

Sim, S. F., Mohamed, M., Lu, N. A. L. M. I., Sarman, N. S. P., & Samsudin, S. N. S. (2012). Computer-assisted analysis of fourier transform infrared (FTIR) spectra for characterization of various treated and untreated agriculture biomass. *BioResources*, **7**(4):5367-5380.

Singh, S. C., & R. Shrestha. (1984). *Investigation on under-exploited plant fibers of Nepal*. Kathmandu: Research Centre for Applied Science and Technology. Tribhuvan University.

Singh, S. C., & R. Shrestha. (1988). *Girardinia diversifolia* (Urticaceae), a non-conventional fiber resource in Nepal. *Economic Botany*, **42**(3):445–447.

Slatkin, M. (1985). Rare alleles as indicators of gene flow. *Evolution*, **39**(1), 53-65.

Sokal, R. R. & Michener, C (1958). A statistical method for evaluating systematic relationships. *University of Kansas Science Bulletin*, **38**:1409-1438.

Souza, S. G. H. D., Carpentieri-Pípolo, V., Ruas, C. D. F., Carvalho, V. D. P., Ruas, P. M., & Gerage, A. C. (2008). Comparative analysis of genetic diversity

among the maize inbred lines (*Zea mays* L.) obtained by RAPD and SSR markers. *Brazilian archives of Biology and Technology*, **51**(1):183-192.

Specht, M. J., Pinto. S. R. R., Albuquerque. P. U.,. Tabarelli. M, & Melo, F. P. L. (2015). Burning biodiversity: Fuelwood harvesting causes forest degradation in human-dominated tropical landscapes. *Global Ecology and Conservation*, **3**:200–09.

Srinivasa, C. V., Arifulla, A., Goutham, N., Santhosh, T., Jaeethendra, H. J., Ravikumar, R. B., Anil, S.G., Kumar, S. D. G. & Ashish, J. (2011). Static bending and impact behaviour of areca fibers composites. *Materials & Design*, **32**(4):2469-2475.

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*, **30**(9):1312-1313.

Subedee, B. R., Tripathi, G. R., Munankarmi, N. N., & Chaudhary, R. P. (2022). Genetic diversity in populations of *Girardinia diversifolia* from Nepal Himalaya using ISSR markers. *Ecological Genetics and Genomics*, **23**: 100120.

Subedee, B. R., Chaudhary, R. P., Uprety, Y., & Dorji, T. (2020). Socio-ecological perspectives of Himalayan giant nettle (*Girardinia diversifolia* (link) Friis) in Nepal. *Journal of Natural Fibers*, **17**(1):1-10

Subedee, B. R., Tripathi, G. R., & Chaudhary, R. P. (2020). DNA isolation and optimization of PCR protocol for ISSR analysis of *Girardinia diversifolia*: A medicinal and economic plant species from Nepal Himalaya. *African Journal of Biotechnology*, **19**(10):747-753.

Subedee, B. R., Chaudhary, R. P. & Shrestha. K. R (2017). Use of Rocket stove for firewood savings and carbon emission reductions by the households involved in Allo (*Girardinia diversifolia*) fiber processing at Khar VDC, Darchula District, Nepal. *International Journal of Latest Engineering and Management Research*, **2** (9):28–35.

Subedee, B. R., Chaudhary, R. P. Dorji. T., & Shrestha. A. J. (2017). Indigenous and local knowledge of conservation and sustainable use of Himalaya Giant Nettle (*Girardinia diversifolia* (Link) Friss) in: Eastern and Far-Western Regions of Nepal. In: Knowing our lands and resources, eds. M. Karki, R. Hill, W. Alangui, K. Ichikawa, & P. Bridgewater, 191–98. Paris: United Nations Educational, Scientific and Cultural Organization (UNESCO).

Suriaman, I., Hendrarsakti, J., Mardiyati, Y., & Pasek, A. D. (2021). The effect of alkali treatment on improving the mechanical properties of ramie (*Boehmeria nivea*), sugar palm (*Arenga Pinnata*), and coir (*Cocos nucifera*) fibers. *ASEAN Engineering Journal*, **11**(3): 177-189.

Surya.S & Hari. N. (2017). Use of Noncoding Plastid Marker trnL-F as DNA Barcode for Identification of True Mangrove Genus Rhizophora. *International Journal of Scientific Research in Science, Engineering and Technology*, **3**(8): 830-834

Swofford, D. L. (2002). Phylogenetic analysis using parsimony (and other methods) V 4.0 b 10. *Sinaur Associates, Sunderland, Massachusetts*. http://citeseer.ist.psu.edu/viewdoc/download;jsessionid=CEA0E4C6999032E08627DEF9DFC37ABE?doi=10.1.1.458.6867&rep=rep1&type=pdf Accessed on (10/03/2020)

Taberlet, P., Gielly, L., Pautou, G., & Bouvet, J. (1991). Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology*, **17**(5): 1105-1109.

Thapa, S. (2012). Medico-ethnobotany of Magar community in Salija VDC of Parbat district, central Nepal. *Our nature*, **10**(1): 176-190.

Thompson, J. D., Higgins, D. G., & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research*, **22(**22):4673-4680.

Tian, H. Z., Han, L. X., Zhang, J. L., Li, X. L., Kawahara, T., Yukawa, T., Pujol, J.L., Kumar, P., Chung, M.G., & Chung, M. Y. (2018). Genetic diversity in the endangered terrestrial orchid *Cypripedium japonicum* in East Asia: Insights into population history and implications for conservation. *Scientific Reports*, **8**(1): 1-13.

Tiwari, K. L., Jadhav, S. K., & Gupta, S. (2012). Modified CTAB technique for isolation of DNA from some medicinal plants. *Research Journal of Medicinal Plant*, **6**(1): 65-73.

Transue, D. K., Fairbanks, D. J., Robison, L. R., & Andersen, W. R. (1994). Species Identification by RAPD analysis of Grain Amaranth Genetic Resources. *Crop Science*, **34**(5): 1385-1389.

Tsuda, Y., Goto, S., & Ide, Y. (2004). RAPD analysis of genetic variation within and among four natural populations of *Betula maximowicziana*. *Silvae Genetica*, **53**(5): 234-239.

Valentini, A., Pompanon, F., & Taberlet, P. (2009). DNA barcoding for ecologists. *Trends in Ecology & Evolution*, **24** (2): 110-117.

Varshney, R. K., Chabane, K., Hendre, P. S., Aggarwal, R. K., & Graner, A. (2007). Comparative assessment of EST-SSR, EST-SNP and AFLP markers for evaluation of genetic diversity and conservation of genetic resources using wild, cultivated and elite barleys. *Plant Science*, **173**(6): 638-649.

Vellend, M., & Geber, M. A. (2005). Connections between species diversity and genetic diversity. *Ecology letters*, **8**(7): 767-781.

Verma, K. S., Haq, S., Kachhwaha, S., & Kothari, S. L. (2017). RAPD and ISSR marker assessment of genetic diversity in *Citrullus colocynthis* (L.) Schrad: a unique source of germplasm highly adapted to drought and high-temperature stress. *3 Biotech*, **7**(5): 1-24.

Weising, K., Nybom, H., Pfenninger, M., Wolff, K., & Kahl, G. (2005). *DNA fingerprinting in plants: principles, methods, and applications*. London.: CRC press,

Wright, S. (1949). The genetical structure of populations, *Annals of Eugenics*.**15**:323-354.

Wu, Z. Y., Milne, R. I., Chen, C. J., Liu, J., Wang, H., & Li, D. Z. (2015). Ancestral state reconstruction reveals rampant homoplasy of diagnostic morphological characters in Urticaceae, conflicting with current classification schemes. *PloS one*, **10**(11): e0141821.

Wu, Z.Y., Monro, A. K., Milne, R. I., Wang, H., Yi, T.S., Liu,J. & Li, D.Z. (2013). Molecular phylogeny of the nettle family (Urticaceae) inferred from multiple loci of three genomes and extensive generic sampling. *Molecular. Phylogenetics and Evolution*. **69** (3): 814–827.

Yang, H. Q., Dong, Y. R., Gu, Z. J., Liang, N., & Yang, J. B. (2012). A Preliminary Assessment of matK, rbcL and trnH—psbA as DNA Barcodes for Calamus (Arecaceae) Species in China with a note on ITS. *Annales Botanici Fennici*, **49**(5): 319-330

Yeh, F. C., Yang, R. C., Boyle T. B. J., Ye, Z., Xiyan, J. M. (2000). PopGene32, Microsoft Windows-Based Freeware for Population Genetic Analysis, Version 1.32. Molecular Biology and Biotechnology Centre, University of Alberta, Edmonton, Alberta, Canada.

Yu, J., Xue, J. H., & Zhou, S. L. (2011). New universal *matK* primers for DNA barcoding angiosperms. *Journal of Systematics and Evolution*, **49**(3):176-181.

Yuan, Q. J., Zhang, B., Jiang, D., Zhang, W. J., Lin, T. Y., Wang, N. H., Chiou, S.J., & Huang, L. Q. (2015). Identification of species and materia medica within *Angelica* L. (Umbelliferae) based on phylogeny inferred from DNA barcodes. *Molecular Ecology Resources*, **15**(2):358-371.

Zhang, L. G., Chang, Y., Zhang, X. F., Guan, F. Z., Yuan, H. M., Yu, Y., & Zhao, L. J. (2014). Analysis of the genetic diversity of Chinese native *Cannabis sativa* cultivars by using ISSR and chromosome markers. *Genetics and Molecular Research*, **13**(4):490-500.

Zhao, H., Kwak, J. H., Zhang, Z. C., Brown, H. M., Arey, B. W., & Holladay, J. E. (2007). Studying cellulose fiber structure by SEM, XRD, NMR and acid hydrolysis. *Carbohydrate Polymers*, **68**(2):235-241.

Zietkiewicz, E., Rafalski, A., & Labuda, D. (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics*, **20(**2):176-183.

APPENDICES

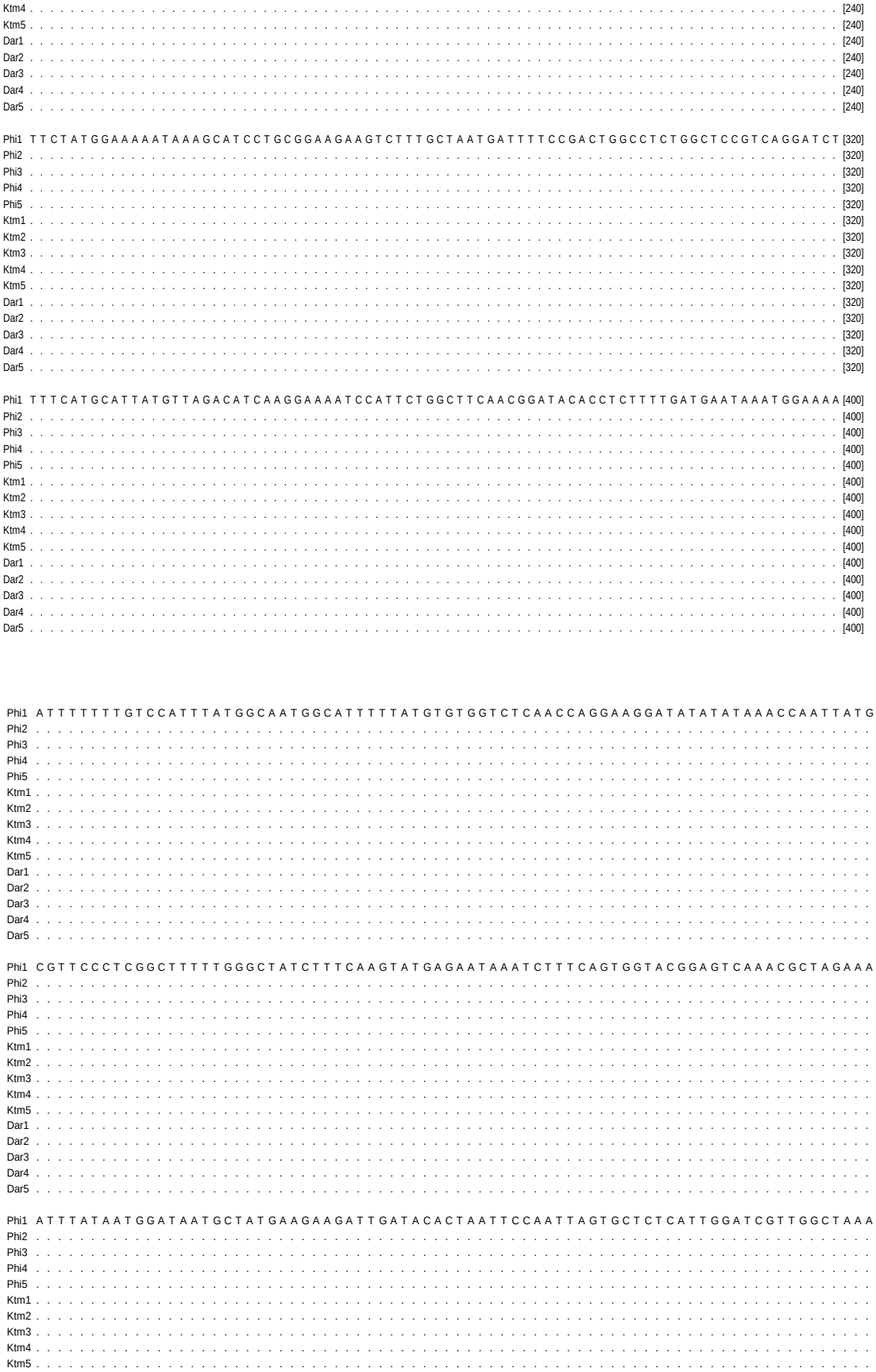
Appendix 1

CLUSTAL W of *matk* sequences from *G.diversifolia*

```
Phi1  GCCTCTTCTTTGCATTTATTAGGGCTTTTTCTTCATAAGTATTATAATTGTAATCGTTTTATTAGTACAAAAAAATTTCC [80]
Phi2  . . . . . [80]
Phi3  . . . . . [80]
Phi4  . . . . . [80]
Phi5  . . . . . [80]
Ktm1  . . . . . T . . . . . [80]
Ktm2  . . . . . T . . . . . [80]
Ktm3  . . . . . T . . . . . [80]
Ktm4  . . . . . T . . . . . [80]
Ktm5  . . . . . T . . . . . [80]
Dar1  . . . . . [80]
Dar2  . . . . . [80]
Dar3  . . . . . [80]
Dar4  . . . . . [80]
Dar5  . . . . . [80]

Phi1  AAATAAATCTATTACTTTTTTTTTCAAAGAGTAATCCAAGATTTTTCTTGTTCCTGTATAATTATCATGTTTGTGGATACG [160]
Phi2  . . . . . [160]
Phi3  . . . . . [160]
Phi4  . . . . . [160]
Phi5  . . . . . [160]
Ktm1  . . . . . [160]
Ktm2  . . . . . [160]
Ktm3  . . . . . [160]
Ktm4  . . . . . [160]
Ktm5  . . . . . [160]
Dar1  . . . . . [160]
Dar2  . . . . . [160]
Dar3  . . . . . [160]
Dar4  . . . . . [160]
Dar5  . . . . . [160]

Phi1  AATCTATCTTACTTTTTCTCTGCAACCAATCTTCTCATTTACGATTAACATCTTCTGGTGTGTTTTTTGAGCGAATATTT [240]
Phi2  . . . . . [240]
Phi3  . . . . . [240]
Phi4  . . . . . [240]
Phi5  . . . . . [240]
Ktm1  . . . . . [240]
Ktm2  . . . . . [240]
Ktm3  . . . . . [240]
```


Ktm4 [240]
Ktm5 [240]
Dar1 [240]
Dar2 [240]
Dar3 [240]
Dar4 [240]
Dar5 [240]
Phi1 TTCTATGGAAAAATAAAGCATCCTGCGGAAGAAGTCTTTGCTAATGATTTTCCGACTGGCCTCTGGCTCCGTCAGGATCT [320]
Phi2 [320]
Phi3 [320]
Phi4 [320]
Phi5 [320]
Ktm1 [320]
Ktm2 [320]
Ktm3 [320]
Ktm4 [320]
Ktm5 [320]
Dar1 [320]
Dar2 [320]
Dar3 [320]
Dar4 [320]
Dar5 [320]
Phi1 TTTCATGCATTATGTTAGACATCAAGGAAAATCCATTCTGGCTTCAACGGATACACCTCTTTTGATGAATAAATGGAAAA [400]
Phi2 [400]
Phi3 [400]
Phi4 [400]
Phi5 [400]
Ktm1 [400]
Ktm2 [400]
Ktm3 [400]
Ktm4 [400]
Ktm5 [400]
Dar1 [400]
Dar2 [400]
Dar3 [400]
Dar4 [400]
Dar5 [400]
Phi1 ATTTTTTTTGTCCATTTATGGCAATGGCATTTTTATGTGTGGTCTCAACCAGGAAGGATATATATAAACCAATTATGTAAG [480]
Phi2 [480]
Phi3 [480]
Phi4 [480]
Phi5 [480]
Ktm1 [480]
Ktm2 [480]
Ktm3 [480]
Ktm4 [480]
Ktm5 [480]
Dar1 [480]
Dar2 [480]
Dar3 [480]
Dar4 [480]
Dar5 [480]
Phi1 CGTTCCCTCGGCTTTTTGGGCTATCTTTCAAGTATGAGAATAAATCTTTCAGTGGTACGGAGTCAAACGCTAGAAAATTC [560]
Phi2 [560]
Phi3 [560]
Phi4 [560]
Phi5 [560]
Ktm1 [560]
Ktm2 [560]
Ktm3 [560]
Ktm4 [560]
Ktm5 [560]
Dar1 [560]
Dar2 [560]
Dar3 [560]
Dar4 [560]
Dar5 [560]
Phi1 ATTTATAATGGATAATGCTATGAAGAAGATTGATACACTAATTCCAATTAGTGCTCTCATTGGATCGTTGGCTAAAATGA [640]
Phi2 [640]
Phi3 [640]
Phi4 [640]
Phi5 [640]
Ktm1 [640]
Ktm2 [640]
Ktm3 [640]
Ktm4 [640]
Ktm5 [640]

```
Dar1 . . . . . [640]
Dar2 . . . . . [640]
Dar3 . . . . . [640]
Dar4 . . . . . [640]
Dar5 . . . . . [640]

Phi1 A A T T T T G T A A C G T A T T A G G G C A T C C C A T T A G T A A G T C G A C C T G G G C A G A T T C A T C A G A T T T T G A T A T T A T T G A C C G A T T T [720]
Phi2 . . . . . [720]
Phi3 . . . . . [720]
Phi4 . . . . . [720]
Phi5 . . . . . [720]
Ktm1 . . . . . [720]
Ktm2 . . . . . [720]
Ktm3 . . . . . [720]
Ktm4 . . . . . [720]
Ktm5 . . . . . [720]
Dar1 . . . . . [720]
Dar2 . . . . . [720]
Dar3 . . . . . [720]
Dar4 . . . . . [720]
Dar5 . . . . . [720]

Phi1 G C G C G T A T A T G C A G A A [736]
Phi2 . . . . . [736]
Phi3 . . . . . [736]
Phi4 . . . . . [736]
Phi5 . . . . . [736]
Ktm1 . . . . . [736]
Ktm2 . . . . . [736]
Ktm3 . . . . . [736]
Ktm4 . . . . . [736]
Ktm5 . . . . . [736]
Dar1 . . . . . [736]
Dar2 . . . . . [736]
Dar3 . . . . . [736]
Dar4 . . . . . [736]
Dar5 . . . . . [736]
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A dot represents the same nucleotide for the samples of *G. diversifolia*

Appendix 2

Dice Coefficient Similarity matrix

	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	J1	J2	J3	J4	J5	J6	J7	J8	J9	J10	k1	k2	k3	k4	k5	k6	k7	k8	k9	k10	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	k1	k2	k3	k4	k5
D1	1.00																																												
D2	0.96	1.00																																												
D3	0.90	0.90	1.00																																										
D4	0.83	0.82	0.89	1.00																																										
D5	0.87	0.87	0.90	0.86	1.00																																								
D6	0.82	0.80	0.85	0.80	0.84	1.00																																							
D7	0.83	0.81	0.86	0.79	0.85	0.97	1.00																																						
D8	0.80	0.78	0.85	0.79	0.83	0.95	0.98	1.00																																					
D9	0.81	0.79	0.84	0.80	0.82	0.94	0.95	0.97	1.00																																				
D10	0.81	0.79	0.86	0.75	0.82	0.92	0.93	0.95	0.96	1.00																																			
J1	0.49	0.51	0.56	0.56	0.57	0.55	0.54	0.53	0.52	0.52	1.00																																		
J2	0.47	0.47	0.55	0.54	0.54	0.53	0.53	0.52	0.51	0.51	0.89	1.00																																	
J3	0.43	0.43	0.47	0.48	0.53	0.48	0.47	0.44	0.43	0.43	0.83	0.81	1.00																																
J4	0.38	0.40	0.46	0.48	0.49	0.47	0.46	0.45	0.44	0.44	0.77	0.76	0.75	1.00																															
J5	0.42	0.46	0.46	0.53	0.51	0.45	0.45	0.45	0.44	0.39	0.63	0.67	0.72	0.91	1.00																															
J6	0.45	0.45	0.53	0.47	0.50	0.50	0.49	0.46	0.44	0.48	0.72	0.75	0.73	0.73	0.57	1.00																															
J7	0.45	0.45	0.53	0.47	0.50	0.50	0.49	0.46	0.44	0.48	0.72	0.75	0.73	0.73	0.57	1.00	1.00																														
J8	0.44	0.46	0.54	0.48	0.49	0.53	0.52	0.51	0.49	0.54	0.78	0.74	0.65	0.77	0.63	0.81	0.81	1.00																														
J9	0.46	0.51	0.55	0.53	0.55	0.54	0.54	0.51	0.50	0.53	0.77	0.71	0.65	0.74	0.65	0.80	0.80	0.90	1.00																												
J10	0.45	0.49	0.54	0.51	0.53	0.53	0.52	0.50	0.48	0.52	0.76	0.70	0.63	0.73	0.65	0.80	0.80	0.91	0.99	1.00																											
k1	0.51	0.52	0.52	0.40	0.54	0.56	0.57	0.56	0.55	0.59	0.49	0.45	0.40	0.48	0.47	0.47	0.47	0.51	0.48	0.49	1.00																									
k2	0.51	0.53	0.52	0.40	0.54	0.52	0.53	0.52	0.51	0.55	0.52	0.48	0.43	0.51	0.50	0.52	0.52	0.51	0.49	0.49	0.94	1.00																								
k3	0.57	0.57	0.58	0.43	0.59	0.55	0.56	0.55	0.54	0.58	0.51	0.47	0.43	0.52	0.56	0.49	0.49	0.50	0.50	0.51	0.90	0.94	1.00																							
k4	0.55	0.53	0.54	0.39	0.55	0.53	0.54	0.53	0.52	0.56	0.51	0.47	0.43	0.51	0.44	0.45	0.45	0.44	0.44	0.43	0.87	0.91	0.88	1.00																						
k5	0.57	0.55	0.55	0.41	0.57	0.54	0.56	0.55	0.53	0.57	0.51	0.49	0.45	0.52	0.49	0.45	0.45	0.46	0.46	0.44	0.84	0.83	0.85	0.93	1.00																					
k6	0.49	0.47	0.46	0.35	0.48	0.46	0.47	0.46	0.46	0.48	0.39	0.38	0.37	0.49	0.43	0.42	0.42	0.38	0.39	0.39	0.67	0.68	0.72	0.72	0.76	1.00																				
k7	0.55	0.53	0.52	0.41	0.53	0.51	0.52	0.51	0.52	0.54	0.43	0.41	0.39	0.51	0.42	0.43	0.43	0.42	0.42	0.43	0.70	0.71	0.74	0.76	0.79	0.95	1.00																			
k8	0.52	0.50	0.49	0.38	0.50	0.48	0.50	0.48	0.49	0.51	0.40	0.38	0.38	0.47	0.42	0.40	0.40	0.39	0.39	0.40	0.70	0.68	0.71	0.73	0.76	0.95	0.98	1.00																	
k9	0.54	0.52	0.49	0.38	0.52	0.50	0.50	0.49	0.49	0.51	0.40	0.38	0.40	0.50	0.41	0.42	0.42	0.39	0.40	0.40	0.67	0.68	0.72	0.74	0.77	0.95	0.96	0.94	1.00																
k10	0.54	0.52	0.51	0.40	0.52	0.50	0.51	0.51	0.51	0.53	0.42	0.38	0.36	0.49	0.43	0.40	0.40	0.41	0.41	0.42	0.71	0.71	0.75	0.77	0.80	0.93	0.96	0.94	0.94	1.00															
P1	0.32	0.30	0.40	0.36	0.34	0.35	0.35	0.34	0.32	0.34	0.46	0.44	0.42	0.43	0.22	0.44	0.44	0.38	0.39	0.39	0.37	0.37	0.43	0.37	0.34	0.42	0.39	0.40	0.38	0.40	1.00														
P2	0.32	0.29	0.40	0.36	0.34	0.35	0.35	0.33	0.32	0.34	0.47	0.46	0.46	0.45	0.27	0.46	0.46	0.42	0.38	0.39	0.39	0.39	0.42	0.39	0.36	0.39	0.37	0.37	0.36	0.37	0.94	1.00													
P3	0.33	0.31	0.42	0.40	0.36	0.41	0.40	0.39	0.38	0.40	0.55	0.56	0.55	0.59	0.39	0.56	0.56	0.53	0.48	0.49	0.49	0.47	0.48	0.45	0.46	0.45	0.45	0.46	0.44	0.46	0.75	0.79	1.00												
P4	0.35	0.35	0.41	0.39	0.41	0.42	0.44	0.42	0.39	0.39	0.50	0.51	0.51	0.45	0.37	0.48	0.48	0.43	0.45	0.46	0.34	0.35	0.37	0.33	0.35	0.42	0.40	0.40	0.39	0.40	0.58	0.62	0.70	1.00											
P5	0.30	0.27	0.37	0.36	0.30	0.34	0.36	0.34	0.30	0.32	0.47	0.48	0.48	0.42	0.33	0.48	0.48	0.41	0.42	0.42	0.29	0.29	0.29	0.31	0.30	0.33	0.31	0.31	0.30	0.31	0.58	0.63	0.69	0.77	1.00										
P6	0.34	0.31	0.39	0.36	0.33	0.36	0.40	0.39	0.35	0.37	0.47	0.45	0.48	0.40	0.33	0.40	0.40	0.36	0.37	0.37	0.35	0.36	0.34	0.38	0.37	0.38	0.40	0.41	0.37	0.41	0.57	0.59	0.67	0.68	0.82	1.00									
P7	0.34	0.30	0.41	0.35	0.35	0.36	0.40	0.38	0.34	0.37	0.51	0.49	0.49	0.41	0.29	0.44	0.44	0.41	0.41	0.42	0.32	0.33	0.36	0.40	0.39	0.40	0.40	0.40	0.39	0.43	0.64	0.67	0.71	0.72	0.82	0.90	1.00							
P8	0.31	0.28	0.37	0.35	0.31	0.32	0.36	0.35	0.31	0.33	0.52	0.50	0.53	0.44	0.34	0.45	0.45	0.42	0.42	0.43	0.34	0.35	0.33	0.36	0.36	0.36	0.38	0.39	0.35	0.39	0.63	0.67	0.73	0.69	0.82	0.92	0.95	1.00						
P9	0.35	0.33	0.38	0.39	0.33	0.30	0.34	0.33	0.31	0.31	0.43	0.39	0.42	0.33	0.34	0.34	0.34	0.33	0.33	0.34	0.31	0.32	0.28	0.31	0.31	0.37	0.39	0.40	0.38	0.40	0.47	0.51	0.61	0.62	0.71	0.86	0.82	0.84	1.00				
P10	0.35	0.32	0.37	0.36	0.33	0.31	0.36	0.34	0.32	0.32	0.42	0.40	0.43	0.33	0.29	0.36	0.36	0.32	0.33	0.33	0.29	0.29	0.29	0.33	0.33	0.36	0.35	0.36	0.37	0.39	0.53	0.58	0.64	0.67	0.81	0.90	0.87	0.87	0.91	1.00			
k1	0.51	0.45	0.47	0.38	0.47	0.46	0.45	0.44	0.42	0.47	0.38	0.40	0.34	0.40	0.35	0.44	0.44	0.45	0.43	0.43	0.46	0.46	0.55	0.53	0.54	0.50	0.49	0.48	0.51	0.53	0.32	0.34	0.39	0.38	0.35	0.38	0.43	0.36	0.36	0.38	1.00		
k2	0.54	0.52	0.53	0.44	0.50	0.49	0.49	0.48	0.46	0.51	0.45	0.46	0.37	0.40	0.33	0.48	0.48	0.49	0.47	0.47	0.39	0.40	0.47	0.45	0.47	0.50	0.49	0.48	0.51	0.48	0.34	0.39	0.38	0.42	0.38	0.37	0.42	0.36	0.36	0.38	0.87	1.00	
k3	0.52	0.49	0.50	0.39	0.48	0.47	0.48	0.49	0.45	0.50	0.41	0.43	0.33	0.38	0.35	0.43	0.43	0.48	0.44	0.44	0.42	0.42	0.51	0.48	0.50	0.48	0.48	0.46	0.49	0.49	0.30	0.33	0.30	0.39	0.39	0.33	0.38	0.32	0.31	0.33	0.85	0.94	1.00
k4	0.45	0.48	0.47	0.40	0.47	0.43	0.45	0.44	0.40	0.42	0.49	0.47	0.44	0.42	0.34	0.47	0.47	0.49	0.48	0.48	0.36	0.37	0.40	0.38	0.40	0.35	0.35	0.33	0.36	0.36	0.37	0.39	0.46	0.56	0.46	0.40	0.48	0.46	0.42	0.46	0.60	0.72	0.70	1.00
k5	0.35	0.37	0.40	0.34	0.40	0.36	0.38	0.39	0.35	0.37	0.45	0.48	0.39	0.44	0.27	0.42	0.42	0.50	0.48	0.48	0.31	0.31	0.35	0.33	0.35	0.33	0.32	0.33	0.34	0.33	0.32	0.31	0.39	0.50	0.38	0.34	0.40	0.38	0.33	0.38	0.53	0.66	0.67	0.86	1.00

Appendix 3

Jaccard coefficient Similarity matrix

	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	J1	J2	J3	J4	J5	J6	J7	J8	J9	J10	k1	k2	k3	k4	k5	k6	k7	k8	k9	k10	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	k1	k2	k3	k4	k5
D1	1																																												
D2	0.91	1.00																																											
D3	0.81	0.81	1.00																																										
D4	0.71	0.69	0.80	1.00																																										
D5	0.77	0.77	0.81	0.75	1.00																																								
D6	0.69	0.66	0.73	0.67	0.73	1.00																																							
D7	0.71	0.68	0.75	0.65	0.75	0.95	1.00																																						
D8	0.67	0.64	0.75	0.65	0.71	0.91	0.96	1.00																																					
D9	0.68	0.66	0.73	0.66	0.70	0.89	0.91	0.94	1.00																																				
D10	0.68	0.66	0.76	0.60	0.70	0.86	0.88	0.91	0.92	1.00																																			
J1	0.32	0.34	0.39	0.38	0.40	0.38	0.37	0.36	0.35	0.35	1.00																																		
J2	0.31	0.31	0.38	0.37	0.37	0.36	0.36	0.35	0.34	0.34	0.80	1.00																																	
J3	0.28	0.28	0.31	0.31	0.36	0.32	0.31	0.28	0.27	0.27	0.70	0.69	1.00																																
J4	0.23	0.25	0.29	0.32	0.33	0.30	0.30	0.29	0.28	0.28	0.62	0.61	0.60	1.00																															
J5	0.27	0.30	0.30	0.36	0.34	0.29	0.29	0.29	0.28	0.24	0.46	0.50	0.57	0.83	1.00																														
J6	0.29	0.29	0.36	0.31	0.33	0.33	0.32	0.30	0.29	0.32	0.56	0.60	0.57	0.58	0.40	1.00																													
J7	0.29	0.29	0.36	0.31	0.33	0.33	0.32	0.30	0.29	0.32	0.56	0.60	0.57	0.58	0.40	1.00	1.00																												
J8	0.28	0.30	0.37	0.32	0.32	0.36	0.35	0.34	0.33	0.37	0.64	0.59	0.48	0.63	0.46	0.68	0.68	1.00																											
J9	0.30	0.34	0.38	0.36	0.38	0.37	0.37	0.34	0.33	0.36	0.62	0.56	0.48	0.59	0.48	0.66	0.66	0.81	1.00																										
J10	0.29	0.32	0.37	0.34	0.36	0.36	0.36	0.33	0.32	0.35	0.61	0.54	0.46	0.57	0.48	0.67	0.67	0.83	0.98	1.00																								
k1	0.34	0.35	0.35	0.25	0.37	0.38	0.40	0.39	0.38	0.42	0.32	0.29	0.25	0.32	0.31	0.31	0.31	0.34	0.32	0.32	1.00																							
k2	0.34	0.36	0.35	0.25	0.37	0.35	0.36	0.35	0.34	0.38	0.35	0.31	0.28	0.34	0.33	0.35	0.35	0.34	0.32	0.33	0.89	1.00																						
k3	0.40	0.40	0.41	0.27	0.42	0.38	0.39	0.38	0.37	0.41	0.34	0.31	0.27	0.35	0.38	0.32	0.32	0.33	0.33	0.34	0.83	0.89	1.00																					
k4	0.38	0.36	0.37	0.24	0.38	0.36	0.37	0.36	0.35	0.39	0.34	0.31	0.28	0.34	0.29	0.29	0.29	0.28	0.29	0.27	0.78	0.83	0.79	1.00																				
k5	0.40	0.38	0.38	0.26	0.39	0.37	0.39	0.38	0.36	0.40	0.34	0.32	0.29	0.35	0.32	0.29	0.29	0.30	0.30	0.29	0.73	0.71	0.75	0.87	1.00																			
k6	0.33	0.31	0.30	0.21	0.32	0.29	0.31	0.30	0.30	0.32	0.24	0.23	0.23	0.32	0.28	0.26	0.26	0.24	0.24	0.24	0.51	0.52	0.57	0.56	0.61	1.00																			
k7	0.38	0.36	0.35	0.26	0.36	0.34	0.36	0.35	0.35	0.37	0.27	0.26	0.24	0.34	0.27	0.28	0.28	0.27	0.27	0.27	0.54	0.55	0.58	0.61	0.65	0.90	1.00																		
k8	0.35	0.33	0.32	0.23	0.34	0.32	0.33	0.32	0.32	0.34	0.25	0.23	0.23	0.31	0.27	0.25	0.25	0.24	0.24	0.25	0.54	0.52	0.55	0.57	0.62	0.90	0.96	1.00																
k9	0.37	0.35	0.32	0.24	0.35	0.33	0.33	0.32	0.32	0.34	0.25	0.24	0.25	0.33	0.26	0.27	0.27	0.24	0.25	0.25	0.51	0.52	0.56	0.59	0.63	0.90	0.92	0.88	1.00															
k10	0.37	0.35	0.34	0.25	0.36	0.33	0.35	0.34	0.34	0.36	0.26	0.23	0.22	0.33	0.28	0.25	0.25	0.26	0.26	0.26	0.55	0.56	0.60	0.63	0.67	0.86	0.92	0.88	0.88	1.00														
P1	0.19	0.17	0.25	0.22	0.21	0.22	0.21	0.20	0.19	0.21	0.30	0.28	0.26	0.27	0.13	0.28	0.28	0.24	0.24	0.24	0.22	0.23	0.27	0.23	0.21	0.26	0.24	0.25	0.24	0.25	1.00													
P2	0.19	0.17	0.25	0.22	0.20	0.21	0.21	0.20	0.19	0.20	0.31	0.30	0.30	0.29	0.16	0.30	0.30	0.27	0.24	0.24	0.24	0.24	0.27	0.24	0.22	0.24	0.22	0.23	0.22	0.23	0.89	1.00												
P3	0.20	0.18	0.26	0.25	0.22	0.26	0.25	0.24	0.23	0.25	0.38	0.39	0.38	0.42	0.24	0.39	0.39	0.36	0.32	0.32	0.32	0.31	0.32	0.29	0.30	0.29	0.29	0.30	0.28	0.30	0.60	0.65	1.00											
P4	0.21	0.21	0.26	0.24	0.26	0.27	0.28	0.27	0.24	0.24	0.33	0.34	0.34	0.29	0.23	0.32	0.32	0.27	0.29	0.30	0.21	0.21	0.22	0.20	0.21	0.27	0.25	0.25	0.24	0.25	0.41	0.44	0.53	1.00										
P5	0.18	0.16	0.23	0.22	0.18	0.20	0.22	0.21	0.18	0.19	0.31	0.31	0.32	0.26	0.20	0.31	0.31	0.26	0.26	0.27	0.17	0.17	0.17	0.18	0.18	0.20	0.18	0.19	0.18	0.19	0.41	0.45	0.53	0.63	1.00									
P6	0.21	0.18	0.24	0.22	0.19	0.22	0.25	0.24	0.21	0.23	0.31	0.29	0.32	0.25	0.20	0.25	0.25	0.22	0.22	0.23	0.22	0.22	0.20	0.23	0.23	0.23	0.25	0.25	0.22	0.26	0.40	0.42	0.50	0.52	0.70	1.00								
P7	0.21	0.18	0.26	0.21	0.21	0.22	0.25	0.24	0.21	0.22	0.34	0.32	0.33	0.26	0.17	0.29	0.29	0.26	0.26	0.26	0.19	0.19	0.22	0.25	0.24	0.25	0.25	0.25	0.24	0.27	0.47	0.51	0.55	0.56	0.70	0.83	1.00							
P8	0.18	0.16	0.23	0.21	0.19	0.19	0.22	0.21	0.18	0.20	0.35	0.33	0.36	0.29	0.21	0.29	0.29	0.26	0.27	0.27	0.21	0.21	0.19	0.22	0.22	0.22	0.24	0.24	0.21	0.24	0.46	0.50	0.57	0.53	0.69	0.85	0.91	1.00						
P9	0.22	0.20	0.23	0.25	0.20	0.18	0.21	0.19	0.18	0.18	0.28	0.24	0.26	0.20	0.21	0.21	0.21	0.19	0.20	0.20	0.18	0.19	0.16	0.19	0.18	0.22	0.24	0.25	0.23	0.25	0.31	0.34	0.44	0.45	0.56	0.75	0.69	0.72	1.00					
P10	0.21	0.19	0.23	0.22	0.19	0.19	0.22	0.21	0.19	0.19	0.27	0.25	0.27	0.19	0.17	0.22	0.22	0.19	0.19	0.20	0.17	0.17	0.17	0.20	0.19	0.22	0.21	0.22	0.23	0.24	0.36	0.40	0.47	0.51	0.67	0.82	0.77	0.77	0.84	1.00				
k1	0.34	0.29	0.31	0.24	0.31	0.30	0.29	0.28	0.27	0.31	0.23	0.25	0.21	0.25	0.21	0.29	0.29	0.29	0.28	0.28	0.30	0.30	0.38	0.36	0.37	0.33	0.33	0.32	0.34	0.36	0.19	0.20	0.24	0.23	0.21	0.23	0.27	0.22	0.22	0.23	1.00			
k2	0.37	0.35	0.36	0.28	0.33	0.33	0.32	0.32	0.30	0.34	0.29	0.30	0.23	0.25	0.20	0.31	0.31	0.32	0.31	0.31	0.24	0.25	0.30	0.29	0.31	0.33	0.33	0.32	0.34	0.32	0.21	0.24	0.24	0.26	0.23	0.23	0.26	0.22	0.22	0.23	0.77	1.00		
k3	0.35	0.32	0.33	0.25	0.31	0.30	0.32	0.33	0.29	0.33	0.26	0.28	0.20	0.24	0.21	0.27	0.27	0.32	0.28	0.28	0.26	0.27	0.34	0.32	0.33	0.32	0.31	0.30	0.32	0.32	0.18	0.19	0.18	0.24	0.24	0.20	0.24	0.19	0.19	0.20	0.74	0.88	1.00	
k4	0.29	0.31	0.30	0.25	0.30	0.28	0.29	0.28	0.25	0.26	0.33	0.31	0.29	0.26	0.21	0.30	0.30	0.33	0.31	0.31	0.22	0.23	0.25	0.23	0.25	0.21	0.21	0.20	0.22	0.22	0.23	0.24	0.30	0.39	0.30	0.25	0.31	0.30	0.26	0.30	0.43	0.56	0.54	1.00
k5	0.21	0.23	0.25	0.21	0.25	0.22	0.24	0.24	0.21	0.23	0.29	0.31	0.25	0.29	0.15	0.27	0.27	0.33	0.32	0.32	0.18	0.18	0.21	0.19	0.21	0.19	0.19	0.20	0.20	0.20	0.19	0.18	0.24	0.33	0.23	0.21	0.25	0.24	0.20	0.23	0.36	0.49	0.50	0.75	1.00

Appendix 4

Simple Matching Coefficient Similarity Matrix

	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	J1	J2	J3	J4	J5	J6	J7	J8	J9	J10	k1	k2	k3	k4	k5	k6	k7	k8	k9	k10	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	k1	k2	k3	k4	k5
D1	1.00																																												
D2	0.97	1.00																																											
D3	0.92	0.92	1.00																																										
D4	0.89	0.88	0.92	1.00																																										
D5	0.90	0.90	0.92	0.90	1.00																																								
D6	0.86	0.85	0.88	0.86	0.87	1.00																																							
D7	0.87	0.85	0.89	0.85	0.88	0.98	1.00																																						
D8	0.85	0.84	0.89	0.85	0.86	0.96	0.98	1.00																																					
D9	0.86	0.85	0.88	0.86	0.85	0.95	0.96	0.98	1.00																																				
D10	0.86	0.85	0.89	0.83	0.85	0.94	0.95	0.96	0.97	1.00																																			
J1	0.63	0.65	0.66	0.68	0.66	0.65	0.64	0.64	0.63	0.63	1.00																																		
J2	0.62	0.63	0.66	0.67	0.63	0.64	0.63	0.63	0.63	0.63	0.92	1.00																																	
J3	0.62	0.62	0.62	0.65	0.64	0.62	0.61	0.60	0.59	0.59	0.88	0.87	1.00																																
J4	0.55	0.56	0.58	0.63	0.59	0.58	0.57	0.57	0.56	0.56	0.82	0.82	0.82	1.00																															
J5	0.67	0.69	0.69	0.73	0.69	0.67	0.67	0.67	0.66	0.63	0.81	0.84	0.85	0.96	1.00																														
J6	0.61	0.61	0.64	0.63	0.60	0.61	0.60	0.59	0.58	0.61	0.79	0.82	0.81	0.80	0.78	1.00																													
J7	0.61	0.61	0.64	0.63	0.60	0.61	0.60	0.59	0.58	0.61	0.79	0.82	0.81	0.80	0.78	1.00	1.00																												
J8	0.61	0.63	0.66	0.64	0.60	0.64	0.63	0.63	0.63	0.66	0.84	0.82	0.76	0.83	0.81	0.86	0.86	1.00																											
J9	0.61	0.64	0.66	0.66	0.63	0.64	0.63	0.62	0.61	0.64	0.82	0.79	0.75	0.80	0.81	0.85	0.85	0.92	1.00																										
J10	0.60	0.63	0.65	0.65	0.63	0.63	0.63	0.61	0.60	0.63	0.82	0.78	0.74	0.79	0.81	0.85	0.85	0.93	0.99	1.00																									
k1	0.67	0.67	0.65	0.59	0.65	0.67	0.68	0.68	0.67	0.71	0.63	0.60	0.59	0.62	0.70	0.62	0.62	0.65	0.62	0.63	1.00																								
k2	0.67	0.68	0.66	0.59	0.66	0.65	0.66	0.66	0.65	0.68	0.65	0.63	0.62	0.64	0.73	0.66	0.66	0.66	0.63	0.63	0.96	1.00																							
k3	0.70	0.70	0.69	0.62	0.68	0.66	0.66	0.66	0.66	0.69	0.64	0.62	0.61	0.65	0.76	0.63	0.63	0.65	0.63	0.64	0.93	0.96	1.00																						
k4	0.68	0.66	0.65	0.58	0.64	0.63	0.64	0.64	0.63	0.66	0.63	0.61	0.60	0.63	0.70	0.60	0.60	0.60	0.58	0.57	0.91	0.93	0.92	1.00																					
k5	0.68	0.67	0.66	0.59	0.65	0.64	0.65	0.65	0.64	0.67	0.63	0.62	0.61	0.63	0.72	0.59	0.59	0.60	0.59	0.58	0.89	0.88	0.89	0.95	1.00																				
k6	0.64	0.63	0.60	0.56	0.59	0.58	0.59	0.59	0.60	0.61	0.55	0.54	0.56	0.62	0.69	0.57	0.57	0.56	0.54	0.55	0.77	0.78	0.80	0.79	0.82	1.00																			
k7	0.68	0.66	0.63	0.60	0.63	0.62	0.63	0.63	0.63	0.65	0.57	0.56	0.57	0.63	0.67	0.58	0.58	0.58	0.56	0.57	0.79	0.80	0.81	0.82	0.84	0.96	1.00																		
k8	0.66	0.65	0.62	0.58	0.61	0.60	0.61	0.61	0.62	0.63	0.56	0.55	0.57	0.61	0.67	0.56	0.56	0.56	0.55	0.56	0.79	0.78	0.79	0.80	0.82	0.96	0.98	1.00																	
k9	0.66	0.65	0.60	0.56	0.61	0.60	0.60	0.60	0.60	0.62	0.54	0.53	0.57	0.61	0.66	0.56	0.56	0.55	0.53	0.54	0.76	0.77	0.79	0.80	0.82	0.96	0.97	0.95	1.00																
k10	0.68	0.66	0.63	0.60	0.63	0.62	0.63	0.63	0.63	0.65	0.57	0.55	0.56	0.63	0.69	0.56	0.56	0.58	0.56	0.57	0.80	0.80	0.82	0.83	0.85	0.95	0.97	0.95	0.95	1.00															
P1	0.55	0.53	0.57	0.58	0.50	0.53	0.52	0.52	0.51	0.53	0.62	0.61	0.62	0.60	0.58	0.61	0.61	0.58	0.56	0.57	0.58	0.59	0.61	0.56	0.53	0.60	0.57	0.59	0.56	0.59	1.00														
P2	0.54	0.52	0.56	0.57	0.50	0.52	0.51	0.51	0.50	0.52	0.63	0.62	0.64	0.60	0.60	0.62	0.62	0.60	0.56	0.56	0.59	0.59	0.60	0.56	0.54	0.57	0.55	0.56	0.53	0.56	0.96	1.00													
P3	0.54	0.53	0.57	0.59	0.50	0.56	0.55	0.55	0.54	0.56	0.68	0.69	0.69	0.70	0.67	0.69	0.69	0.67	0.63	0.63	0.64	0.63	0.64	0.60	0.61	0.61	0.60	0.62	0.59	0.62	0.83	0.85	1.00												
P4	0.54	0.54	0.56	0.58	0.53	0.56	0.56	0.56	0.54	0.54	0.63	0.64	0.66	0.60	0.64	0.63	0.63	0.60	0.60	0.60	0.54	0.54	0.55	0.51	0.52	0.58	0.56	0.57	0.54	0.57	0.71	0.73	0.79	1.00											
P5	0.57	0.55	0.58	0.61	0.51	0.55	0.56	0.56	0.53	0.55	0.66	0.66	0.69	0.62	0.70	0.66	0.66	0.63	0.62	0.63	0.56	0.57	0.56	0.55	0.54	0.57	0.55	0.56	0.53	0.56	0.75	0.77	0.81	0.85	1.00										
P6	0.58	0.56	0.58	0.58	0.52	0.56	0.58	0.58	0.56	0.58	0.64	0.63	0.68	0.59	0.64	0.60	0.60	0.58	0.57	0.58	0.58	0.58	0.58	0.58	0.58	0.59	0.59	0.61	0.57	0.62	0.73	0.74	0.78	0.79	0.90	1.00									
P7	0.56	0.53	0.58	0.58	0.51	0.53	0.56	0.56	0.53	0.55	0.66	0.65	0.67	0.59	0.64	0.62	0.62	0.60	0.59	0.60	0.55	0.56	0.57	0.58	0.57	0.59	0.58	0.60	0.56	0.61	0.76	0.79	0.81	0.81	0.89	0.94	1.00								
P8	0.55	0.53	0.56	0.58	0.50	0.52	0.54	0.54	0.52	0.53	0.67	0.66	0.70	0.62	0.66	0.63	0.63	0.62	0.60	0.61	0.56	0.57	0.56	0.56	0.56	0.57	0.58	0.60	0.55	0.60	0.76	0.79	0.82	0.79	0.89	0.95	0.97	1.00							
P9	0.61	0.60	0.60	0.63	0.54	0.53	0.56	0.56	0.55	0.55	0.64	0.62	0.66	0.57	0.66	0.59	0.59	0.59	0.57	0.58	0.57	0.58	0.56	0.56	0.56	0.60	0.61	0.63	0.60	0.63	0.69	0.71	0.76	0.76	0.85	0.92	0.89	0.91	1.00						
P10	0.59	0.58	0.58	0.61	0.53	0.53	0.56	0.56	0.55	0.55	0.63	0.62	0.66	0.56	0.63	0.59	0.59	0.57	0.56	0.56	0.55	0.56	0.56	0.56	0.56	0.59	0.58	0.60	0.58	0.61	0.72	0.74	0.78	0.79	0.89	0.94	0.92	0.92	0.95	1.00					
k1	0.69	0.66	0.64	0.63	0.62	0.62	0.61	0.61	0.61	0.64	0.58	0.60	0.58	0.59	0.67	0.62	0.62	0.63	0.61	0.61	0.66	0.67	0.70	0.68	0.69	0.66	0.66	0.66	0.66	0.69	0.56	0.57	0.61	0.58	0.62	0.63	0.64	0.61	0.64	0.64	1.00				
k2	0.68	0.67	0.66	0.63	0.62	0.63	0.62	0.62	0.61	0.64	0.61	0.62	0.58	0.57	0.64	0.63	0.63	0.65	0.62	0.63	0.59	0.60	0.63	0.61	0.62	0.65	0.64	0.64	0.64	0.64	0.56	0.59	0.58	0.60	0.62	0.61	0.62	0.59	0.62	0.62	0.92	1.00			
k3	0.69	0.66	0.65	0.62	0.61	0.61	0.62	0.64	0.61	0.65	0.59	0.61	0.55	0.56	0.67	0.60	0.60	0.64	0.60	0.60	0.62	0.63	0.66	0.64	0.65	0.64	0.63	0.63	0.63	0.65	0.54	0.55	0.53	0.57	0.63	0.59	0.60	0.56	0.60	0.60	0.91	0.96	1.00		
k4	0.63	0.65	0.61	0.61	0.60	0.58	0.59	0.59	0.56	0.58	0.64	0.62	0.62	0.58	0.65	0.61	0.61	0.64	0.61	0.61	0.56	0.57	0.57	0.55	0.56	0.54	0.53	0.53	0.53	0.55	0.57	0.58	0.63	0.69	0.66	0.61	0.65	0.65	0.65	0.66	0.74	0.81	0.80	1.00	
k5	0.58	0.61	0.59	0.60	0.57	0.55	0.56	0.58	0.55	0.57	0.63	0.65	0.61	0.62	0.67	0.61	0.61	0.66	0.64	0.64	0.55	0.56	0.56	0.55	0.55	0.55	0.54	0.55	0.54	0.55	0.56	0.55	0.61	0.66	0.64	0.61	0.62	0.62	0.62	0.64	0.71	0.78	0.79	0.91	1.00

Appendix 5

Herbarium Specimens of *G. diversifolia*

A **B**

Photo: Herbarium specimens of *G. diversifolia* from (A) Dolakha District, (B) Kathmandu District

Appendix 6

List of publications

Adhikari, L., Shrestha, A. J., Dorji, T., Lemke, E., &**Subedee, B. R.** (2018). Transforming the lives of mountain women through the Himalayan nettle value chain: a case study from Darchula, far west Nepal. *Mountain research and development*, **38**(1): 4-13. https://doi.org/10.1659/MRD-JOURNAL-D-17-00074.1

Singh Gaur., Uprety Yadav., **Subedee, B. R**., & Chaudhary, R.P. (2019). Allo: The Himalayan Giant Nettle. In: *Poverty Reduction through Non-Timber Forest Products.* (Eds.: Pullanikkatil, D., & Shackleton, C. M), Springer nature Switzerland, 115-118. Springer, Cham. https://doi.org/10.1007/978-3-319-75580-9

Subedee, B. R., Tripathi, G. R., Munankarmi, N. N., & Chaudhary, R. P. (2022). Genetic diversity in populations of *Girardinia diversifolia* from Nepal Himalaya using ISSR markers. *Ecological Genetics and Genomics*, 100120. https://doi.org/10.1016/j.egg.2022.100120

Subedee, B. R., Tripathi, G. R., & Chaudhary, R. P. (2020). DNA isolation and optimization of PCR protocol for ISSR analysis of *Girardinia diversifolia:* A medicinal and economic plant species from Nepal Himalaya. *African Journal of Biotechnology*, **19(**10): 747-753. https://doi.org/10.5897/AJB2020.17228

Subedee, B. R., Chaudhary , R. P., Uprety Y & Dorji, T.(2020). Socio-ecological perspectives of Himalayan Giant Nettle (*Girardinia diversifolia* (Link) Friis) in Nepal, *Journal of Natural Fibers,* **17(**1): 9-17. https://doi.org/10.1080/15440478.2018.1458684

Subedee, B. R.,Chaudhary, R. P., Dorji, T., & Shrestha, A.J. (2017). Indigenous and local knowledge of conservation and sustainable use of Himalayan Giant Nettle (*Girardinia diversifolia* (Link) Friss) in Eastern and Far-Western Regions of Nepal. In: *Knowing our lands and resources* (Eds.: M. Karki, R. Hill, W. Alangui, K. Ichikawa and P. Bridgewater), United Nations Educational, Scientific and Cultural Organization (UNESCO), Paris, 191-198.

Appendix 7

Representative specimens.

Province 1. Sankhuwasabha District: Balaute, 960m (Shakya, P. R., 9524,20 Dec 1990; KATH 045295).

Province 3. Dolakha District: Phedi Kharka, 2200 m (Rajbhandari, K. R., 10191, 13 Sep 1983; KATH 044947); Jiri, 7200 ft [2194 m] (Singh, S. C. and Shrestha, R, 27 Sep1985; KATH 045290); Singati – Manthali, 1000 m (Bhattarai, N. K. and Kattel, L. P., 88/225, 19 Feb 1988; KATH 045289). **Sindhupalchok District:** Melamchi, 2450 m (Bhattarai, N. K, 86/382, 16 Jun 1986; KATH 045288). **Ramechhap District:** Lapchane, 2700 m (Rajbhandari, K. R. and Roy, B. 2198, 10 Aug 1977; KATH 045283). **Kathmandu District**: Shiwapuri Summit 2,650 m (Suzuki, M., Maeda, T., Naruhashi, N., Watanabe, R., Subedi, M. N., Minaki, M., Noshiro, S. and Ikeda, H. 8882023, 14 Sep, 1988; KATH 045282). **Makwanpur District**: [Makwanpur] 1700 m, (Pendry, C. A., Shrestha, K. K., Dahal, S., Giri, A., Miller, A. G., Pandey, N., Pullan, M.R., Shakya, L.R., Shrestha, S. and Siwakoti, M., DNEP2 B208, 29 Nov 2004; KATH 044945); Torke, 1590 m (Manandhar, N. P., 12682, 1 Dec 1988: KATH 045279). **Rasuwa District:** Dhunche, 2000m (Joshi, N. and Shrestha, I. 699/2001, 5 Sept 2001, KATH 045294); Rimiche, 2500m (Subedi, M.N., 400414, 30 Sep 2000; KATH 045291); Rimiche, on the way to Lama Hotel to Syaphrubesi, 2500m (Subedi, M.N. 400414, 30 Aug 2000; KATH 036709). **Chitwan District:** Jarwang, 1050 m (Manandhar, N. P., 13954, 16 Nov 1989; KATH 045280).

Province 4. Myagdi District: GodepaniDeurali- Shika, 2830- 2010 m (Suzuki, M.,Maeda, T., Naruhashi, N., Watanabe, R., Subedi, M. N., Minaki, M., Noshiro, S. & Ikeda, H., 8881363, 25 Aug 1988; KATH 015474); Banthanti, 2600 m (Subedi, M.N, 8890809, 23 Aug 1988; KATH 045293); Bhainse Kharka, 2600 m (Subedi, M.N. 8890809, 23 Aug 1988; KATH 045292); ChimKhola village, 1770 m (Metz, J. J. 483, 25 Oct 1986; KATH 045286); Bhakiaamlo, 1700 m (Manandhar, N. P, 1031-91, 9 Sep 1991; KATH 045285). **Gorkha District:** Gyachok VDC, above G. VILAGE, E- Facing, 1850 m (Olsen 322, 4898, 16 Sep 1995; KATH 045276). **Kaski District:** Ghorepani 2760 m- Bainthati 2180 m - Ulleri 1950 m - Tikhe Dhunga 1470 m- Ramghai 1200 m- Birethanti Bazar 1030 m, (Mikage, M., Fujii, N., Kajita, T., Kondo, N., Noshiro, S. and Yoda, K., 9460485, 28 Aug 1994; KATH 045267).

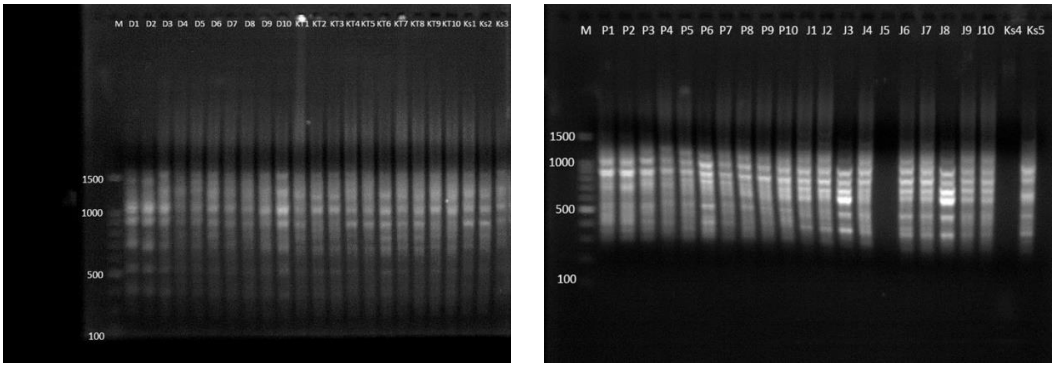
Province 5. Rolpa District: Kolgam-Tiha, 1,550 m (Bhattarai, N. K., 88/486, 25 Sep 1988; KATH 004528); **Salyan District:** Makhantakuri, 2000 m (Kurmi, P. P, KB 317, 8 Jul 1992; KATH 045277).

Province 6. Jumla District: Chaurkot, 10,000 ft [3048](Polunin, O., Skyes, W. R. and Williams, L. H. J. 5433, 27 Sep 1952; KATH 044948). **Jajarkot District:** Dhaulakot, 2200 m (Rajbhandari, K. R. and Roy, B. 4649, 13 Aug 1979; KATH 044939).

Province 7. Bajura District: Birseni-Porakya, 2250m (Rajbhandari, K. R., 14822, 8 Dec 1991; KATH 016032). **Bajhang District:** Gorkhali Village, 2000 m (Bhattarai, K. R., 90/1249, 2 Feb 1990; KATH 045153). **Doti District**: Ghanteswor, 2100 m (Rajbhandari, K. R., Regmi, P. M. and Malla, K. J., 5471, 17 Aug 1980; KATH 044940).

Appendix 8

A **B**



C **D**

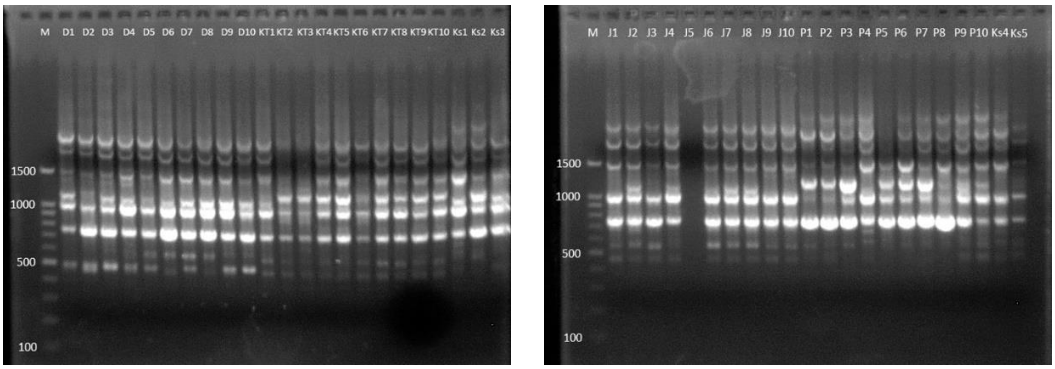
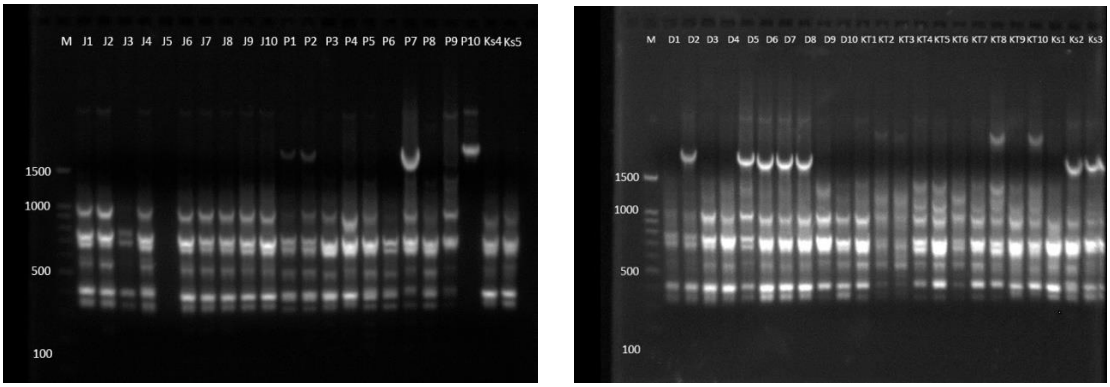


Plate 2: ISSR profiles of *G. diversifolia* with **UBC 808**. Lanes marked with 100plus DNA ladder. A Lanes D1-D10 (samples from Darchula District); K1- K10, (samples from Kathmandu District) Kas1-Kas3 (sample from Kaski District). B Lanes S1-S10 (samples from Panchthar District); J1-J10 (samples from Dolakha District). Kas4-Kas5 (samples from Kaski District) C with **UBC (ATC)6C** Lanes D1-D10 (samples from Darchula District); K1-K10 (samples from Kathmandu District) Kas1-Kas3 (Kaski District). D with **UBC (ATC)6C** Lane J1-J10 (samples from Dolakha District); S1-S10 (Samples from Panchthar District) Kas4-Kas5 (Sample from Kaski District.).

A **B**



C **D**

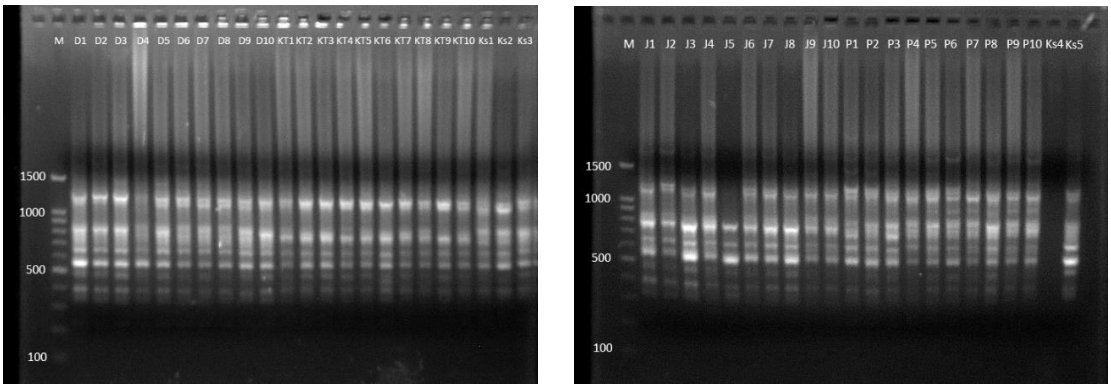


Plate 2: ISSR profiles of *G. diversifolia* with **UBC (ATC)6T**. Lanes marked with 100plus DNA ladder. A Lanes J1-J10 (samples from Dolakha District); S1- S10, (samples from Panchthar District) Kas4- Kas5 (sample from Kaski District). B **UBC (ATC)6T** Lanes D1-D10 (samples from Darchula District); K1-K10 (samples from Kathmandu District). Kas1-Kas3 (samples from Kaski District) C with **UBC 826** Lanes D1-D10 (samples from Darchula District); K1-K10 (samples from Kathmandu District) Kas1-Kas3 (Kaski District). D with **UBC 826** Lane J1-J10 (samples from Dolakha District); S1-S10 (Samples from Panchthar District) Kas4-Kas5 (Sample from Kaski District.).

Appendix 9

Figure : A:preparation for PCR. B: gel electrophoresis at Institute of Botany , Chinese Academy of Sciences (IBCAS), Beijing, China

Figure: Researcher with Allo workers

Appendix 10

Photocopies of published journals and certificates of paper presentation

Nepal Academy of Science and Technology

CERTIFICATE

Awarded to

BIJAY RAJ SUBEDEE

for active participation/paper presentation/~~poster presentation~~

in

THE 7th NATIONAL CONFERENCE ON SCIENCE AND TECHNOLOGY

SCIENCE, TECHNOLOGY AND INNOVATION FOR NEPAL'S GRADUATION TO DEVELOPING COUNTRY STATUS

March 29-31, 2016

Kathmandu, Nepal

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Prof. Dr. Jiba Raj Pokharel
Vice-Chancellor

Institute of Botany
Chinese Academy of Sciences

Add: No. 20 Nanxincun, Xiangshan, Beijing 100093, China

2022-05-03

To whom it may concerned,

Subject: Presentation of Nepal plant resources: Use and Conservation

I am pleased to recall that as the team leader of Sino-Nepal 2017 expedition, I have organized a symposium on 10th January 2018 in Beijing in my institution, and Mr. Bijay Raj Subedee from Tribhuvan University, Kirtipur, Nepal have made a presented on the topic "Use and Conservation of Plant Resources including Girardinia diversifolia under the project Kailash Sacred Landscape Conservation and Development Initiative (KSLCDI)".

If you need more information, feel free to contact me.

Sincerely yours,

Haining Qin

Research Professor &
Dr. HainingQin
Institute of Botany, Chinese Academy of Sciences

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Ecological Genetics and Genomics 23 (2022) 100120

Contents lists available at ScienceDirect

Ecological Genetics and Genomics

journal homepage: www.elsevier.com/locate/egg

Genetic diversity in populations of *Girardinia diversifolia* from Nepal Himalaya using ISSR markers

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ARTICLE INFO

Keywords:
Himalayan giant nettle
Girardinia diversifolia
Genetic variation
Bast fibers
Nepal Himalaya

ABSTRACT

Girardinia diversifolia (Link) Friis from Urticaceae family is a perennial herb, commonly known as the Himalayan giant nettle. It has cultural, medicinal, and economic values among the indigenous peoples and local communities living in the mountains of Nepal and India. Prior knowledge of genetic diversity in plant species may contribute to conservation and sustainable utilization of important genotype. The aim of this study is to assess the genetic diversity within and among different populations of the 45 accessions of *G. diversifolia* collected from Far-western, Central, and Eastern regions of Nepal. The amplification of genomic deoxyribonucleic acid (DNA) with ten inter-simple sequence repeat (ISSR) primers yielded 131 clear DNA bands, of which 98.09% were found to be polymorphic. The mean effective number of alleles (ne), Nei's gene diversity (h) and Shannon's information index (I) were 1.598, 0.349, and 0.523, respectively. The total genetic diversity Ht (0.350 ± 0.04), low intra-population genetic diversity Hs (0.141 ± 0.03) and low estimated gene flow Nm (0.355 ± 0.11) reflected high genetic differentiation among population Gst (0.594 ± 0.08). The analysis of molecular variance among and within five populations of *G. diversifolia* showed that the value of genetic variation among the population was 60% whereas the value of genetic variation within the population was 40%. The results show that the ISSR markers are informative for the study of genetic diversity, which may help in the conservation and sustainable utilization of *G. diversifolia* and allied taxa.

1. Introduction

Natural fiber harvested in an environment-friendly and sustainable way is becoming popular among textile industries, scientists and consumers [1]. *Girardinia diversifolia* (Link) Friis is a perennial herb that belongs to the Urticaceae family and is commonly known as the Himalayan giant nettle. *G. diversifolia* is an important natural fiber-producing plant with great cultural, economic, and medicinal value among the indigenous peoples and local communities (Rais, Gurung, Sherpa, Magar, Tamang, Lepcha, etc.) living in the Hindu Kush Himalayan region of Nepal and India [2–5]. This plant is widely distributed in subtropical and temperate regions of the Himalayas between the altitudes of 1,200 to 3,000 m above sea level [6–8]. The fiber obtained from the stem of this plant is among the important livelihood options for the indigenous peoples and local communities of Nepal from which clothes, fishing nets, bags, coats, and many other textile products are prepared [9]. Different products (textiles and souvenirs) from the fiber of *G. diversifolia* are in increasing demand in national and international markets [4,10,11].

Many bioactive compounds have been found in plants such as β-sitosterol, 7- hydroxysitosterol and 3-hydroxystigmast-5-en-7-one [12] trans syringin, linoleic and linolenic acid [13]. The plant is used in traditional medicine for the treatment of gastritis, joint pain, headache, and skin allergies [2,3,9]. *G. diversifolia* has potential application in pharmaceuticals showing significant ability to inhibit acetylcholinesterase, downregulation of low-density lipoprotein receptor affecting the hepatocarcinoma cells, a crucial regulator of cellular cholesterol homeostasis [13].

Morphological study of *G. diversifolia* showed high level of variation mostly on its leaf lobes [6,8,14]. Leaves are alternate, petiolate, and elliptic to ovate, but consist of varying degrees of division in the same plant or within the population [2,6,8,15,16]. Genetic analysis of plants provides broader knowledge on their diversity and basis to study important metabolites that they produce [17]. Genetic diversity can be

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https://doi.org/10.1016/j.egg.2022.100120
Received 17 July 2021; Received in revised form 8 March 2022; Accepted 10 April 2022
Available online 14 April 2022

determined based on morphological, biochemical, and molecular information of the plant [18]. Molecular markers detect genetic diversity at the DNA level and help to determine the differences not shown by morphological and biochemical markers [19].

Molecular markers have advantages over other kinds of markers because they show genetic differences on a more detailed level without interferences from environmental factors [20]. Molecular markers based on polymerase chain reaction (PCR) are widely used to study genetic diversity of plants because they are effective, and require a small amount of DNA. The popular markers are random amplified polymorphic DNA (RAPD) [21], simple sequence repeat (SSR) [22], and inter-simple sequence repeat (ISSR) [23]. ISSR markers have shown high potential for assessing the genetic diversity of wild species which are based on di-, tri-, tetra-, or penta-nucleotide repeats and used for various purposes including genetic diversity study [24,25]. The ISSR markers were also utilized to study genetic diversity in some of the natural fiber-producing plants like *Boehmeria* species [26], *Cannabis sativa* [27,28] which have become revolutionary tools for both applied and basic studies. ISSR molecular markers have been selected for this study where ISSR follows relatively simple procedure with high reproducibility and stability [24,25]. Generic phylogeny and character evaluation in Urticaceae including *G. diversifolia* have been studied based on analysis of nuclear ribosomal internal transcribed spacer (nrITS) and two plastid DNA regions *rbcL* exon and *trnL-F* spacer [16]. However, there have been no studies so far on the genetic diversity of *G. diversifolia* from the Himalayan region. Thus, there is a need to study genetic diversity of the plant species.

Despite the multipurpose utility of *G. diversifolia*, existing genetic diversity requires conservation and sustainable utilization, the plant has not received adequate attention from conservation biologists. Therefore, the objective of the study is to determine genetic relationship and diversity among and within the populations of *G. diversifolia* occurring in Himalayan region using ISSR markers which can facilitate conservation and sustainable utilization of *G. diversifolia* and allied taxa.

2. Materials and methods

2.1. Plant materials

A total of forty-five germplasms have been collected from the population of *G. diversifolia* at an interval of approximately 900 m altitudinal gradients beginning from 1000 m to 2700 m based on uniform distribution in selected districts. The forty-five accessions representing five populations of the *G. diversifolia* collected from different districts representing four regions: Far-Western region (Darchula District); Western region (Kaski District); Central region (Kathmandu District and Dolakha District); and Eastern region (Panchthar District) of Nepal (Table 1; Fig. 1). The herbarium was prepared in the field and brought to Kathmandu. The herbarium specimen was first identified with the help of Prof. Dr. Ram Prasad Chaudhary, Professor of Botany (plant systematics), who is also one of the co-authors. The collected herbarium specimens were later reconfirmed by comparing morphological characteristics of herbarium specimens deposited at National Herbarium and Plant Laboratories (KATH), Department of Plant Resources, Nepal. During the DNA extraction process, large amount of exudates were observed, which inhibited PCR reaction. The same plants were planted and maintained at Truffle Research Centre, Coronation Garden of Tribhuvan University, Kirtipur, Kathmandu, Nepal (27°40′50″N, 85°17′26.5″E). DNA was extracted from fresh leaf. The samples were collected from August to October 2019 during autumn which is the plant's flowering season. Herbarium specimens were deposited at Tribhuvan University Central Herbarium (TUCH), Kathmandu, Nepal.

2.2. DNA isolation and quantification

Young leaf samples (100 mg) from each forty-five accessions were

Table 1
Sample summary of *G. diversifolia* used in the study.

Sl. No	Collection site	District	Plant accession no.	Latitude/Longitude
1	Hopari, Dhulighad	Darchula	Dar1	29° 46′ 4.5″ N 80° 39′ 31″ E
2	Dhulighad	Darchula	Dar2	29° 46′ 51″ N 80° 38′ 40″ E
3	Okhaldhar	Darchula	Dar3	29° 47′ 0.6″ N 80° 37′ 28″ E
4	Malephar	Darchula	Dar4	29° 47′ 13″ N 80° 37′ 02″ E
5	Godhyan	Darchula	Dar5	29° 47′ 06″ N 80° 36′ 52″ E
6	Godhyan	Darchula	Dar6	29° 47′ 12″ N 80° 36′ 37″ E
7	Pangdhunga	Darchula	Dar7	29° 47′ 17″ N 80° 36′ 21″ E
8	Pangdhunga	Darchula	Dar8	29° 47′ 16″ N 80° 36′ 11″ E
9	Pangdhunga dhar	Darchula	Dar9	29° 47′ 20″ N 80° 36′ 08″ E
10	Pangdhunga dhar	Darchula	Dar10	29° 47′ 34″ N 80° 36′ 23″ E
11	Ghandruk	Kaski	Kas1	28° 23′ 14″ N 83° 48′ 45″ E
12	Ghandruk	Kaski	Kas2	28° 22′ 31″ N 83° 48′ 12″ E
13	Ghandruk	Kaski	Kas3	28° 22′ 29″ N 83° 48′ 08″ E
14	Panchase	Kaski	Kas4	28° 14′ 03″ N 83° 49′ 27″ E
15	Panchase	Kaski	Kas5	28° 13′ 51″ N 83° 49′ 08″ E
16	Machhegau	Kathmandu	Kat1	27° 39′ 22″ N 85° 14′ 51″ E
17	Machhegau	Kathmandu	Kat2	27° 39′ 21″ N 85° 14′ 45″ E
18	Machhegau	Kathmandu	Kat3	27° 39′ 08″ N 85° 14′ 45″E
19	Machhegau	Kathmandu	Kat4	27° 39′ 07″ N 85° 14′ 34″ E
20	Machhegau	Kathmandu	Kat5	27° 39′ 05″ N 85° 14′ 23″ E
21	Machhegau	Kathmandu	Kat6	27° 39′ 02″ N 85° 14′ 22″ E
22	Machhegau	Kathmandu	Kat7	27°39′ 02″ N 85° 14′ 14″ E
23	Nagarjun	Kathmandu	Kat8	27° 44′ 10″ N 85° 15′ 56″ E
24	Nagarjun	Kathmandu	Kat9	27° 44′ 23″ N 85° 15′ 37″E
25	Nagarjun	Kathmandu	Kat10	27° 44′ 30″ N 85° 15′ 14″ E
26	Jiri	Dolakha	Jir1	27° 36′ 57″ N 86° 12′ 48″ E
27	Jiri	Dolakha	Jir2	27° 37′ 03″ N 86° 12′ 34″ E
28	Jiri	Dolakha	Jir3	27° 37′ 13″ N 86° 11′ 59″ E
29	Jiri	Dolakha	Jir4	27° 37′ 36″ N 86° 11′ 30″ E
30	Jiri	Dolakha	Jir5	27° 37′ 47″ N 86° 11′ 24″ E
31	Jiri	Dolakha	Jir6	27° 38′ 06″ N 86° 11′ 19″ E
32	Malung	Dolakha	Jir7	27° 31′ 05″ N 86° 02′ 56″ E
33	Malung	Dolakha	Jir8	27° 30′ 59″ N 86° 02′ 42″ E
34	Dandakharkha	Dolakha	Jir9	27° 31′ 08″ N 86° 00′ 28″ E
35	Dandakharkha	Dolakha	Jir10	27° 31′ 10″ N 86° 00′ 11″ E
36	Siddin	Panchthar	Phi1	27° 09′ 41″ N 87° 54′ 01″ E
37	Siddin	Panchthar	Phi2	

(continued on next page)

Table 1 (*continued*)

Sl. No	Collection site	District	Plant accession no.	Latitude/Longitude
				27° 09′ 25″ N 87° 53′ 43″ E
38	Siddin	Panchthar	Phi3	27° 09′ 08″ N 87° 53′ 28″ E
39	Siddin	Panchthar	Phi4	27° 08′ 47″ N 87° 53′ 08″ E
40	Siddin	Panchthar	Phi5	27° 08′ 31″ N 87° 52′ 47″ E
41	Siddin	Panchthar	Phi6	27° 08′ 19″ N 87° 52′ 35″ E
42	Prangbung	Panchthar	Phi7	27° 10′ 15″ N 87° 54′ 59″ E
43	Prangbung	Panchthar	Phi8	27° 10′ 36″ N 87° 56′ 07″ E
44	Prangbung	Panchthar	Phi9	27° 10′ 11″ N 87° 58′ 15″ E
45	Prangbung	Panchthar	Phi10	27° 10′ 01″ N 87° 58′ 26″ E

taken, inserted into liquid nitrogen, and ground until a fine homogenous powder was obtained. Total genomic DNA was extracted using a modified Doyle and Doyle (1984) method [29]. Varying concentrations of sodium chloride (NaCl), β-mercaptoethanol, and polyvinylpyrrolidone (PVP) were used in the modified DNA isolation method utilized [29]. The concentration and purity of the extracted DNA was quantified in Nano Spectrophotometer (Bio-spec nano, Shimadzu, Japan). Final concentration of the isolated DNA was adjusted to 50 ng μL^{-1} for polymerase chain reactions. All extracted DNA samples were kept at −20 °C until PCR amplification.

2.3. ISSR PCR optimizations and amplifications

All 18 ISSR primers used in this research, including the ISSR-18 and ISSR-19 markers were obtained from the University of British Columbia (UBC), and were initially screened using five randomly selected accessions. Ten ISSR primers were found more polymorphic with strong reproducible bands and were used for amplification of PCR (Table 2). The total reaction volume of 15 μL for PCR amplifications consisting of the concentration of 1X buffer (Vivantis, Malaysia), (0.6) mM dNTPs (Vivantis, Malaysia), 4 mM $MgCl_2$ (Vivantis, Malaysia), 0.7 μM primer (Vivantis, Malaysia), 2 unit *Taq* polymerase (Vivantis, Malaysia), and 50 ng μL^{-1} of DNA template. The thermocycler (Biorad T100) was programmed and optimized by testing various conditions: 3 min at 93 °C, followed by 45 cycles for 30 s at 93 °C, 45 s at different annealing temperature (45–52) °C, extension at 72 °C for 2 min, final extension at 72 °C for 10 min, and finally holding temperature at 4 °C [27]. After PCR reaction, electrophoresis of the PCR products were carried out in 1.8% (w/v) agarose gel containing 10 mg/mL of ethidium bromide, 1X TAE buffer at 80 V for 1 h. The 100 base pair (bp) plus DNA ladder (New England biolabs) was used for determining the molecular weight. The DNA bands were observed under ultraviolet light using unified gel documentation system WGD 30 - POA (Fig. 2).

2.4. Statistical analysis

Clear, unambiguous, and strong bands were used to score DNA bands (Fig. 2). Experiments have been performed twice for the purpose of scoring strong and clear DNA bands. Bands obtained from the ISSR markers were scored in a binary matrix as 1 for presence and 0 for absence of all the bands obtained relative to 100 bp plus DNA ladder. The obtained binary data matrix was investigated using MS-Excel 2007 for assessment of total number of bands (TNB), number of polymorphic

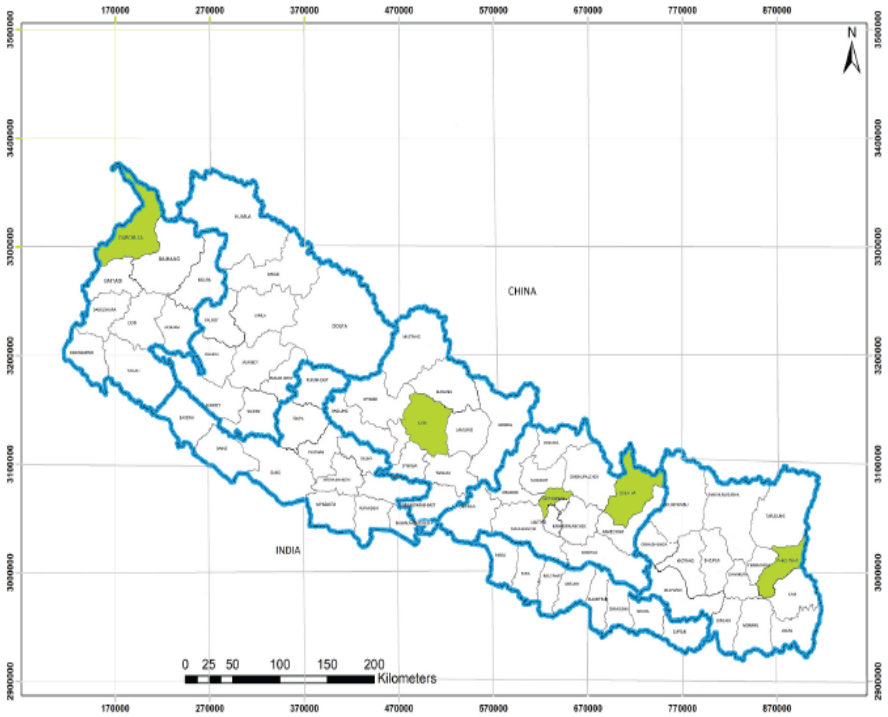

Fig. 1. Map of Nepal showing five sampling sites from Far-western region to Eastern region: Darchula District, Kaski District, Kathmandu District, Dolakha District and Panchthar District.

Table 2
Primer code, sequence and annealing temperature of selected ten ISSR primers.

S.N	Primers	Primer sequence (3′-5′)	Annealing temperature (°C)	TNB	NPB	P(%)	PIC	Rp
1	UBC 812	(GA)8A	52	18	18	100	0.266	8.177
2	UBC 834	(AG)8 YT	45	18	18	100	0.252	7.733
3	UBC 808	(AG)8C	45	14	14	100	0.381	9.288
4	UBC 824	(TC)8G	45	12	12	100	0.414	9.244
5	UBC 811	(GA)8C	48	14	14	100	0.396	10.400
6	UBC 828	(TG)8A	45	15	15	100	0.428	11.911
7	UBC 826	(AC)8C	50	11	10	90.91	0.272	9.822
8	UBC 817	(CA)8A	52	11	11	100	0.388	10.044
9	ISSR-18	(ATC)6T	45	8	8	100	0.409	8.711
10	ISSR-19	(ATC)6C	45	10	9	90	0.295	9.511
	Total			**131**	**129**			
	Mean			**13.1**	**12.9**	**98.09**	**0.350**	**9.484**

TNB = Total number of bands; NPB= Number of polymorphic bands; P(%) = Polymorphism percentage; PIC= Polymorphic information content; Rp = Resolving power; Single letter abbreviation for mixed base Y= (C,T).

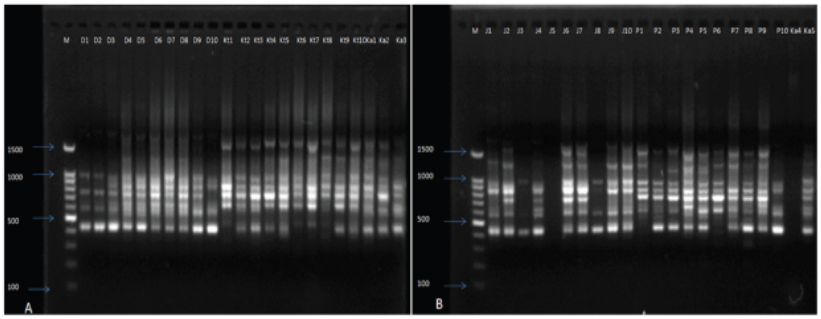

Fig. 2. ISSR amplification results of 45 accessions (A and B) of *G. diversifolia* with primer UBC 824. Lane M = DNA markers (from bottom to top, the bands represent 100 to 1500bp).

bands (NPB), percent polymorphism (P%) (Table 2), polymorphic information content (PIC), band informativeness (BI), and resolving power (RP) for each primer (Table 2). PIC was calculated using the formula [30] given below:

$$PIC_i = 2f_i(1\text{-}f_i)$$

Where PIC*i* is the polymorphic information content of marker *i*, *fi* is the frequency of the marker bands which were present, and *1-fi* is the frequency of marker bands which were absent [30]. Resolving power was calculated according to the formula

$$Rp = \sum I_b$$

Where, I_b is the band informativeness with $I_b = 1 - [2 \times (0.5\text{-}p)]$ and where '*p*' is the proportion of clones containing the band [31].

Cluster analysis was performed using Unweighted Pair-Group Method with Arithmetic means (UPGMA) and similarity indices were computed using a Similarity for Qualitative Data (SIMQUAL) computer algorithm via NTSYS-PC (Numerical Taxonomy and Multivariate System, version 2.02i, Exeter software; Setauket, New York, USA). Genetic similarities between *G. diversifolia* accessions were estimated using Jaccard coefficients and the corresponding similarity matrix [32]. PopGene ver. 1.32 [33] was applied to compute genetic diversity indices, effective number of alleles, Nei's (1973) gene diversity, Shannon's information index, total heterozygosity, mean heterozygosity, genetic differentiation and gene flow (Table 3). Analysis of Molecular variance (AMOVA) was investigated using GenAlEx 6.5 considering variation (Table 7) among and within the population [34].

Table 3
Genetic differentiation and diversity within and between the populations of *G. diversifolia*.

S.N.	Primers	ne	H	I	Ht	H_s	G_{ST}	Nm
1	UBC 812	1.406	0.265	0.423	0.265	0.142	0.462	0.581
2	UBC 834	1.387	0.252	0.404	0.266	0.094	0.646	0.273
3	UBC 808	1.649	0.381	0.565	0.37	0.164	0.559	0.393
4	UBC 824	1.713	0.405	0.592	0.397	0.112	0.718	0.195
5	UBC 811	1.648	0.385	0.571	0.384	0.182	0.524	0.453
6	UBC 828	1.642	0.378	0.561	0.364	0.136	0.625	0.299
7	UBC 826	1.559	0.322	0.484	0.332	0.101	0.695	0.218
8	UBC 817	1.649	0.364	0.536	0.363	0.170	0.531	0.440
9	ISSR-18	1.744	0.416	0.603	0.408	0.180	0.558	0.395
10	ISSR-19	1.581	0.327	0.489	0.347	0.130	0.623	0.302
	Mean	**1.598**	**0.349**	**0.523**	**0.350**	**0.141**	**0.594**	**0.355**
	SD	**0.118**	**0.056**	**0.069**	**0.049**	**0.032**	**0.080**	**0.119**

ne = effective number of alleles; h = Nei's (1973) gene diversity; I = Shannon's information index.; Ht = Total heterozygosity/diversity; Hs = mean heterozygosity/gene diversity within population; Gst = genetic differentiation between population; Nm = gene flow.

3. Results

3.1. Markers analysis and genetic diversity in G. diversifolia

Genetic diversity assessment of 45 accessions of *G. diversifolia* using 10 ISSR primers generated 131 bands of which 98.09% were found polymorphic. The average amplification was 13 bands per primer using 45 *G. diversifolia* accessions. The highest number of DNA bands 18 was generated by primer UBC 812 and primer UBC 834 showing 100% polymorphism. The lowest number of DNA bands i.e. eight DNA bands was produced by ISSR-18. The PIC ranged from 0.252 to 0.428. The primer UBC 828 showed the maximum PIC value of 0.428 and was found to be the most informative primer revealing a good amount of polymorphism information content (Table 2).

The obtained mean value of effective number of alleles was 1.598. The highest number of effective alleles was obtained from the primer ISSR-18 (1.744) while the lowest value was obtained from the primer UBC 834 (1.387). The mean value of Nei's gene diversity was 0.349, with the highest value for the primer ISSR-18 (0.416) and the lowest value for the primer UBC 834 (0.252). The mean value of Shannon's information index as a measure of genetic diversity was 0.523 for all the primers, with the highest value for the ISSR-18 (0.603) and the lowest value for the primer UBC 834 (0.404) (Table 3).

Total heterozygosity/diversity (0.350), mean heterozygosity/gene diversity within population (0.141), genetic differentiation between population (0.594) and gene flow (0.355) were obtained indicating greater efficiency of ISSR primers (Table 3).

The genetic similarity coefficient based on the Jaccard's similarity ranged from 0.98 to 0.16. The highest similarity coefficient was found between accessions from Jiri 6 and Jiri 7, whereas the lowest similarity was found between accessions Phi 8 and Dar 2 showing genetic variations among 45 accessions of *G. diversifolia* (Fig. 3).

3.2. Dendrogram analysis by ISSR primers

Genetic relationships among the 45 accessions of *G. diversifolia* were observed based on the Jaccard's coefficients from 131 amplified loci and a dendrogram was constructed using UPGMA method. A cluster analysis was performed on the basis of Jaccard's similarity (Fig. 3). The genetic similarity calculated from 45 accessions of *G. diversifolia* ranged from 0.24 to 1.00. Jaccard's coefficient generated five major clusters (i.e. A, B, C, D, E) and has the similarity coefficient of 0.50. Cluster A compared to the accessions from Darchula was further sub-clustered at the similarity coefficient level of 0.74 and 0.90, Cluster B from Kathmandu was further sub-clustered at 0.75 and 0.89, Cluster C from Dolakha at 0.56 and 0.83, Cluster D from Kaski at 0.75 and 0.74, and Cluster E from Panchthar at 0.61 and 0.53.

UPGMA tree construction methods were used to construct the dendrogram of five populations. The dendrogram obtained from UPGMA analysis of ten ISSR primers revealed five major groups of *G. diversifolia* (Fig. 3).

The result obtained from Mantel test using NTSYSpc version 2.02i showed the highest and most significant correlation between Jaccard and Dice similarity matrices 0.99094 (Table 4). The highest correlation value, comparison of standard chart of goodness of fit and Jaccard's coefficient of similarity with UPGMA clustering method was found most suitable for studying relationship among *G. diversifolia* accessions.

3.3. Genetic variability in G. diversifolia

Among the populations, the highest degree of variation was recorded in the population of Dolakha with Nei's genetic diversity of 0.192 and Shannon's information index of 0.285. The lowest degree of variation was recorded on the population of Darchula with Nei's genetic diversity of 0.110 and Shannon's diversity of 0.162 (Table 5).

The highest genetic identity was (0.756), and found between the populations of Dolakha and Panchthar, with the shortest genetic distance (0.278). The maximum genetic distance was found between the populations of Darchula and Panchthar (0.448) with the minimum genetic identity (0.638) (Table 6).

3.4. Analysis of molecular variance (AMOVA) of G. diversifolia

The partitioning of variations within and among the populations was

Table 4
Correlation coefficients from Mantel test of original matrices (2 way).

	Simple Matching	Jaccard	Dice
Simple matching	******	–	–
Jaccard	0.96536	******	–
Dice	0.96213	0.99094	******

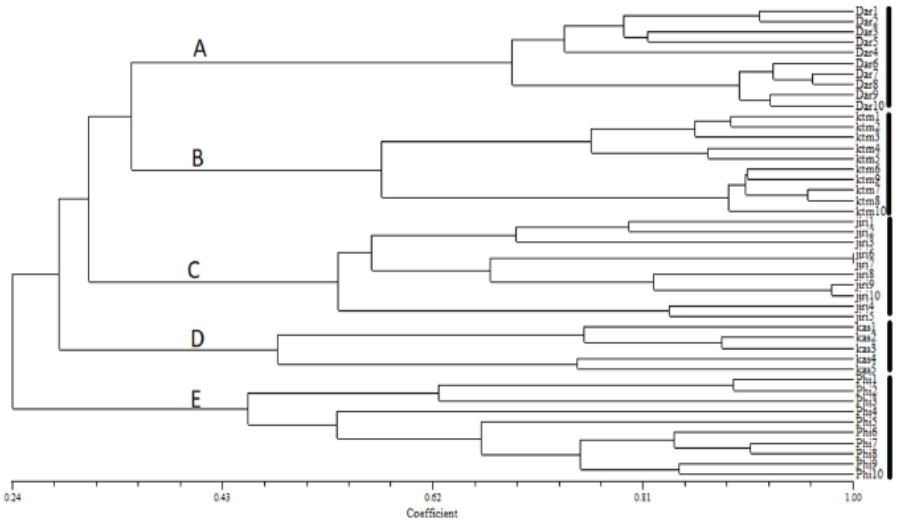

Fig. 3. UPGMA based dendrogram showing the genetic relationship among 45 accessions of *G. diverisifolia* using 10 ISSR primers.

Table 5
Genetic variability within the population of *G. diversifolia*.

Population	Sample size	No of polymorphic band	PPB %	h	I
Darchula	10	38	29.01	0.111	0.162
Kaski	5	45	34.35	0.125	0.186
Kathmandu	10	44	33.59	0.126	0.187
Dolakha	10	68	51.91	0.192	0.285
Panchthar	10	62	47.33	0.153	0.232

Percentage of polymorphic bands/loci (PPB), Nei's (1973) genetic diversity (h), Shannon's information index (I).

Table 6
Nei's unbiased measure of genetic identity (above diagonal) and genetic distance (below diagonal).

Population	Darchula	Dolakha	Kathmandu	Panchthar	Kaski
Darchula	****	0.728	0.721	0.638	0.697
Dolakha	0.316	****	0.714	0.756	0.746
Kathmandu	0.326	0.335	****	0.682	0.714
Panchthar	0.448	0.278	0.382	****	0.731
Kaski	0.360	0.292	0.336	0.312	****

analyzed by AMOVA, which revealed occurrence of 60% total genetic variation among the different populations and 40% total genetic variation within populations (Table 7). The difference between the individuals within and among the populations was statistically significant with the P value < 0.001 (Table 7).

3.5. Principal Coordinate Analysis (PCoA)

All the data obtained using ten ISSR primers were used in principal coordinate analysis (PCoA) using Jaccard's coefficient of similarity. The first PC1, PC2, PC3 explained total variation of 52.73 (22.07, 17.28, 13.38, respectively). The grouping of individuals is indicated in Fig. 4 using two coordinates. PCoA analysis categorized genotypes into four different groups without following their genetic information (Fig. 4).

4. Discussions

Knowledge on the genetic diversity of plant species will help to select genotypes for conservation and development programs [35]. Molecular characterization is one of the reliable tools which have shown important role in estimating relation between cultivars [36]. It uses molecular markers like ISSR [23,25]. ISSR markers are highly reproducible and sensitive markers [25]. The selected ISSR primers (Table 2) in this study indicated the existence of microsatellite regions of (GA)n, (TC)n, (AT)n, (AC)n in *G. diversifolia* which were also found in *Urtica dioica* [37] and *Boehmeria nivea* [26]. Genetic polymorphism observed from 10 ISSR markers for *G. diversifolia* was high (98.09%) in comparison to the results observed for *Urtica dioica* (68%) [37], *Cannabis sativa* (85.8%) [26], *Boehmeria nivea* (96.3%) [26], and lower than *Chenopodium quinoa* (99%) [38]. Since the selected ISSR primers showed high genetic polymorphism, these primers appear to be useful in determining the genetic diversity of *G. diversifolia*.

Polymorphism information content (PIC) is a widely used metric of usefulness of molecular markers and calculated based on the number of alleles and their distribution in population [39–41]. The maximum value of PIC in dominant marker is 0.5 [42]. The average value of PIC of the primers was found to be 0.350 in this study. The ten primers selected in this study had relatively proper distribution in population of *G. diversifolia*. UBC primer 828 gave relatively high PIC value (0.428) in the study. A study conducted in one of the highly used natural fiber producing plant *Linum usitatissimum* showed the PIC value of 0.367 [43]. The obtained values of PIC also suggested that high level of genetic diversity is present in analyzed germplasm.

ISSR markers system evaluated genetic variation and similarities among and within the five populations of *G. diversifolia* from Far-western, Western, Central and Eastern Nepal. The results obtained from the similarity coefficient indicated that genetic variation ranged from 0.98 to 0.16. High level of genetic diversity was recorded in the population of Dolakha PPB (51.91%), H (0.1928), I (0.2852), and lower level was recorded in the population of Darchula PPB (29.01%), H (0.111), I (0.162) (Table 5).

The study obtained the effective number of alleles (1.38–1.74) (Table 3). Nei and Li classify genetic differentiation between population (Gst) into three classes: low (Gst<0.05), moderate (0.05< Gst<0.15) and high (Gst>0.15) [44]. The genetic structure obtained in the study suggested that the differentiation coefficients (Gst 0.594) is higher than the average coefficients (Table 3). The Shannon's index varies from 0 to 1 and the values closer to zero represent lower genetic diversity [45]. The Shannon's diversity index obtained in the study was in the average of 0.349. The Shannon's diversity index from Dolakha (0.285) was the most diverse population compared to Panchthar (0.232), Kathmandu (0.187), Kaski (0.186) and Darchula (0.162). High genetic differentiation in this species suggests that the individual populations have been reproductively isolated and have little current gene flow between them. The value of Nm was found to be 0.355. The value of gene flow (Nm) < 1 which denotes less than one migrant per generation into a population is the threshold value at which the differentiation occurs in population in a significant amount [46]. An Nm of less than one suggests that the diversity maintained in the population is prone to genetic drift [47].

It is generally agreed that plant genetic diversity changes with time and distance [48]. The extent and distribution of genetic diversity in a plant species depends on different factors like its evolution and breeding system, ecological and geographical factors, past bottlenecks, and time and again, by many human factors [49].

Analysis of molecular variance (AMOVA) of *G. diversifolia* showed that higher distribution of genetic variation is present among the populations (60%) compared to within the populations (40%) with the significance value of p < 0.001. The genetic diversity is closely correlated with different factors like effective population size; breeding system; natural selection; and life history traits (including life form, ecological tolerance, seed dispersal, and gene flow) [46]. For *G. diversifolia*, the plant harbors shady moist habitat, commonly found in high altitudes above 1,000 m to 3,000 m between mountain gorges. A similar analysis was conducted on *Stylosanthes scabra* [50]; *Zingiber officinale* [51]; *Oryza granulata* [52]; and *Cypripedium japonicum* [53]. Genetic relationship of *Boehmeria* species from Urticaceae Family using 37 accessions showed ISSR markers are more informative in assessment of ramie diversity collected from different agroecological conditions [26]. The study showed that there is clear association between

Table 7
Analysis of molecular variance (AMOVA).

Source of variation	DF	SS	MS	Estimated variance	Total variance	P value
Among populations	4	593.856	148.464	15.540	60%	<0.001
Within populations	40	413.300	10.333	10.333	40%	<0.001
Total	44	1007.156	–	25.872	100%	

Degree of freedom (DF), Sum of Square (SS), Mean Sum of Square (MS), Estimated Variance, Percentage of Variation and Significance based on permutation across the full data set.

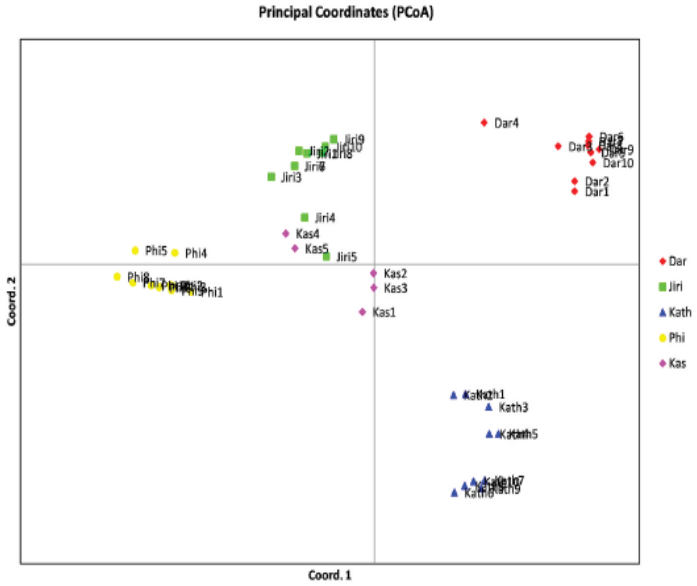

Fig. 4. Principal coordinates analysis (PCoA) among 45 *G. diversifolia* accessions using 10 ISSR primers: Dar (Darchula); Kas (Kaski); Kath (Kathmandu); Jiri (Dolakha); Phi (Panchthar).

geographical origin and genetic similarity in populations distributed in different regions. The present study also revealed that high level of genetic differentiation is present among populations of *G. diversifolia* and increase with an increase in geographical distance. Gst value 0.594 also strengthens the result of the study. Principal coordinate analysis (PCoA) confirmed the clustering of 45 *G. diversifolia* accessions into five main populations using 10 ISSR primers.

The Nm value less than 1, Gst value more than 0.15, and clear cluster formation depending upon geographic distance suggested that high level of genetic variance is present among the population of *G. diversifolia*. Further, overexploitation and massive destruction of its natural habitat [5] indicate that conservation and sustainable utilization of *G. diversifolia* is essential for cultural identity and economic benefits for the indigenous peoples and local communities living in the Hindu Kush Himalayan region of Nepal and India.

5. Conclusions

ISSR markers allowed determination of the genetic variability in *G. diversifolia* by grouping them according to their geographical locations in Nepal Himalaya. Comprehensive molecular analysis reveal that *G. diversifolia* has low genetic diversity within population and high genetic differentiation among the population. This genetic variability potential provides scientific basis for sustainable utilization and conservation efforts.

CRediT authorship contribution statement

Bijay Raj Subedee: Conceptualization, Methodology, Software, Data curation, Writing – original draft. **Giri Raj Tripathi:** Writing – review & editing, Supervision, Methodology. **Nabin Narayan Munankarmi:** Visualization, Investigation. **Ram Prasad Chaudhary:** Supervision, Conceptualization, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgments

This study was supported by Kailash Sacred Landscape Conservation and Development Initiative (KSLCDI) Nepal, a collaborative programme between Ministry of Forests and Environment (MoFE), Government of Nepal; Research Centre for Applied Science and Technology (RECAST), Tribhuvan University; and International Centre for Integrated Mountain Development (ICIMOD); the German Federal Ministry for Economic Cooperation and Development (BMZ) through the Deutsche Gesellschaft für INTERNATIONALE ZUSAMMENARBEIT (GIZ) GmbH, and the United Kingdom's Department for International Development (DFID) – UK Aid, as well as by the core funds of ICIMOD contributed by the governments of Afghanistan, Australia, Austria, Bangladesh, Bhutan, China, India, Myanmar, Nepal, Norway, Pakistan, Switzerland, and the United Kingdom. The views and interpretation in this publication are those of the authors and should not be ascribed to MoFE, RECAST, ICIMOD or their donors.

References

[1] A. Mohanty, S. Vivekanandhan, J. Pin, M. Misra, Composites from renewable and sustainable resources: challenges and innovations, Science 362 (2018) 536–542, https://doi.org/10.1126/science.aat9072.
[2] T.P. Barakoti, K.P. Shrestha, Commercial utilization of allo (*Girardinia diversifolia*) by the rais of sankhuwasabha for income generation, Banko Janakari 18 (2008) 18–24, https://doi.org/10.3126/banko.v18i1.2162.
[3] A. Gurung, H. Flanigan, A.K. Ghimeray, R. Karki, R. Bista, O.P. Gurung, Traditional knowledge of processing and use of the himalayan giant nettle (*Girardinia diversifolia* (Link) Friis) among the Gurungs of Sikles, Nepal, Ethnobot. Res. Appl. 10 (2012) 167–174, https://doi.org/10.17348/era.10.0.167-174.
[4] A. Pandey, U.P. Lepcha, K.S. Gaira, R. Joshi, N. Chettri, Nettle plant (*Girardinia diversifolia*) fibre extraction and yarn making, NIHE (2020) 1–18.
[5] B.R. Subedee, R.P. Chaudhary, Y. Uprety, T. Dorji, Socio-ecological perspectives of himalayan giant nettle (*Girardinia diversifolia* (link) Friis) in Nepal, J. Nat. Fibers 17 (2018) 9–17, https://doi.org/10.1080/15440478.2018.1458684.
[6] I. Friis, A synopsis of *girardinia* (Urticaceae), Kew Bull. 36 (1981) 143–157, https://doi.org/10.2307/4119014.
[7] O. Polunin, A. Stainton, Flowers of the Himalaya, Oxford University Press, Delhi, 1984.
[8] S.C. Singh, R. Shrestha, *Girardinia diversifolia* (Urticaceae), a non-conventional fiber resource in Nepal, Plants, Econ. Bot. 42 (1988) 445–447.
[9] B.R. Subedee, R.P. Chaudhary, T. Dorji, A.J. Shrestha, Indigenous and local knowledge of conservation and sustainable use of himalayan giant nettle

(*Girardinia diversifolia* (link) Friis) in eastern and far-western regions of Nepal, in: M. Karki, R. Hill, W. Alangui, K. Icikawa, P. Bridgewater (Eds.), In Knowing Our Lands and Resources, United Nations Educational, Scientific and Cultural Organization (UNESCO), France, 2017, pp. 191–198.

[10] L. Adhikari, A.J. Shrestha, T. Dorji, E. Lemke, B.R. Subedee, Transforming the lives of mountain women through the himalayan nettle value chain: a case study from Darchula, far west Nepal, Mt. Res. Dev. 38 (2018) 4–13, https://doi.org/10.1659/MRD-JOURNAL-D-17-00074.1.

[11] P.L. Bhandari, Value chain analysis of Allo (*Girardinia diversifolia*) in Solukhumbhu district of Nepal, AAES 4 (2019) 410–423, https://doi.org/10.26832/24566632.2019.040407.

[12] P.M. Njogu, G.N. Thoithi, J.W. Mwangi, F.N. Kamau, I.O. Kibwage, S.T. Kariuki, A. Yenesew, H.N. Mugo, J.M. Mwalukumbi, Phytochemical and antimicrobial investigation of *girardinia diversifolia* (Link)Friis (Urticaceae), east cent, Afr. J. Pharm. Sci. 14 (2011) 89–94.

[13] S.S. Shrestha, S. Sut, I. Ferrarese, S.B. Marco, G. Zengin, M.D. Franco, D.R. Pant, M. F. Mahomoodally, N. Ferri, N. Biancorosso, F. Maggi, S.D. Acqua, S. Rajbhandary, Himalayan nettle *Girardinia diversifolia* as a candidate ingredient for pharmaceutical and nutraceutical applications—phytochemical analysis and in vitro bioassays, Molecules 25 (2020) 1563, https://doi.org/10.3390/molecules25071563.

[14] J. Chen, Q. Lin, I. Friis, C.M. Wilmot-Dear, A.K. Monro, Urticaceae, in: Z.Y. Wu, P. H. Raven, D.Y. Hong (Eds.), Flora of China: Ulmaceae through Basellaceae, Science Press, Beijing and Missouri Botanical Garden Press, St. Louis, 2003, pp. 76–189.

[15] C. Kim, T. Deng, M. Chase, D.-G. Zhang, Z.-L. Nie, H. Sun, Generic phylogeny and character evolution in Urticeae (Urticaceae) inferred from nuclear and plastid DNA regions, Taxon 64 (2015) 65–78, https://doi.org/10.12705/641.20.

[16] Z.-Y. Wu, A.K. Monro, R.I. Milne, H. Wang, T.-S. Yi, J. Liu, D.-Z. Li, Molecular phylogeny of the nettle family (Urticaceae) inferred from multiple loci of three genomes and extensive generic sampling, Mol. Phylogenet. Evol. 69 (2013) 814–827, https://doi.org/10.1016/j.ympev.2013.06.022.

[17] A.R. Fernie, H.J. Klee, The use of natural genetic diversity in the understanding of metabolic organization and regulation, Front. Plant Sci. 2 (2011) 59, https://doi.org/10.3389/fpls.2011.00059.

[18] A. Mohammadi, B.M. Prasanna, Analysis of genetic diversity in crop plants—salient statistical tools and considerations, Crop Sci. 43 (2003) 1235–1248, https://doi.org/10.2135/cropsci2003.1235.

[19] G.S. Erzurumlu, N. Sultana, M. Vural, S. Serce, Genetic and phenotypic variation among Turkish terrestrial orchid species as revealed by RAPD and morphological characteristics, Turk. J. Agric. For. 42 (2018) 227–236, https://doi.org/10.3906/tar-1711-37.

[20] J. Baruah, S.K. Pandey, T. Begum, N. Sarma, M. Paw, M. Lal, Molecular diversity assessed amongst high dry rhizome recovery Ginger germplasm (*Zingiber officinale* Roscoe) from NE-India using RAPD and ISSR markers, Ind. Crop. Prod. 129 (2019) 463–471, https://doi.org/10.1016/j.indcrop.2018.12.037.

[21] Z. Li, R.L. Nelson, RAPD marker diversity among cultivated and wild soybean accessions from four Chinese provinces, Crop Sci. 42 (2002) 1737–1744, https://doi.org/10.2135/cropsci2002.1737.

[22] X. Qi, T.S. Pittaway, S. Lindup, H. Liu, E. Waterman, F.K. Padi, C.T. Hash, J. Zhu, M.D. Gale, K.M. Devos, An integrated genetic map and a new set of simple sequence repeat markers for pearl millet, *Pennisetum glaucum*, Theor. Appl. Genet. 109 (2004) 1485–1493, https://doi.org/10.1007/s00122-004-1765-y.

[23] M.P. Reddy, N. Sarla, E.A. Siddiq, Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding, Euphytica 128 (2002) 9–17, https://doi.org/10.1023/A:1020691618797.

[24] J.G.K. Williams, A.R. Kubelik, K.J. Livak, J.A. Rafalski, S.V. Tingey, DNA polymorphism amplified by arbitrary primers are useful as genetic markers, Nucleic Acids Res. 18 (1990) 6531–6535, https://doi.org/10.1093/nar/18.22.6531.

[25] E. Zietkiewicz, A. Rafalski, D. Labuda, Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification, Genomics 20 (1994) 176–183, https://doi.org/10.1006/geno.1994.1151.

[26] L. Liu, Z.Q. Meng, B. Wang, X. Wang, J. Yang, D. Peng, Genetic diversity among wild resources of the genus *Boehmeria* jacq. From west China determined using inter-simple sequence repeat and rapid amplification of polymorphic DNA markers, Plant Prod. Sci. 12 (2009) 88–96, https://doi.org/10.1626/pps.12.88.

[27] L.G. Zhang, Y. Chang, X.F. Zhang, F.Z. Guan, H.M. Yuan, Y. Yu, L.J. Zhao, Analysis of the genetic diversity of Chinese native *Cannabis sativa* cultivars by using ISSR and chromosome markers, Genet. Mol. Res. 13 (2014) 490–500, https://doi.org/10.4238/2014.December.12.10.

[28] Z.K. Punja, G. Rodriguez, S. Chen, Assessing genetic diversity in *Cannabis sativa* using molecular approaches, in: S. Chandra, H. Lata, M. Elsohly (Eds.), *Cannabis Sativa* L.-Botany and Biotechnology, Springer, Cham, 2017, pp. 395–418, https://doi.org/10.1007/978-3-319-54564-6_19.

[29] B.R. Subedee, G.R. Tripathi, R.P. Chaudhary, DNA isolation and optimization of PCR protocol for ISSR analysis of *Girardinia diversifolia*: a medicinal and economic plant species from Nepal Himalaya, Afr. J. Biotechnol. 19 (2020) 747–753, https://doi.org/10.5897/AJB2020.17228.

[30] I. Roldan-Ruiz, J. Dendauw, E.V. Bockstaele, A. Depicker, M.D. Loose, AFLP markers reveal high polymorphic rates in ryegrasses (*Lolium* spp.), Mol. Breed. 6 (2000) 125–134, https://doi.org/10.1023/A:1009680614564.

[31] A. Prevost, M.J. Wilkinson, A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars, Theor. Appl. Genet. 98 (1999) 107–112, https://doi.org/10.1007/s001220051046.

[32] F.J. Rohlf, NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System, New York, 2000.

[33] F.C. Yeh, R.C. Yang, T.B.J. Boyle, Z. Ye, J.M. Xiyan, PopGene32, Microsoft Windows-Based Freeware for Population Genetic Analysis, Version 1.32. Molecular Biology and Biotechnology Centre, University of Alberta, Edmonton, Alberta, Canada, 2000.

[34] R. Peakall, P.E. Smouse, GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update, Bioinformatics 28 (2012) 2537–2539, https://doi.org/10.1093/bioinformatics/bts460.

[35] M.A. Monfared, D. Samsampour, G.R. Sharifi-Sirchi, F. Sadeghi, Assessment of genetic diversity in *Salvadora persica* L. based on inter simple sequence repeat (ISSR) genetic marker, J. Genet Eng. Biotechnol. 16 (2018) 661–667, https://doi.org/10.1016/j.jgeb.2018.04.005.

[36] J. Baruah, B. Gogoi, K. Das, N.M. Ahmed, D.K. Sarmah, M. Lal, B.S. Bhau, Genetic diversity study amongst *Cymbopogon* species from NE-India using RAPD and ISSR markers, Ind. Crop. Prod. 95 (2017) 235–243, https://doi.org/10.1016/j.indcrop.2016.10.022.

[37] M. Haghpanah, S.K. Kazemitabar, S.H. Hashemi, S. Alavi, Comparison of ISSR and AFLP markers in assessing genetic diversity among Nettle (*Urtica dioica* L.) populations, J. Plant Mol. Breed. 4 (2016) 10–16, https://doi.org/10.22058/JPMB.2016.19659.

[38] M.C. Ana-Cruz, M.E. Helena, M.C. Yacenia, Molecular characterization of *Chenopodium quinoa* Willd. using inter-simple sequence repeat (ISSR) markers, Afr. J. Biotechnol. 16 (2017) 483–489, https://doi.org/10.5897/AJB2017.15925.

[39] J.A. Anderson, G.A. Churchill, J.E. Autrique, S.D. Tanksley, M.E. Sorrells, Optimizing parental selection for genetic linkage maps, Genome 36 (1993) 181–186, https://doi.org/10.1139/g93-024.

[40] D. Botstein, R.L. White, M. Skolnick, R.W. Davis, Construction of a genetic linkage map in man using restriction fragment length polymorphisms, Am. J. Hum. Genet. 32 (1980) 314–331.

[41] S. Nagy, P. Poczai, I. Cernák, A.M. Gorji, G. Hegedűs, J. Taller, PICcalc: an online program to calculate polymorphic information content for molecular genetic studies, Biochem. Genet. 50 (2012) 670–672, https://doi.org/10.1007/s10528-012-9509-1.

[42] Y.V. Chesnokov, A.M. Artemyeva, Evaluation of the measure of polymophism information of genetic diversity, Agriculture Biology 50 (2015) 571–578, https://doi.org/10.15389/agrobiology.2015.5.571eng.

[43] A. Kumari, S. Paul, V. Sharma, Genetic diversity analysis using RAPD and ISSR markers revealed discrete genetic makeup in relation to fibre and oil content in *Linum usitatissimum* L. genotypes, Nucleus 61 (2018) 45–53, https://doi.org/10.1007/s13237-017-0206-7.

[44]] Z. Hashemifar, M. Rahimmalek, Genetic structure and variation in *Perovskia abrotanoides* Karel and *P. atriplicifolia* as revealed by molecular and morphological markers, Sci. Hortic. 230 (2018) 169–177, https://doi.org/10.1016/j.scienta.2017.11.029.

[45] M.A. Monfared, D. Samsampour, G.R.S. Sirchi, F. Sadeghi, Assessment of genetic diversity in *Salvadora persica* L. based on inter simple sequence repeat (ISSR) genetic marker, J. Genet Eng. Biotechnol. 16 (2018) 661–667, https://doi.org/10.1016/j.jgeb.2018.04.005.

[46] M. Slatkin, Rare alleles as indicators of gene flow, Evolution 39 (1985) 53–65, https://doi.org/10.1111/j.1558-5646.1985.tb04079.x.

[47] S. Wright, The genetical structure of populations, Ann. Eugen. 15 (1949) 323–354, https://doi.org/10.1111/j.1469-1809.1949.tb02451.x.

[48] M.D. Loveless, J.L. Hamrick, Ecological determinants of genetic structure in plant populations, Annu. Rev. Ecol. Systemat. 15 (1984) 65–95, https://doi.org/10.1146/annurev.es.15.110184.000433.

[49] V.R. Rao, T. Hodgkin, Genetic diversity and conservation and utilization of plant genetic resources, Plant Cell Tissue Organ Cult. 68 (2002) 1–19, https://doi.org/10.1023/A:1013359015812.

[50] J.C. Costa, G.G.M. Fracetto, F.J.C. Fracetto, T.C. Souza, M.V.F. Santos, M.A. L. Junior, Genetic diversity in natural populations of *Stylosanthes scabra* Fabaceae using ISSR markers, Genet. Mol. Res. 17 (2018) 1–9, https://doi.org/10.4238/gmr18219.

[51] A. Das, M. Gaur, D.P. Barik, E. Subudhi, Genetic diversity analysis of 60 ginger germplasmcore accessions using ISSR and SSR markers, Plant Biosyst. 151 (2016) 822–832, https://doi.org/10.1080/11263504.2016.1211197.

[52] W. Qian, S. Ge, D.Y. Hong, Genetic variation within and among populations of a wild rice *Orysa granulata* from China detected by RAPD and ISSR markers, Theor. Appl. Genet. 102 (2001) 440–449, https://doi.org/10.1007/s001220051665.

[53] H.Z. Tian, L.X. Han, J.L. Zhang, X.L. Li, T. Kawahara, T. Yukawa, J.L. Pujol, P. kumar M.G. Chung, M.Y. Chung, Genetic diversity in the endangered terrestrial orchid *Cypripedium japonicum* in East Asia: insights into population history and implications for conservation, Sci. Rep. 8 (2018) 1–13, https://doi.org/10.1038/s41598-018-24912-z.

Vol. 19(10), pp.747-753, October, 2020
DOI: 10.5897/AJB2020.17228
Article Number: 6A612D665169
ISSN: 1684-5315

http://www.academicjournals.org/AJB

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African Journal of Biotechnology

Full Length Research Paper

DNA isolation and optimization of PCR protocol for ISSR analysis of *Girardinia diversifolia*: A medicinal and economic plant species from Nepal Himalaya

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Received 20 August, 2020; Accepted 9 October, 2020

***Girardinia diversifolia* (Link) Friis is a perennial herb commonly known as Himalayan giant nettle which belongs to the family Urticaceae. The plant has cultural, medicinal and economic importance. The plant contains high concentration of polysaccharides, polyphenols and secondary metabolites which obstructs the process of isolation of the Deoxyribonucleic Acid (DNA) and inhibits downstream Polymerase Chain Reaction (PCR) amplifications. This study protocol developed a DNA extraction protocol from leaf-tissue based on the Cetyltrimethylammonium bromide, and optimized the PCR protocol for Inter Simple Sequence Repeat (ISSR) analysis. Genomic DNA extraction process was conducted using modified Doyle and Doyle method to obtain good quality DNA. The method yielded 445 ng/µL of DNA, where the purity ranged from 1.8-2.0 indicating minimum contamination of metabolites. The optimum condition for ISSR analysis was established using 4 mM $MgCl_2$, 0.6 mM dNTPs, 2.0 U *Taq* polymerase, 50 ng template DNA, and 0.7 µM primer. PCR program was optimized in the sequence of denaturation at 94°C for 3 min, subsequently followed by 45 cycles at 94°C for 30 s, annealing temperature at 45°C for 30 s, extension at 72°C for 2 min, and final extension at 72°C for 10 min. The modified technique was found to be ideal for isolation of genomic DNA and optimization of PCR process for ISSR analysis of *G. diversifolia*. The results of the research are beneficial for future molecular characterization and genetic diversity analysis of allied taxa.**

Key words: *Girardinia diversifolia*, Himalayan nettle, DNA isolation.

INTRODUCTION

*Girardinia di*versifolia (Link) Friis is commonly known as Himalayan giant nettle and locally known as 'Allo' in Nepal. The plant belongs to family Urticaceae which contains approximately 54 genera and has more than 2000 species with high concentration of genera and species in tropical Asia (Wu et al., 2013). This plant is widely distributed in the subtropical and temperate Himalayas (Polunin and Stainton, 1984) and its habitat is found between the altitudes of 1,200 to 3,000 metres above sea level (Friis, 1981; Shrestha, 1997; Singh and

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Shrestha, 1988). Molecular genetic markers reflect the variation at the level of DNA (Anne, 2006). Inter Simple Sequence Repeat (ISSR)-PCR is a technique, which utilizes microsatellite sequence in polymerase chain reaction to generate multilocus markers (Reddy et al., 2002). This technique with great reproducibility has been widely used in classification and systematic comparison of species (Amom et al., 2018; Nilkanta et al., 2017), evolutionary relationship of species and identification of genetic varieties (Mohamad et al., 2017).

Himalayan giant nettle has great cultural, economic and medicinal significance among the indigenous people and local communities (IPLCs) of Nepal (Barakoti and Shrestha, 2008; Subedee et al., 2020). Fiber obtained from the stem of this plant is used to make clothes, fishing nets, bags, coats and many other textile products. The plant is used in traditional medicine for treating gastritis, joint pain, headache, and skin allergies (Barakoti and Shrestha, 2008; Subedee et al., 2020). Market demand of the products made from this plant is increasing year by year, and created a risk of over exploitation from its natural habitat. Generic phylogeny and character evaluation in Urticaceae including *G. diversifolia* from China has been studied based on analysis of nuclear ribosomal internal transcribed spacer (nrITS) and two plastid DNA regions *rbcL* exon and *trnL-F* spacer (Wu et al., 2013). However genetic diversity of *G. diversifolia* by using ISSR has not been conducted yet. Thus, there is a need to study molecular characteristics of this plant by using ISSR.

G. diversifolia contains many bioactive compounds such as β-sitosterol, 7- hydroxysitosterol and 3-hydroxystigmast-5-en-7-one (Njogu et al., 2011) trans syringin, linoleic and linolenic acid (Shrestha et al., 2020). Genetic analysis of plants provides broader knowledge on their diversity and basis to study important metabolites produced by them (Fernie and Klee, 2011). Evaluation of genetic diversity in plants requires high quality and quantity of DNA (Sá et al., 2011) for which *G. diversifolia* presented a great challenge. Secondary metabolites present in plants can interfere in genomic DNA extraction, purification and downstream applications (Friar, 2005). Urticaceae family members produce large amounts of exudate, which obstruct DNA extraction (Sarrazola and Alzate, 2019). Various methods of DNA extraction has been carried out on many species of Urticaceae family (Bharmauria et al., 2009; Wu et al., 2013) which has indicated that further modifications are essential to obtain good quality genomic DNA for PCR based analysis (Aboul-Maaty and Oraby, 2019). Despite high value, very few information is available on extraction of its genomic DNA. Isolation of DNA from leaf tissue was found to contain high degree of phytochemical while using standard protocol by Doyle and Doyle (1987). These phytochemicals inhibited PCR reaction. Hence, modification in the extraction protocol was a requisite to obtain good quality DNA. The objective of this study is to develop DNA extraction protocol and optimization of PCR protocol for ISSR-PCR analysis of *G. diversifolia*.

MATERIALS AND METHODS

Plant material

Seeds of *G. diversifolia* were collected from Naugad rural municipality of Darchula District, Far-western region of Nepal (29°47'34.9"N, 80°36'23.5"E). The seeds were thoroughly rinsed with distilled water, allowed to germinate on top of moist absorbent paper in plastic petri dishes using top-of-paper method (Rao et al., 2006) and transplanted to small pot after 12 days. The germinated plants were maintained at Truffle Research Centre, Coronation Garden of Tribhuvan University, Kirtipur, Kathmandu, Nepal (27°40'50"N, 85°17'26.5"E). Young fresh juvenile leaves of the plants were collected prior to extraction of DNA.

Reagents used in isolation of genomic DNA

Cetyltrimethylammonium bromide (CTAB) was modified which consists of extraction buffer 2% (w/v) CTAB (Sigma, Sintra, Portugal), 1 M Tris Hcl (pH 8.0), 0.5 M EDTA (pH 8.0), 5 M NaCl, with 5% PVP (w/v) Polyvinlypyrrolidone (Sigma, Sintra, Portugal), 5% β-mercaptoethanol, Ammonium acetate 7.5 M, (25:24:1) Phenol:Chloroform:Isoamyl alcohol (Sigma-aldrich). Ethanol 70% and 100% were used for isolation of DNA.

Isolation of genomic DNA

Freshly harvested young leaves (100 mg) were ground in liquid nitrogen into fine powder with the help of mortar and pestle. The study followed CTAB, DNA isolation protocol of Doyle and Doyle (1987), and modified the DNA extraction method. The modification of Doyle and Doyle protocol to extract genomic DNA was followed as described below.

Prior to DNA extraction, 5% β-mercaptoethanol and 5% PVP (Polyvinylpyrrolidone) was added in CTAB buffer. Freshly harvested young leaf samples (100 mg) were ground in liquid nitrogen in a pre-chilled mortar and pestle. CTAB buffer (500 µL) was added quickly and transferred into sterile 1.5 mL centrifuge tubes. The tubes were incubated at 55°C for 1 h with occasional shaking for every 10 min. Phenol:chloroform:isoamyl alcohol (25:24:1) was mixed well (500 µL) to form an emulsion by shaking tubes. Centrifugation was carried out at 14000 Revolution Per Minute (RPM). Phenol:chloroform:isoamyl alcohol step was repeated twice. The supernatant was carefully decanted and transferred to new tubes, pre-chilled 0.08 volumes of 7.5 M Ammonium acetate and 0.54 volumes of cold isopropanol was added and mixed well.

The samples were kept in -20°C for 1 h and centrifuged at 14000 RPM for 3 min. The pellet was washed twice with 70% and once in 100% ethanol. The supernatant was decanted and DNA pellet was air-dried at room temperature until the white pellet turned transparent. The DNA pellet was resuspended in 100 µL of TE buffer.

Quantification of extracted DNA and testing for purity

The yield of extracted DNA was measured in a nanospectrometer (Bio-spec nano) at 260 nm. The purity of DNA was measured by estimating the ratio of absorbance at 260 to 280 nm. The DNA purity was determined by running the sample in 0.8% agarose gel.

Table 1. Primers used in screening of ISSR-PCR of *G. diversifolia*.

S/N	Primers name	Primer sequence
1	UBC 812	5'-GAG AGA GAG AGA GAG AA-3'
2	UBC 834	5'-AGA GAG AGA GAG AGA GYT-3'
3	UBC 808	5'-AGA GAG AGA GAG AGA GC-3'
4	UBC 824	5'-TCT CTC TCT CTC TCT CG-3'
5	UBC 811	5'-GAG AGA GAG AGA GAG AC-3'
6	UBC 828	5'-TGT GTG TGT GTG TGT GA-3'
7	(ATC)6C	5'-ATC ATC ATC ATC ATC ATC C-3'
8	(ATC)6T	5' ATC ATC ATC ATC ATC ATC T-3'
9	UBC 826	5'- ACA CAC ACA CAC ACA CC-3'
10	UBC 817	5'-CAC ACA CAC ACA CAC AA-3'
11	UBC809	5'-AGA GAG AGA GAG AGA GG-3'
12	UBC 815	5'-CTC TCT CTC TCT CTC TG-3'
13	UBC 820	5'-GTG TGT GTG TGT GTG TT-3'
14	UBC 823	5'-TCT CTC TCT CTC TCT CC-3'
15	UBC827	5'-ACA CAC ACA CAC ACA CG-3'
16	(GA)9C	5'-GAG AGA GAG AGA GAG AGA-3'

The size of each fragments was estimated using 100 bp plus DNA ladder.

Optimization of Polymerase Chain Reaction (PCR)

The optimization of ISSR-PCR was carried out with the extracted genomic DNA from *G. diversifolia*. Five major factors; *Taq* DNA polymerase (Vivantis, Malaysia), dNTPs (Vivantis, Malaysia), primers (University of British Columbia, Canada), $MgCl_2$ (Vivantis, Malaysia), template DNA, and their concentrations were considered highly as shown in Table 4. The ISSR primer obtained from University of British Columbia (UBC) were used for optimization of PCR condition.

The reaction was carried out in the DNA thermocycler (Biorad T100). Total volume of 15 µL PCR reaction mixture was used which contained 1X buffer, (0.1-0.6) mM dNTPs, (1.5-4) mM $Mgcl_2$, (0.1-0.9) µM primer, (0.5-3.0) unit *Taq* polymerase and (25-100) ng of DNA template. The thermocycler was programmed and optimized by testing various conditions: 3 min at 93°C, followed by 45 cycles for 30 s at 93°C, 45 s at different annealing temperature (45-52)°C, extension at 72°C for 2 min, final extension at 72°C for 10 min and finally holding temperature at 4°C. After PCR reaction, electrophoresis of The PCR products were carried out in 1.8% agarose gel containing 10 mg/mL of ethidium bromide, 1X TAE buffer at 80 V for 1 h. DNA ladder of 100 base pair was used for determining the molecular weight. The DNA bands were observed under ultraviolet light using Gel documentation system.

RESULTS

Isolation and purity detection of DNA

The mean concentration and purity of DNA samples extracted from the leaves of *G. diversifolia* are presented in Table 2.

Yield of DNA from Doyle and Doyle method leaf tissue was found to have high degree of phytochemical and thus needed modification of the method. The modified method yielded 445 ng/µL of DNA, the purity ranged between 1.8 -2.0 indicating minimum contamination of metabolites.

High concentration of β-mercaptoethanol and PVP in extraction buffer played a major role in neutralization of polyphenols, tannins and oxidation of secondary metabolites (Porebski et al., 1997). The modified protocol showed high efficiency to extract good quality DNA by varying the concentration of NaCl, β-mercaptoethanol and PVP. The modified conditions are listed in Table 3.

Optimization of PCR-ISSR and screening primers

For optimization of ISSR-PCR, various concentrations were considered including template DNA, primers, *Taq* polymerase, dNTPs, $MgCl_2$, annealing temperature. The optimized conditions for ISSR-PCR protocol are given in Table 4.

The optimized condition for ISSR-PCR reaction for *G. diversifolia* was found to be 50 ng of genomic DNA, 4 mM Magnesium chloride, 0.6 mM dNTPs, 0.7 µM primer concentration, 2 U *Taq* polymerase in total 15 µL PCR reaction volume. The reproducible clear bands in agarose gel electrophoresis is shown in Figure 1.

DNA extracted from *G. diversifolia* using the optimized PCR parameters are shown in Table 4. Among 16 screened ISSR primers (Table 1), seven ISSR primers (UBC811, UBC812, UBC817, UBC824, UBC826, UBC827, UBC 834) showed the clear, reproducible bands and the experiment was repeated twice (Figure 2). Total volume of 15 µL PCR reaction mixture which contained 1× buffer, 0.6 mM dNTPs, 4 mM $Mgcl_2$, 0.7 µM primer, 2 unit *Taq* polymerase and 50 ng of DNA template were optimized

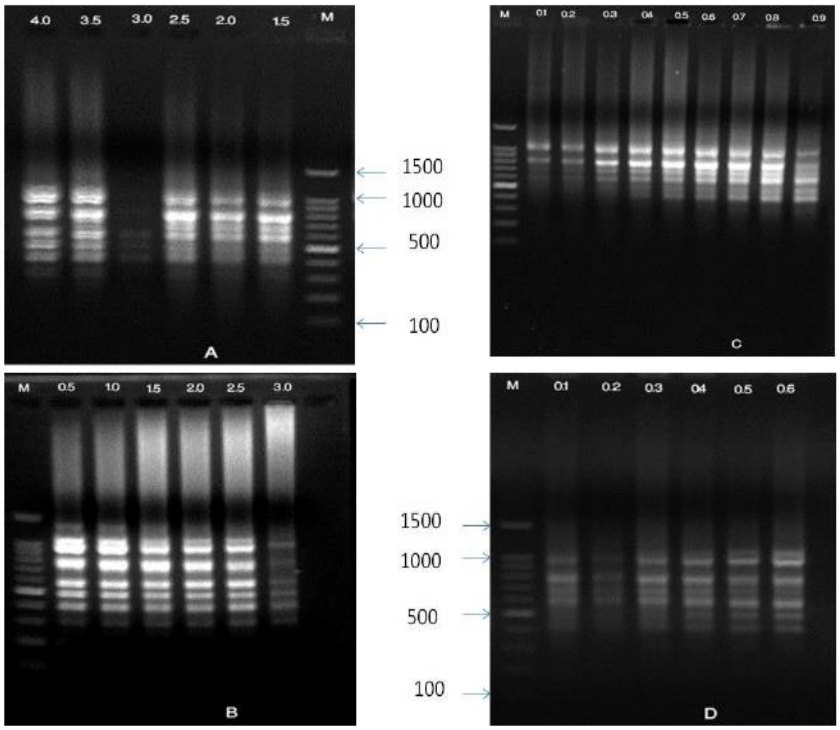


Figure 1. Optimization pattern of DNA sample of *Girardinia diversifolia* using the ISSR primer (UBC 817): **A**, Optimization of $MgCl_2$; **B**, Optimization of *Taq* polymerase; **C**, Optimization of primer; **D**, Optimization of dNTPs.

Table 2. Quantification and quality analysis of extracted DNA.

Protocol	Mean of A 260/280	Mean of A 260/230	Mean of concentration (ng/µL)	Color/Viscosity
Doyle and Doyle	2.208	1.10	331.39	Dark/viscous
Modified protocol	1.83	2.20	445	Clear/nonviscous

Table 3. Standardized condition of DNA extraction.

S/N	Parameters	Standardized condition	Inference
1	NaCl	5 M	Helped in removal of polysaccharides
2	PVP	5%	Absorbed polyphenols
3	β-mercaptoethanol	5%	Extracted clear DNA pellet
4	Ammonium acetate	7.5 M	Neutralized charges on the sugar phosphate backbone

(Figure 1 and Table 4). The thermocycler was optimized by testing conditions: 3 min at 93°C, followed by 45 cycles for 30 second at 93°C, 45 second at different annealing temperature 45°C, extension at 72°C for 2 min, final extension at 72°C for 10 min and finally holding temperature at 4°C. After PCR reaction, electrophoresis of the PCR products were carried out in 1.8% agarose gel containing 10 mg/mL of ethidium bromide, 1× TAE buffer at 80 V for 1 h. DNA ladder of 100 basepair was used for determining the molecular weight. The optimal

Table 4. PCR parameter tested and selected optimized condition.

S/N	PCR parameter	Tested range	Optimum condition
1	DNA template concentration (ng)	25,37.5, 50, 62.5, 75, 87.5, 100	50
2	Magnesium chloride (mM)	1.5, 2, 2.5, 3, 3.5, 4	4
3	Deoxynucleotide triphosphate(dNTPs) (mM)	0.1, 0.2, 0.3, 0.4, 0.5, 0.6	0.6
4	Primer concentration (µM)	0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9	0.7
5	Taq DNA polymerase (Units)	1, 1.5, 2, 2.5, 3	2
6	Annealing temperature (°C)	45-52	45

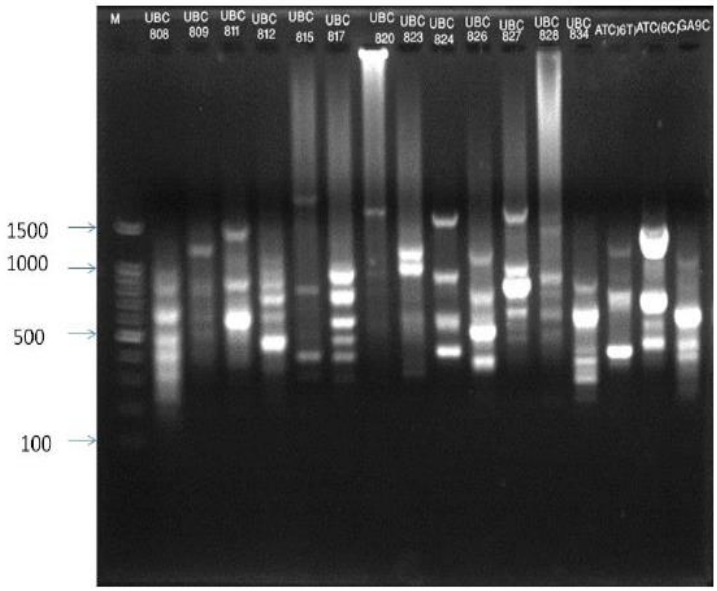


Figure 2. Amplification patterns of 16 ISSR primers for a specimen of *G. diversifolia*.

ISSR-PCR reaction conditions were used for the study of genetic diversity of *G. diversifolia* separately.

DISCUSSION

Genomic DNA isolation from *G. diversifolia* using Doyle and Doyle (1987) standard protocol was conducted. Many obstacles were encountered from the very first step of genomic DNA extraction, including cell lysis, DNA elution and subsequent PCR reactions. The biochemical composition in plant tissues of different species is expected to vary considerably and may not yield optimal DNA from one isolation protocol (Khanuja et al., 1999; Kumari et al., 2020). Due to the presence of PCR inhibitors, protocol of Doyle and Doyle (1987) did not show good quality DNA. It resulted in the absence of DNA band in electrophoresis technique. Thus, procedure described by Doyle (1987) was modified by altering the parameters: increased concentration of NaCl from 2 to 5M, increased concentration of β-mercaptoethanol, increased concentration PVP, addition of phenol to the deproteinization process.

The recommended values for the A260/A280 ratio ranged from 1.8-2.0 and absorption ratio at A260/230 is 2.0-2.22 for impurity free DNA (Arruda et al., 2017). Higher value of absorbance from 2.0 indicated the contamination of phenol in extracted DNA while lower value indicated the presence of proteins. The optimized protocol presented in the study showed a mean DNA concentration of 400 ng/µl extracted from the leaf of *G. diversifolia*. The method resulted in the mean value of 1.83 which confirmed the extraction of pure DNA at A260/A280 and A260/A230 ratios.

High concentration of NaCl (5 M) effectively removed polysaccharides during DNA extraction of *G. diversifolia*

(Table 3). Studies in other plants species like *Mangifera indica*, *Capsicum sp*. *Eclipta alba, Aegle marmelos*, *Grewia asiatica*, supported that this modification allowed an efficient elimination of polysaccharides (Devi et al., 2018; Kit and Chandran, 2010; Kumar et al., 2018; Mujeeb et al., 2017; Shukla et al., 2018). High concentration of NaCl helped in the elimination of polyphenols from the leaf of *G. asiatica* (Shukla et al., 2018).

Use of high concentration of PVP improved the quality of DNA by removing secondary metabolites during genomic DNA extraction process (John, 1992; Osena et al., 2017). The modified protocol used 5% PVP in 2% CTAB buffer which was effective in the elimination of polyphenols that resulted in the extraction of clear DNA pellets. Some studies also suggested the use of 5% PVP in CTAB buffer *Vigna* sp. (Choudhary et al., 2008) and *Mimosa tenuiflora* (Arruda et al., 2017) to obtain good quality DNA pellet.

β-mercaptoethanol enhances denaturation of protein (Tiwari et al., 2012). In the modified protocol, the concentration of β-mercaptoethanol was increased to 5%. The high concentration of β-mercaptoethanol is important for the reduction of polyphenols during extraction of genomic DNA of plants containing high content of secondary metabolites (Arruda et al., 2017). The modified protocol contains 5% β-mercaptoethanol instead of 2% as used by Doyle and Doyle (1987). The modified protocol helped to reduce brown coloured DNA pellet. Other study carried out in *Litchi chinensis* (Arruda et al., 2017; Puchooa, 2004) also reported that increase in the concentration of β-mercaptoethanol helped to extract clear DNA pellet. Polyphenols often damage extracted genomic DNA and make some enzymes inaccessible (Anerao et al., 2016). The use of phenol: choloroform: isoamyl alcohol effectively removed polyphenols and yielded pure genomic DNA.

For the identification of polymorphic characteristics and genetic diversity of plant species, ISSR acts as a powerful method since it depends on the quality and quantity of extracted DNA. Selection of primers is an important factor because the same primer may exhibit different amplification results in different species.

Conclusion

The Doyle and Doyle (1987) protocol was successfully modified to isolate genomic DNA by increasing the concentration of PVP, β-mercaptoethanol, NaCl and addition of Phenol:chloroform:isoamyl alcohol (25:24:1) in the extraction buffer. These changes made it possible to obtain high quality genomic DNA from *G. diversifolia*. The extracted DNA was used to optimize PCR based ISSR protocol which gave clear and amenable DNA bands. The obtained results confirm that the modified protocol is suitable with *G. diversifolia* and other plant species containing high concentration of secondary metabolites. The research is beneficial for future molecular characterization, genetic diversity analysis of allied taxa and genetic improvement works.

ACKNOWLEDGMENTS

This study was supported by Kailash Sacred Landscape Conservation and Development Initiative (KSLCDI) Nepal, a collaborative programme between Ministry of Forests and Environment, Government of Nepal; Research Centre for Applied Science and Technology, Tribhuvan University; and International Centre for Integrated Mountain Development (ICIMOD); the German Federal Ministry for Economic Cooperation and Development (BMZ) through the Deutsche Gesellschaft für Internationale Zusammenarbeit (GIZ) GmbH and the United Kingdom's Department for International Development (DFID) – UK Aid, as well as by the core funds of ICIMOD contributed by the governments of Afghanistan, Australia, Austria, Bangladesh, Bhutan, China, India, Myanmar, Nepal, Norway, Pakistan, Switzerland, and the United Kingdom. The views and interpretation in this publication are those of the authors and should not be ascribed to MoFE, RECAST, ICIMOD or their donors.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Aboul-Maaty NF, Oraby HAS (2019). Extraction of high-quality genomic DNA from different plant orders applying a modified CTAB-based method. Bulletin of the National Research Centre 43(1):25.

Amom T, Tikendra L, Rahaman, Nongdam P (2018). Evaluation of genetic relationship between 15 bamboo species of North-East India based on ISSR marker analysis. Molecular Biology Research Communications 7(1):7-15.

Anerao J, Jha V, Desai N (2016). Optimization of DNA Extraction Methods from *Garcinia* species for ISSR-PCR, RAPD-PCR and DNA Barcoding. Asian Journal of Biotechnology 9(1):35-42.

Anne C (2006). Choosing the right molecular genetic markers for studying biodiversity: From molecular evolution to practical aspects. Genetica 127:101-120.

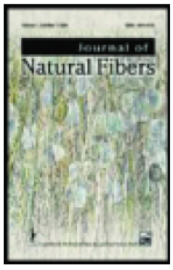
Arruda SR, Pereira DG, Silva-Castro MM, Brito MG, Waldschmidt AM (2017). An optimized protocol for DNA extraction in plants with a high content of secondary metabolites, based on leaves of *Mimosa tenuiflora* (Willd.) Poir. (Leguminosae). Genetics and Molecular Research 16(3):1-9.

Barakoti TP, Shrestha KP (2008). Commercial utilization of Allo (*Girardinia diversifolia*) by the Rais of Sankhuwasabha for income generation. Banko Janakari 18(1):18-24.

Bharmauria V, Narang N, Verma V, Sharma S (2009). Genetic variation and polymorphism in the Himalayan nettle plant *Urtica dioica* based on RAPD marker. Journal of Medicinal Plants Research 3(3):166-170.

Choudhary K, Mathur N, Choudhary OP, Pillai U (2008). Protocol for Isolation of Genomic Dna from Dry and Fresh Leaves of *Vigna*

Species Suitable for Rapd and Restriction Digestion. Advances in Biological Research 2(5-6):83-89.
Devi AA, Brajendra N, Dinachandra M (2018). Genetic Diversity Analysis in Chilli (*Capsicum annuum* L.) Found in Manipur Using RAPD Markers. International Journal of Current Microbiology and Applied Sciences 7(10):257-262.
Doyle JJ, Doyle JL (1987). A Rapid DNA Isolation Procedure for Small Quantities of Fresh Leaf Tissue. Phytochemical Bulletin 19:11-15.
Fernie AR, Klee HJ (2011). The Use of Natural Genetic Diversity in the Understanding of Metabolic Organization and Regulation. Frontiers in Plant Science 2:59.
Friar EA (2005). Isolation of DNA from Plants with Large Amounts of Secondary Metabolites. In Methods in Enzymology 395:1-12.
Friis I (1981). A Synopsis of *Girardinia* (Urticaceae). Kew Bulletin 36(1):143-157.
John ME (1992). An efficient method for isolation of RNA and DNA from plants containing polyphenolics. Nucleic Acids Research 20(9):2381-2381.
Khanuja SP, Shasany AK, Darokar MP, Kumar S (1999). Rapid isolation of DNA from dry and fresh samples of plants producing large amounts of secondary metabolites and essential oils. Plant Molecular biology Reporter 17(1):74.
Kit YS, Chandran S (2010). A simple, rapid and efficient method of isolating DNA from Chokanan mango (*Mangifera indica* L.). African Journal of Biotechnology 9(36):5805-5808.
Kumar A, Singh I, Badari NS (2018). Extraction of Genomic DNA from Dried mature leaves of *Eclipta alba* (L.) suitable for ISSR-PCR and other DNA fingerprinting and Barcoding techniques. International Journal of Molecular Biotechnology 4(2):23-30.
Kumari M, Jadhav AS, Chahande RV (2020). Isolation of genomic DNA from groundnut plant by modified, rapid and efficient protocol. Journal of Pharmacognosy and Phytochemistry 9(1):2268-2271.
Mohamad A, Alhasnawi AN, Kadhimi AA, Isahak A, Wan Yusoff WM, Che Radziah CMZ (2017). DNA Isolation and Optimization of ISSR-PCR Reaction System in *Oryza sativa* L. International Journal on Advanced Science, Engineering and Information Technology 7(6):2264.
Mujeeb F, Bajpai P, Pathak N, Verma SR (2017). Genetic Diversity Analysis of Medicinally Important Horticultural Crop *Aegle marmelos* by ISSR Markers. In L. Domingues (Ed.), PCR. Springer New York. pp. 195-211. Available at: https://link.springer.com/protocol/10.1007/978-1-4939-7060-5_14
Nilkanta H, Amom T, Tikendra L, Rahaman H, Nongdam P (2017). ISSR Marker Based Population Genetic Study of *Melocanna baccifera* (Roxb.) Kurz: A Commercially Important Bamboo of Manipur, North-East India. Scientifica 2017:1-9.
Njogu PM, Thoithi GN, Mwangi JW, Kamau FN, Kibwage IO, Kariuki ST, Mwalukumbi JM (2011). Phytochemical and Antimicrobial Investigation of *Girardinia diversifolia* (Link)Friis (Urticaceae). East and Central African Journal of Pharmaceutical Sciences 14(3):89-94.
Osena G, Nyaboga E, Amugune N (2017). Rapid and Efficient Isolation of High Quality DNA from Cassava (*Manihot esculenta* Crantz) Suitable for PCR Based Downstream Applications. Annual Research and Review in Biology 12:1-10.
Polunin O, Stainton A (1984). Flowers of the Himalaya. Oxford University Press.
Porebski S, Bailey LG, Baum BR (1997). Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. Plant Molecular Biology Reporter 15(1):8-15.
Puchooa D (2004). A simple, rapid and efficient method for the extraction of genomic DNA from lychee (*Litchi chinensis* Sonn.). African Journal of Biotechnology 3(4):253-255.
Reddy MP, Sarla N, Siddiq EA (2002). Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. Euphytica 128(1):9-17.
Sá O, Pereira JA, Baptista P (2011). Optimization of DNA Extraction for RAPD and ISSR Analysis of *Arbutus unedo* L. Leaves. International Journal of Molecular Sciences 12:4156-4164.
Sarrazola JH, Alzate FA (2019). Obtaining DNA from Urticaceae: Overcoming the challenges associated with chemical compounds and herbarium specimens. International Journal of Molecular Biology 4(5):158-165.
Shrestha SS, Sut S, Ferrarese I, Barbon Di Marco S, Zengin G, De Franco M, Pant DR, Mahomoodally MF, Ferri N, Biancorosso N, Maggi F, Dall Acqua S, Rajbhandary S (2020). Himalayan Nettle *Girardinia diversifolia* as a Candidate Ingredient for Pharmaceutical and Nutraceutical Applications—Phytochemical Analysis and In Vitro Bioassays. Molecules 25:1563.
Shrestha R (1997). Cytological Studies in *Girardinia Diversifolia* (Link) Friis. Pakistan Journal of Botany 29(2):263-269.
Shukla R, Sharma DC, Pathak N, Bajpai P (2018). Genomic DNA Isolation from High Polyphenolic Content *Grewia asiatica* L. Leaf Without Using Liquid Nitrogen. Iranian Journal of Science and Technology, Transactions A: Science 42:347-351.
Singh SC, Shrestha R (1988). *Girardinia diversifolia* (Urticaceae), a non-conventional fiber resource in Nepal. Economic Botany 42:445-447.
Subedee BR, Chaudhary RP, Uprety Y, Dorji T (2020). Socio-ecological perspectives of Himalayan Giant Nettle (*Girardinia diversifolia* (Link) Friis) in Nepal. Journal of Natural Fibers 17(1):9-17.
Tiwari KL, Jadhav SK, Gupta S (2012). Modified CTAB Technique for Isolation of DNA from some Medicinal Plants. Research Journal of Medicinal Plant 6(1):65-73.
Wu ZY, Monro AK, Milne RI, Wang H, Yi TS, Liu J, Li DZ (2013). Molecular phylogeny of the nettle family (Urticaceae) inferred from multiple loci of three genomes and extensive generic sampling. Molecular Phylogenetics and Evolution 69(3):814-827.



Journal of Natural Fibers

ISSN: 1544-0478 (Print) 1544-046X (Online) Journal homepage: http://www.tandfonline.com/loi/wjnf20

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To cite this article: Bijay Raj Subedee, Ram Prasad Chaudhary, Yadav Uprety & Tashi Dorji (2018): Socio-ecological perspectives of Himalayan Giant Nettle (Girardinia diversifolia (Link) Friis) in Nepal, Journal of Natural Fibers, DOI: 10.1080/15440478.2018.1458684

To link to this article: https://doi.org/10.1080/15440478.2018.1458684

Published online: 03 Apr 2018.

JOURNAL OF NATURAL FIBERS
https://doi.org/10.1080/15440478.2018.1458684

Socio-ecological perspectives of Himalayan Giant Nettle (*Girardinia diversifolia* (Link) Friis) in Nepal

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ABSTRACT

Natural fibers have attracted considerable attention in recent years because of their low cost, renewable, and eco-friendly nature. The study investigated the economic, social, medicinal, and traditional use of Himalayan Giant Nettle commonly called "Allo" (*Girardinia diversifolia*) in Nepal. The fiber extracted from "Allo" is one of the income sources for people living in rural mountainous region of Nepal. The traditional fiber processing techniques, use of locally available materials, and medicinal value helped to preserve "Allo." Nevertheless, the resource stock is declining from its natural habitat due to changes in traditional grazing practices, rapid expansion of plantation of cash crops like large cardamom (*Amomum subulatum*) and Chirayito (*Swertia chirayita*) where "Allo" grows naturally. Cultivation, sustainable harvesting, and policy formulation on "one-door taxation" system are important to preserve this important natural fiber species.

摘要

天然纤维以其低成本、 可再生和环保的特性近年来受到人们的广泛关注。研究经济、社会、 医疗和传统使用的喜马拉雅巨型荨麻一般称为"异"（girardiniadiversifolia）在尼泊尔。从尼泊尔提取的纤维是农村山区居民的收入来源之一。传统的纤维加工技术，利用当地可获得的材料和药用价值，有助于保存"异体"。然而，资源存量下降，由于在传统的放牧方式改变其自然栖息地，对喜欢大小豆蔻的现金作物的种植快速扩张（amomumsubulatum）和Chirayito（swertiachirayita），"异"自然生长。在"一道税"制度下，种植、可持续采伐和政策制定对于保护这一重要的天然纤维物种十分重要。

KEYWORDS

Himalayan Giant Nettle; bast fibers; Kailash Sacred Landscape; economic potential; traditional fiber; Allo

关键词

喜马拉雅荨麻; 韧皮纤维; 神山圣景; 经济潜力; 传统的纤维

Introduction

The growing interests from industry, scientists, and consumers on eco-friendly natural resources have attracted considerable attention toward natural fibers (Silva, Chawla., and Filho 2008). There are more than 70 fiber plant species that grow from tropical to temperate forest of Nepal. Jute (*Corchorus capsularis*), hemp (*Cannabis sativa*), and Himalayan Giant Nettle (*Girardinia diversifolia*) popularly called "Allo" in Nepal are predominantly used fiber plants compared to other species (Singh and Shrestha 1984).

"Allo" belongs to family Urticaceae. It grows across Nepal between 1200 and 3000 m above sea level. It is also found in China, India, Bhutan, and East Africa induding Madagascar (Chen et al. 2003; Shrestha & Hoshion 1998; Friis 1981). The plant is a shade-loving shrub, 1.5–3 m tall with the perennial rootstock having stinging spikes. Fiber is found in the inner bark of the stalk with high strength and length (Singh and Shrestha 1988). It has longest fiber length reported for any bast fiber

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(Lanzilao, Goswami, and Blackburn 2016). The fiber is stronger, environment friendly than cotton, and finer than other bast fibers such as hemp (Summerscales et al. 2010).

Socio-ecological arrangement utilizes natural, socioeconomic, and cultural resources which are harmonized by the combination of ecological and social systems (Redman, Grove., and Kuby 2004). Indigenous peoples and local communities (IPLCs) of Nepal utilize the fiber for domestic use and produce commercial items. "Allo" is an important plant for IPLCs living in Kailash Sacred Landscape (KSL) Nepal, which comprises four districts in Far-Western Nepal namely Darchula, Baitadi, Bajhang, and Humla (Zomer and Oli 2011), and in Sankhuwasabha and Solukhumbhu districts of Eastern Nepal. The IPLCs of Nepal are involved in "Allo" processing and product development from time immemorial (Singh and Shrestha 1988; Deokota & Chhetri 2009). But very few studies are available on socioeconomic and ecological dynamics of this plant species. This article investigated economic, social, medicinal, and traditional use of "Allo" and identified technological and policy gaps and provided recommendation for sustainable use and management of "Allo".

Methods

Study area

The present study was conducted in Nepal. Nepal is a landlocked Himalayan country in South Asia, bordering China in the north and India in the south, east, and west. It lies parallel to the Himalayan ranges and stretches 800 km from 80°05′ to 88°10′E, with an average width about 180 km, with latitudes ranging from 26°20′ N in the east to 30° 25′N in the west. Owing to its climatic and geographical variations, Nepal is rich in biological diversity (Miehe, Pendry, and Chaudhary 2015).

The study was conducted in two districts, namely Darchula and Sankhuwasabha, from two different regions (Far-Western and Eastern) of Nepal where "Allo" is abundantly available in forests and has sociocultural values (Figure 1). Local communities of Darchula district have recently started

Figure 1. Study area with KSL Nepal in Far-Western region and Sankhuwasabha district in Eastern region of Nepal (Source: ICIMOD).

commercialization of "Allo" products, whereas the communities of Sankhuwasabha district had established the "Allo" market since long time. The following Village Development Committees (VDCs) were selected to document traditional use, processing, and resource management practices of "Allo": Khar, Airkot, Katae, Septi, Sitola of Darchula district located in Api-Nampa Conservation Area, KSL Nepal and Bala, Sisuwa, Tamku, and Mangtewa of Sankhuwasabha districts located in Makalu-Barun National Park, Eastern region of Nepal.

Field survey and data collection

Field surveys were conducted from 2014 to 2016 at different seasons. Key informant interviews, focus group discussions, informal meetings, and field observations were used as a primary method of data collection. Semi-structured questionnaire was used to collect information after obtaining informed consent with the local communities. Non-timber forest products (NTFPs) collectors, traders, "Allo" processers, and community members were invited in focus group discussions and informal meetings. A total of 110 informants (30 male, 80 female) representing different ethnic groups including Kulung Rai of Eastern Nepal and Thagunna, Dhami, Manyal, and Bohora communities from Far- Western Nepal had participated in the study.

Results

Cultural use of "Allo" by different ethnic groups of Nepal

Kulung Rai community of Eastern Nepal perform a rite for *nagi* (a supernatural water serpent) (Dougal 1973) and offer cloth made from "Allo." During the entry of new house, during marriage ceremony, and death funeral, "Allo" cloth is an important cultural item of Kulung Rai. Kulung Rai communities have a strong belief that "Allo" was associated by creating the world, and the traditional red and blue [green as shown in Figure 2] stripped "Allo" cloth called lalachaar or lukspa are still used during Shaman's ceremonies (Dunsmore 1998). During funeral, dyed "Allo" cloth is the most important requirement. "Allo" cloth is divided into two halves, male and female. The ingredients required for dyeing of "Allo" cloth is only known to few aged men who remain sanctity at the time of dyeing process. After dyeing cloth, corpse should be wrapped with the "Allo" cloth but the cloth should be removed before cremation. The ingredients of natural dye for "Allo" cloth could have a commercial value and could provide market opportunity for "Allo." In these communities, "Allo" is also used as fodder for livestock, bedding material, and firewood.

Figure 2. Kulung Rai communities of Eastern Nepal showing naturally dyed "Allo" cloth.

Use of "Allo" fiber to make cloth is also reported from other parts of Nepal, for example, Gurung communities living in Annapurna Conservation Area prepare "Allo" cloth known as *bhangra*. Two types of *bhangra* are available in the area, namely soft *masino kuldu* and rough *gerdu kuldu* (Gurung et al. 2012). In Gurung community during feasts and festivals such as Tamu loshar, men wear soft *bhangra*, a white shirt like cloth opened at the back for carrying things. They use rough *bhangra* as sack for harvesting crops, fodder collection, and firewood collection (Gurung et al. 2012). Chhantyal communities living in Myagdi and Baglung district of Nepal exchange "Allo" cloth with surrounding villagers to develop social relation called *Gharpati* (house owner) and *Sangina* (guest). "Allo" cloth is also used as gift and is considered to bring good fortune to the family (Pun 2011).

Traditional use and processing of "Allo"

Different parts of the plant like root, stem, leaf, and inflorescence are used by local healers (*Vaidhya*); local communities such as Bohora, Dhami, and Thagunna of Darchula district use "Allo" for treating gastritis, joint pain, headache, tuberculosis, and asthma. Indigenous communities (Kulung Rai, Gurung) utilize the fiber of the plant for making different articles for their daily use (Table 1 and Figure 3). Of the total 110 informants, 77% ($N = 85$) know the collection and processing techniques for "Allo". These informants at least know a single use of "Allo" or have heard about its uses.

Table 1. Traditional use of "Allo."

Parts used	Traditional use
Root	Juice is used to treat gastritis. Paste is applied in dog bite and joint pain
Bark	Fiber obtained from the bark is applied for production of different articles such as porter strap *Namlo*, fishing nets, bags, sacks clothing materials, weaving rugs, jackets. Cloth of "Allo" is believed to have an anti-allergic effect on skin
Leaf	Juice is used to treat headache, joint pain, tuberculosis, and asthma. It is also used as a fresh and dried vegetable.
Stem	It is heated and wrapped around the leg and hand to treat bone fractures
Inflorescence	Used as vegetable and soup and considered as high nutritional value

Figure 3. "Allo" processing techniques and equipment used in Far-Western and Eastern regions of Nepal. (a) Harvesting of 'Allo' bark. (b) Cooking of 'Allo' bark in iron drum. (c) 'Allo' bark drying with white clay (Kamero). (d) Spinning of fiber (e) Use of wooden handloom for weaving of 'Allo' clothes. (f) Products made from 'Allo'fiber.

Table 2. Traditional "Allo"-processing equipment, materials, and technology used by the people of Eastern and Far-Western regions of Nepal.

Harvesting	Iron sickle (*Hasiya, Khurpa, Khukuri*) is used for harvesting of "Allo" stem from community and national forests (Deokota and Chhetri 2009). Porter strap *Namlo* is used by local people to carry heavy loads
Wrapping cloth	"Allo" plant has stinging spikes. For wrapping purpose, inoperative cloth is used to remove the bark from "Allo" stem
Cooking drum	Iron drum is preferred for cooking purpose. Aluminum vessel is prohibited because wood ash and caustic soda or sodium hydroxide (NaOH) may react with the vessel
Cooking stove	Traditional cooking stove made up of three stones locally called *Chulo* are used, which consumes large quantity of firewood
Firewood	"Allo" bark soaked for 24 h in water is mixed with wood ash and cooked for 3–4 h. Five kilograms of firewood is needed to cook per kilogram of "Allo" in the traditional cooking stove
Wood ash	
Caustic soda	"Allo" fiber is tightly bound with the pectin molecule. It is hard to remove non-fibrous part. Caustic soda helps to remove non-fibrous part of the bark. About 2 g of caustic soda is required for 1 kg of "Allo."
Wooden hammer	Traditional wooden hammer *Mungro* is manually used for beating "Allo" to remove non-fibrous part of the bark. After cooking, beating of "Allo" stem bark near the water sources is preferred
White clay, talc (*Kamero*)	Locally available soil called *Kamero* is mostly used for the softening of fiber. It helps to remove the non-fibrous part of the bark
Hand spindle (*Katuwa*)	Wool extracted after cooking process, "Allo" is used to prepare fiber. A *Katuwa* is a portable yarn spinning equipment which is used to prepare fiber
Handloom	Locally made wooden handlooms are utilized. Communities use locally available wooden source of *Pinus wallichiana, Prunus cerasoides, Quercus lanata,* and *Dendrocalamus* species to prepare handloom

There are some differences in the processing techniques followed by local communities of Eastern and Far-Western regions. Processing techniques and equipment used by various communities are listed in Table 2.

Harvesting

Local communities annually harvest "Allo" during November from both community-managed forest and government-managed national forest. Mature stems are selected and bark are removed manually by using knife and hand gloves. People of Far-Western region harvest and carry whole stem and peel off the bark at home but in Eastern region people peel off "Allo" bark at forest after harvesting.

Income generation by "Allo"

Collection and trading of NTFPs are the main income-generating source for the people living in Far-Western and Eastern regions of Nepal. Among the NTFPs, "Allo" is a good income source for the people living in Darchula and Shankhuwashbha districts. Mostly women are engaged in collection, processing, and product designing. Women sell dry raw bark, fiber, bags, sacks, weaving rugs, jackets, and clothes made from "Allo" in local and national markets (Subedee et al. 2017a).

Cost-benefit analysis during harvesting, processing, and weaving of "Allo" bark

The cost analysis was conducted after consulting with "Allo" entrepreneurs of Khar VDC, Darchula district. There are various steps of "Allo" products starting from one to seven. The detailed cost analysis of steps from one to seven is given in Table 3.

Availability of Allo in natural habitats

During the study, in total 110 respondents, 89% (n = 98) respondents reported on disturbance of natural habitat of "Allo." Growth of plant species and yield of fiber use were found to be in

Table 3. Cost analysis of "Allo" during harvesting, processing, and weaving.

Steps	Activities	Weight (kg)	Day/man	Cost NRs	Selling price per kg	Selling price (NRs)	Profit %	Loss %
1.	Green, fresh bark collection from forest	25	1 day/man	400				
2.	Drying "Allo" bark	5 kg dry bark (20 kg get lost in the form of moisture after drying)			Dried bark NRs 100/kg	500	25	
3.	Firewood for cooking "Allo"	50 kg		400				
4.	Cooking, beating, washing, and mixing with *Kamero*	4 kg of wool produced (1 kg gets wasted while beating)	2 days/man	800				
5.	Spinning yarn	3.5 kg (0.5 gm get wasted while spinning)	7 days/man	2800 + 1600 = 4400	1000/kg	3500		20.45
6.	Preparation of loom		1 day/man	400				
7.	Weaving of "Allo" cloth	3.5 kg yarn (1 kg yarn produces 3m cloth)	4 days/men	1600 + 4800 = 6400	1000/m	10,500	64	
	Total		15 days/man	12,800		14,500	13	

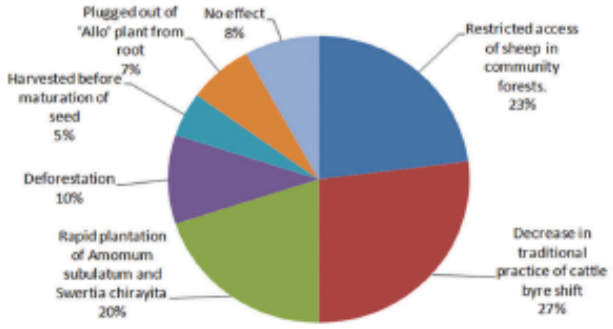

Figure 4. Causes of decrease of natural habitat of "Allo."

declining stage due to the destruction of natural habitat. The detailed analysis is given in Figure 4.

Sheep husbandry is an important livelihood activity of people in Darchula for meat (food), wool, and as source of manure. The excreta of sheep is a good source of fertilizer for "Allo" plants. The "Allo" seeds attached on wool of sheep during grazing help in seed dispersal in forest and thus contribute to plant regeneration. In most of the VDCs of Darchula district, sheep are still used as means of transportation to carry goods. There are several traditional routes along the forests. The issue is that sheep are not allowed to graze in community-managed forests; thus, the lack of access to traditional grazing in the forests has affected sheep husbandry and also greatly affected natural regeneration of "Allo." About 23% of respondents mentioned that the loss of "Allo" in natural habitat is due to decrease of sheep numbers. About 27% of the respondents mentioned that the loss of natural habitat of "Allo" is due to decrease in traditional practice of cattle shifting in byre. About 20% of the respondents reported that the loss of "Allo" habitat is due to the increase in cultivation of large cardamom (*Amomum subulatum*) and a medicinal herb Chirayita (*Swertia chirayita*) farming in previously "Allo"-growing land; about 10%

reported that the reason is deforestation. "Allo" is a shade-loving plant; thus, cutting down shade-giving trees also affect the "Allo" regeneration. About 7% responded that the removal of whole "Allo" plant to support fodder grass regeneration for domesticated animal contributes to "Allo" loss while about 8% responded no effect on the natural habitat and only 5% realized destruction due to the harvest of plant before the maturation of seed (Figure 4).

Community practice for sharing natural resources

Kulung communities in Sankuwasabha are following the calendar for harvest of "Allo" resource. They harvest "Allo" in particular month and day of the year. "Allo" collection and processing groups have been formed by Kulung communities. Every year Kulung community collect "Allo" from forest starting on first day of *Mangsir* (mid-November) and spend a week inside the forest for collecting "Allo." They equally share the collected "Allo" bark among the members. But no such community-managed system is found in Far-Western region. People of this region harvest "Allo" from forest randomly with no certain month and date. Haphazard collection and overexploitation of "Allo" have resulted in decline of resource in natural habitat.

Discussion

This study revealed that IPLCs from Far-Western and Eastern regions follow different traditional methods of harvesting and processing of "Allo." Peoples are aware about the cultural, medicinal, and economic importance of "Allo."

The study documented that "Allo" is mainly used for joint pain, skin allergies, and gastritis. Similar observations conducted in other parts of Nepal add more evidence about its medicinal properties. Root juice is used for treating gastritis, constipation, and is applied on swelling (Barakoti and Shrestha 2008; Gurung et al. 2012; Malla, Gauchan, and Chhetri 2014).

"Allo" root is mixed with *Centella asiatica* and boiled about 10 minutes in water and the prepared juice about four teaspoons twice a day is used for treating gastritis Juice of root about six teaspoons is used twice a day for treating constipation (Manandhar 2002). Juice of "Allo" root is used for treating gastritis and constipation and as is an antiulcer agent. Juice of leaf is used for headache, fever, and joint pains (Garg and Saggu 2017; Uniyal and Shiva 2005). Thami ethnic group of Eastern Nepal has a belief that "Allo" spines can stimulate milk production. (Turin 2003). Stem of "Allo" is used to produce blue and green natural dyes (Shrestha 1994).

At present, the traditional processing practices have been altered by the use of caustic soda instead of wood ash. Though caustic soda replaces non-fibrous part faster than the ash, it pollutes water, degrades fiber quality, and affects the health of "Allo" farmers. Traditional three stone cooking stoves are used to cook "Allo," which needs more firewood, exhausts more smoke, and is less efficient (Specht et al. 2015). Rocket stove can be a good alternative (Subedee et al. 2017b). Regeneration from seed is not easy if the plant is harvested earlier or later than the maturation time. Ideal season for "Allo" harvest starts in mid-November (Chanysi 2011) as practiced by the communities of Eastern region.

Use of high-quality clay *Kamero* is an important step for softening of fiber. But softening potential varies from place to place. The effect of various qualities of *Kamero* needs to be studied. *Katuwa* is a portable yarn spinning equipment but it is less efficient. Modern spinning machine (motorized Charkhas) can be an alternative option which will enable fiber spinning faster and efficiently (Shrestha 1994). Locally made wooden handlooms are utilized in traditional processing which are difficult to operate and hardly weave 3 m of cloth per day. Traditional handlooms could be replaced by metallic handlooms. It is more profitable to sell refined "Allo" cloth than raw "Allo" fiber. The demand of shawl and sweater in national and international markets is high which could be tapped by local women (MEDEP 2010). Training in knitting for the products like shawl and sweater

would be more beneficial for adding value to the "Allo" products for local people (Chauhan, Bahti and Juyal 2013).

People are harvesting "Allo" directly from the forest. They have to travel a long distance and have to spend a week in the forest to collect "Allo" bark. Another threatening issue is massive plantation of large cardamom (*Amomum subulatum*) and Chirayita (*Swertia chirayita*), where people have replaced "Allo" plant and have planted large cardamom. Large cardamom has been one of the income-generating options which has occupied the habitat of "Allo." High exploitation rate of "Allo" and massive destruction of its natural habitat indicates that conservation of "Allo" natural habitat is necessary for cultural achievements and economic benefits.

Tax policy on transportation of raw "Allo" bark and finished products from village to national and international markets is undear. Farmers/collectors in Nepal have to pay tax at several points while carrying both "Allo" bark and its products (Dunsmore 1998). Since these taxation systems are ambiguous for most of the NTFPs including "Allo," stakeholder's engagement and dialogue could only resolve the issues.

Conclusions

The present study reveals that "Allo" has medicinal, economic, and cultural importance for the people living in Far-Western and Eastern regions of Nepal. Traditional and cultural beliefs helped "Allo" to sustain in its natural habitat. "Allo" can provide livelihood opportunities in terms of economic opportunity and medicinal value. Though people are trying to improve their income through "Allo," lack of technological know-how is limiting them. This artide concludes that the technological intervention, research and development on cultivation, sustainable harvesting, and policy formulation on "one-door taxation" system are important for improving livelihood opportunities for rural people through "Allo."

Acknowledgment

The views and interpretations expressed in this publication, however, are those of the authors.

Funding

This study has been undertaken jointly by the Ministry of Forests and Soil Conservation (MoFSC) Nepal, Research Center for Applied Science and Technology (RECAST) under Tribhuvan University, Nepal and the International Center for Integrated Mountain Development (ICIMOD) under the Kailash Sacred Landscape Conservation and Development Initiative (KSLCDI). KSLCDI is a joint effort to promote transboundary cooperation on landscape conservation among China, India and Nepal through the respective governments and partners which is supported by the Ministry for Economic Cooperation and Development (BMZ), Germany, the DFID/UKAid, and core funds of ICIMOD contributed by the governments of Afghanistan, Australia, Austria, Bangladesh, Bhutan, China, India, Myanmar, Nepal, Norway, Pakistan, Switzerland, and the United Kingdom.

References

Barakoti, T., and K. Shrestha. 2008. Commercial utilization of 'Allo' (*Girardinia diversifolia*) by the Rais of Sankhuwasabha for income generation. *Banko Janakari* 18 (1):18–24. doi:10.3126/banko.v18i1.2162.

Chanysi, H. G. 2011. *A resource manual on 'Allo' cultivation, conservation, processing, weaving and market management*. Kathmandu: Shree Manjushree press.

Chauhan, S., S. Bahti, and M. Juyal. 2013. *Value chain solutions to bridge the rural urgan divide in Uttarkhand India*. India: GIZ-RED Programme, Uttarakhand, Sustainable Economic Development.

Chen, C. J., Q. Lin, I. Friis, C. M. Wilmot-Dear, and A. K. Monro. 2003. Urticaceae. *Flora of China* 5:76–189.

Deokota, R., and R. B. Chhetri. 2009. Traditional knowledge on wild fiber processing of 'Allo' in Bhedetar of Sunsari District, Nepal. *Kathmandu University Journal of Science, Engineering and Technology* 5 (1):136–42. doi:10.3126/kuset.v5i1.2853.

Dougal, M. C. 1973. Structure and division in Kulung Rai society. *Kailash* 1:205–24.
Dunsmore, J. 1998. Microenterprise development: Traditional skills and the reduction of poverty in highland Nepal. *Himalaya, Journal of the Association for Nepal and Himalayan Studies* 18 (2):22–27.
Friis, I. 1981. A synopsis of *Girardinia* (Urticaceae). *Kew Bulletin* 36 (1):143–57. doi:10.2307/4119014.
Garg, N., and K. H. Saggu. 2017. Mapping and profile of Himalayan nettle (*Girardinia diversifolia*) units in Uttrakhand. *International Journal of Home Science* 3 (1):146–49.
Gurung, A., H. Flanigan., A. Ghimeray., R. Karki., R. Bista., and O. P. Gurung. 2012. Traditional knowledge of processing and use of the himalayan giant nettle (*Girardinia diversifolia* (Link) Friis) among the Gurungs of Sikles, Nepal. *Ethnobotany Research and Applications* 10:167–74. doi:10.17348/era.10.0.167-174.
Lanzilao, G., P. Goswami, and R. S. Blackburn. 2016. Study of the morphological characteristics and physical properties of Himalayan giant nettle (*Girardinia diversifolia* L.) fibre in comparison with European nettle (Urtica dioica L.) fibre. *Materials Letters* 181:200–03. doi:10.1016/j.matlet.2016.06.044.
Malla, B., D. P. Gauchan, and R. B. Chhetri. 2014. Medico-ethnobotanical investigations in Parbat district of Western Nepal. *Journal of Medicinal Plants Research* 8 (2):95–108. doi:10.5897/JMPR2013.5228.
Manandhar, N. P. 2002. *Plants and people of Nepal*. Portland: Timber press.
MEDEP (Micro-Enterprise Development Programme). 2010. *Value chain based approach to micro-enterprise development: Value chain analysis-'Allo'. Value chain analysis series*. Bakhundole, Lalitpur. http://medep.org.np/epanel/publication/docs/Value%20Chain%20Analysis.pdf
Miehe, G., C. A. Pendry, and R. Chaudhary. 2015. *Nepal: An introduction to the natural history, ecology and human environment of the Himalayas*. Edinburgh: Royal Botanic Garden Edinburgh.
Pun, Y. B. 2011. *Indigenous knowledge on 'Allo' management, a study on Chhantyal community of Mayadhi District*. Lalitpur: Social Inclusion Research Fund (SIRF) SNV.
Redman, C. L., M. J. Grove., and H. L. Kuby. 2004. Integrating social science into the Long Term Ecological Research (LTER) network: Social dimensions of ecological change and ecological dimensions of social change. *Ecosystems* 7 (2):161–71. doi:10.1007/s10021-003-0215-z.
Shrestha, B. 1994. *Dye-Yielding Plants of Nepal*. Kathmandu: Research Centre for Applied Science and Technology, Tribhuvan University.
Shrestha, R., and T. Hoshion. 1998. Karyomorphological studies in *Girardinia diversifolia* (Link) Friis (Urticaceae) collected from Nepal. *Journal of Japanese Botany* 73 (3):125–27.
Silva, F. A., N. Chawla., and R. D. T. Filho. 2008. Tensile behavior of high performance natural (sisal) fibers. *Composites Science and Technology* 68:3438–43. doi:10.1016/j.compscitech.2008.10.001.
Singh, S. C., and R. Shrestha. 1984. *Investigation on under-exploited plant fibers of Nepal*. Kathmandu: Research Centre for Applied Science and Technology. Tribhuvan University.
Singh, S. C., and R. Shrestha. 1988. *Girardinia diversifolia* (Urticaceae), a non-conventional fiber resource in Nepal. *Economic Botany* 42 (3):445–47.
Specht, M. J., S. R. R. Pinto., P. U. Albuquerque., M. Tabarelli., and F. P. L. Melo. 2015. Burning biodiversity: Fuelwood harvesting causes forest degradation in human-dominated tropical landscapes. *Global Ecology and Conservation* 3:200–09. doi:10.1016/j.gecco.2014.12.002.
Subedee, B. R., R. P. Chaudhary, T. Dorji, and A. J. Shrestha. 2017a. Indigenous and local knowledge of conservation and sustainable use of Himalayan Giant Nettle (Girardinia diversifolia (Link) Friss) in Eastern and Far-Western Regions of Nepal. In *Knowing our lands and resources*, eds. M. Karki, R. Hill, W. Alangui, K. Ichikawa, and P. Bridgewater, 191–98. Paris: United Nations Educational, Scientific and Cultural Organization (UNESCO.
Subedee, B. R., R. P. Chaudhary, and K. R. Shrestha. 2017b. Use of Rocket stove for firewood savings and carbon emission reductions by the households involved in Allo (*Girardinia diversifolia)* fiber processing at Khar VDC, Darchula District, Nepal. *International Journal of Latest Engineering and Management Research* 2:28–35.
Summerscales, J., N. P. J. Dissanayake, S. A. Virk, and W. Hall. 2010. A review of bast fibers and their composites. Part 1-Fibers as reinforcements. *Composites: Part A* 41:1329–35. doi:10.1016/j.compositesa.2010.06.001.
Turin, M. 2003. Ethnobotanical notes on Thangmi plant names and their medicinal and ritual uses. *CNAS Journal* 30:19–52.
Uniyal, B., and V. Shiva. 2005. Traditional Knowledge on medicinal plants among rural women of the Garhwal Himalaya, Uttaranchal. *Indian Journal of Traditional Knowledge* 4 (3):259–66.
Zomer, R., and K. P. Oli, eds. 2011. *Kailash sacred landscape conservation initiative – Feasibility assessment report*. Kathmandu: ICIMOD.

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Author(s): Lipy Adhikari, Anu Joshi Shrestha, Tashi Dorji, Eileen Lemke, and Bijay Raj Subedee
Source: Mountain Research and Development, 38(1):4-13.
Published By: International Mountain Society
https://doi.org/10.1659/MRD-JOURNAL-D-17-00074.1
URL: http://www.bioone.org/doi/full/10.1659/MRD-JOURNAL-D-17-00074.1

Mountain Research and Development (MRD)
An international, peer-reviewed open access journal
published by the International Mountain Society (IMS)
www.mrd-journal.org

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Transforming the Lives of Mountain Women Through the Himalayan Nettle Value Chain: A Case Study From Darchula, Far West Nepal

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Naugad is a remote rural municipality in the mountains of far west Nepal with poor accessibility and limited economic opportunities, especially for women and marginalized communities. Promotion of the natural resource-based value chain for allo (the Himalayan nettle, Girardinia diversifolia) was identified as an innovative livelihood strategy by the local community. Value chain development started in 2014. The project was designed to focus on women and include participation by the private sector. This paper analyzes the impact of the project, especially on women's lives, using primary and secondary data. A community-owned enterprise was established with private-sector support from the South Asian Association of Regional Cooperation's Business Association of Home Based Workers (SABAH) Nepal. The enterprise now has 82 members (69 of them women), with 150 households benefiting directly and indirectly. SABAH Nepal provided training in sustainable harvesting and processing techniques and promotes the products in high-end international markets. A buyback guarantee scheme provides security to local artisans. The quality and range of allo products have increased markedly, as has the share in benefits for local people. Skills training and visits to trade fairs have helped women build their capacity and take a leading role in the value chain process. The community-owned enterprise members have earned up to NPR 4000 per month from sewing, more than the local rate for day labor and sufficient to cover general household expenses. More than 25 women entrepreneurs have started microbusinesses related to allo. Allo has become an important economic asset, transforming the lives of mountain women in this village area. The approach has potential for scaling up across the subtropical to temperate areas of the Himalayan region in Bhutan, China, India, Myanmar, and Nepal.

Keywords: *Allo; Himalayan nettle; Girardinia diversifolia; mountain women; common facility center; poverty; sustainable livelihoods; value chain; private sector.*

Peer-reviewed: *December 2017* ***Accepted:*** *January 2018*

Introduction

Naugad is a small rural municipality in Darchula district in far west Nepal with poor accessibility, low economic development, and limited livelihood opportunities. As a result of its remoteness and lack of basic facilities, the proportion of villagers living in poverty is greater than the national average. Residents have traditionally depended on subsistence farming supplemented by collection and sale of non-timber forest products (NTFPs), such as the caterpillar fungus *yarshagumba (Ophiocordyceps sinensis)*, for their livelihoods (Negi 2007). *Yarshagumba* is a high-value medicinal herb, mostly found in alpine and subalpine pastures of the Tibetan plateau and the Himalayas, that is becoming one of the biggest contributors to the cash economy in high mountain areas of Nepal (Shrestha and Bawa 2013; Pant et al 2017). Men, women, and children risk their lives on the high mountain slopes to collect it (Pant et al 2017).

In recent years, considerable competition has emerged between local residents and outsiders, as well as among local collectors, for control over *yarshagumba* and other NTFPs, which has threatened the local people's livelihoods (Pant et al 2017). At the same time, men have started to migrate to India and other countries in search of employment, leaving women with extra responsibilities as heads of household (Sharma 2011). The cultural and social norms of the area also tend to limit the possibilities for women and members of other marginalized groups to explore new opportunities. Traditionally, women marry early and are confined to the household. Working outside

the home and earning income to support the family are not encouraged.

The Himalayan nettle *allo* (*Girardinia diversifolia*, also called *puwa* in western Nepal) is a fiber-yielding, self-sustaining perennial herb that can grow up to 1.5 m high (Singh and Shrestha 1988; Barakoti and Shrestha 2009; Gurung et al 2012; Radhakrishnan and Preeti 2015). *Allo* is widely distributed in the subtropical and temperate Himalayas and grows in mountain and hill areas across Nepal at elevations between 1200 and 3500 masl (Singh and Shrestha 1989; Shrestha 1997). After cutting, the dry bark is peeled off and fiber is extracted by boiling, washing, and beating. *Allo* fiber has physical and mechanical properties that are better suited to textile work than other commonly used fibers and the longest fiber length of any natural fiber (Lanzilao et al 2016).

Traditionally, the fiber has been processed, spun, and woven to make coarse products—jackets, cloth, headbands for carrying, rope, mats, fishnets, grain sacks, bags, and blankets (Singh and Shrestha 1985; Shrestha 1997; Clarke 2007; Barakoti and Shrestha 2009; Pyakurel and Baniya 2011; Gurung et al 2012)—both for direct use and for exchange for grain and other goods (Duthie 1960; Singh and Shrestha 1988). However, there is a growing interest among the local people, government, and private sector in the development of finer commercial products from *allo* (Subedee et al 2017), and the plant has been recognized as an NTFP with high potential for generating rural income, especially in mountain and hill areas (Gurung et al 2012). There is a high demand for clothes made from woven nettle in national and international markets, and they are a prime Nepalese souvenir product (ICIMOD 2015; Subedee et al 2017). At the same time, the textile industry worldwide is looking for alternative sources of natural fiber to reduce dependence on cotton and silk. India has already recognized the potential of naturally occurring nettle and has added the plant to the list of potential plants for use in producing commercial fiber because of its abundance (Radhakrishnan and Preeti 2015). To increase quality and demand, Uttarakhand has initiated scouring and softening of nettle without the use of chemicals and blending with organic cotton or bamboo (at a 50:50 ratio) to make it suitable for open-end spinning (Radhakrishnan and Preeti 2015).

Around 1805 tons of nettle thread are produced in Nepal annually (MEDEP 2010), with half the production consumed in country and half exported. It is mostly used for making carpets and textiles. The total export value of *allo* products from Nepal was NPR 7 million (about US$ 68,000) in fiscal year 2012–2013 alone (TEPC 2014). An estimated 20 tons of raw *allo* bark is produced annually in Darchula, with 8–9 tons of this coming from Naugad (ICIMOD 2015). Despite *allo*'s substantial availability, until recently, local people had limited knowledge and awareness of the possibility of developing sustainable *allo*-based enterprises and effective value chains.

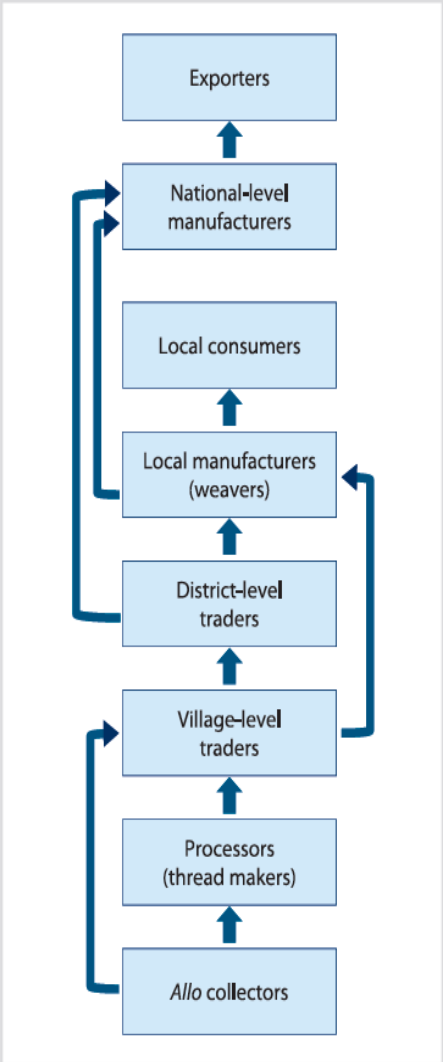
Kaplinsky and Morris (2001) defined value chain as the "full range of activities which are required to bring a product or service from conception, through the different phases of production (involving a combination of physical transformation and the input of various producer services), delivery to final consumers, and final disposal after use." According to Mitchell et al (2009), the development of value chains should use a practical approach that supports specific target groups and is useful in understanding how poor people in rural areas of developing countries can efficiently engage in domestic, regional, or international trade. It is a stepwise process to create a sustainable approach to enable local producer communities to generate employment and gain an equitable share of benefits from their local products (Figure 1). Actors who are part of the value chain mechanism include collectors, processers, manufacturers, traders, and consumers who work together for improving the supply of inputs, extension services, and access to market facilities (ICIMOD 2015; Rasul et al 2016). The value chain approach is arguably one of the most effective ways to improve linkages between businesses and poor communities, tackle poverty, and develop a local resource-based enterprise that benefits local people (Mitchell et al 2009; IFPRI 2016).

However, researchers have found that value chain development often denies access to women and does not recognize their knowledge and contributions (Bhattarai et al 2010; USAID n.d.). Similarly, communities can use small and medium enterprises to generate employment in extractive operations, but only about a third of the workers in these enterprises are women (World Bank 2014), and the women working in them are often paid inadequately (USAID n.d.).

One way to ensure the inclusion of women is to develop women-owned enterprises. This can increase women's financial independence, help them overcome their lack of assets, empower them, and provide them with a voice in the community. According to a recent study (Gurung et al 2012), women have traditionally been responsible for all stages of processing *allo* fiber and weaving *allo* products in Nepal. *Allo* has a high cultural and religious value in the Kulung Rais community, where the cloth made of *allo* is offered to God in special religious events and presented to brides by their parents during wedding ceremonies (Barakoti and Shrestha 2009; Gurung et al 2012). Thus, *allo* has particular potential for supporting women's development in Nepal (Gurung et al 2012).

In developing countries, job creation is a high priority for government and development partners and other public and private institutions whose aim is to reduce poverty and achieve pro-poor growth (OECD 2009). In most cases, development partners emphasize policies and projects that improve the competitiveness of the private sector to create jobs (OECD 2009). Private-sector entities

FIGURE 1 Links in the *allo* value chain in Naugad. (Based on ICIMOD 2017)



include large multinational companies, medium- and small-scale local enterprises, and farmers; they provide necessary goods and services to improve people's lives and are key to stimulating economic growth (OECD/WTO 2015). The private sector plays an important role in enabling business organizations to expand their production frontiers, achieve cost advantages, and enhance their competitiveness and strength (OECD/WTO 2015). Global value chains provide the private sector in developing countries with access to networks, new markets, capital, knowledge, and technology that lead to more diversified and powerful economic growth (OECD/WTO 2015). By promoting low-cost innovations, the private sector can contribute greatly to sustainable development (GIZ 2013), while private-sector engagement helps to ensure continuity of activities after completion of a project (IFPRI 2016). However, attracting private-sector engagement in remote mountain areas is difficult, and it is important for development partners to identify private-sector entities with a social and environmental "sound" approach rather than simply focusing on economic viability.

In 2014, the Kailash Sacred Landscape Conservation and Development Initiative (KSLCDI) Nepal—a joint initiative of Nepal's Ministry of Forest and Soil Conservation, the Research Centre for Applied Science and Technology, and the International Centre for Integrated Mountain Development (ICIMOD), aiming to improve the livelihoods of poor communities in selected far-western districts of Nepal while ensuring efficient use of natural resources—identified development of the *allo* value chain as a high-priority livelihood strategy for communities in Naugad (ICIMOD 2015). The project was designed to achieve the goal with the participation of the private sector and specific focus on women. To ensure a sustainable local supply of *allo* products in Naugad, a private-sector entity partnered with community groups formed by women. Women members were trained in order to improve their skills to meet the required quality and volume specifications. The project ended in December 2017, and this paper analyzes the project's impact on local livelihoods, and especially on women.

Methodology

The methodology was based on the modified value chain analysis framework (Kaplinsky and Morris 2001; Hoermann et al 2010), which emphasizes inclusiveness and mountain specificity and follows the guidance for pro-poor value chain development with a focus on gender mainstreaming offered by Joshi et al (2016).

Study site

Naugad (known as Khar Village Development Committee until 2017, when it was renamed under Local Government Act 2074) is a small rural municipality in Darchula district in far west Nepal (29°45′40.06″ to 29°49′2.33″N, 80°35′51.11″ to 80°41′0.11″E) within the Api Nampa Conservation Area, 12 km from the district headquarters at Dallekh to the northeast. It is around 1650 masl and covers an area of 26 km^2. It has 698 households with a total population of 4272 (2056 male and 2216 female) (NHPC 2011). According to a baseline survey conducted as part of the study in 2015, the 2 main population groups in

Naugad are the Chettri (89%) and the Dalit (7.3%). In Nepal, the Chettri are generally considered socially and economically strong. However, because Naugad lies in the remotest part of the country, even members of its Chettri community are economically weak compared to urban dwellers. Women and Dalits are both identified as marginalized groups in Nepal's Constitution. Marginalized communities are those with less access to political, economic, and social opportunities because of their caste, culture, or sex.

Situation analysis and value chain selection

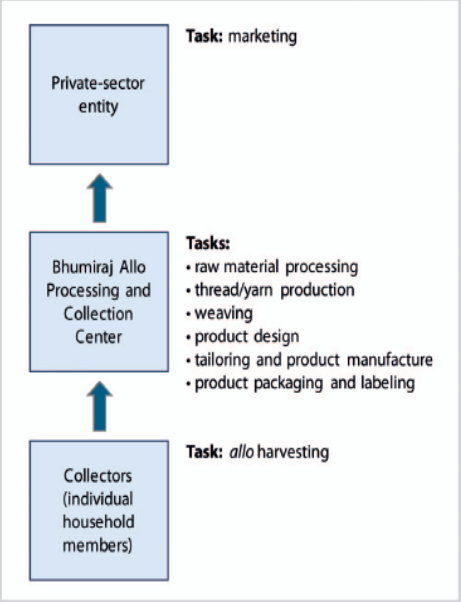
Allo was selected as a potential livelihood source, especially for women, following a stakeholder consultation carried out in 2014 in Naugad with local residents, existing *allo* producers, local entrepreneurs, and local government representatives. The consultation included a household survey conducted with members of *allo* producer groups in Naugad, 5 focus group discussions held with the members of *allo* producer groups, local government agencies, and women's groups at the community level in Naugad, followed by in-depth interviews with women and members of other marginalized groups in Naugad. Skills testing was carried out on yarn processing, weaving, and sewing, and the results were used as a basis for designing the skills-training part of the project.

Description of the private sector's involvement

In 2015, the KSLCDI team held a consultation meeting with the private-sector entities on potential areas for collaboration on strengthening the *allo* value chain in Naugad. One of the major criteria set by the initiative was to select a private-sector entity considering social and environmental aspects, in addition to economic outcomes in business. The South Asian Association of Regional Cooperation (SAARC) Business Association of Home Based Workers (SABAH) Nepal, a national organization possessing a South-Asian wide network that is promoted by SAARC, was selected for the *allo* value chain-strengthening project in Naugad. SABAH-Nepal is registered as a social business organization. Unlike other private-sector entities, SABAH Nepal's approach is to develop common facility centers in which members of the facility centers work together to add value to products at the local level. This gave SABAH Nepal an advantage in enhancing the quality of products and in marketing products made by rural women and men to ensure the sustainability of the project.

In 2016, with support from the KSLCDI, SABAH Nepal and the Naugad community established the Bhumiraj Allo Processing and Collection Center (BHPC). The BHPC is registered as a community-owned enterprise and is the focus of this study. SABAH Nepal provides skills training in sustainable *allo* harvesting, processing, and

FIGURE 2 Role of the Bhumiraj Allo Processing and Collection Center in the *allo* value chain.



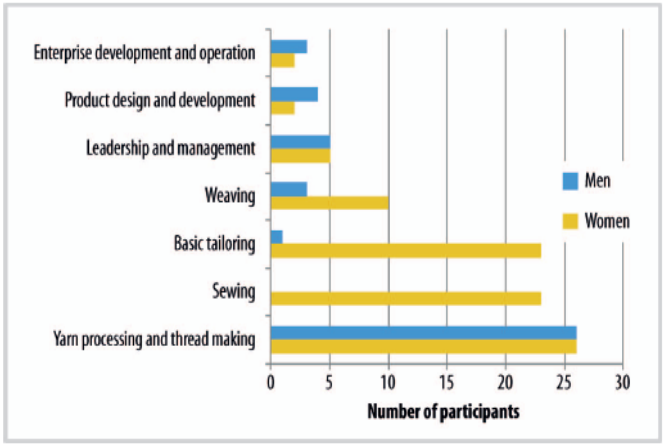
manufacturing and then promotes the *allo* products in high-end international markets. The BHPC started with 23 members in 2016; as of March 2017, its membership had grown to 82 members, including 69 women. In total, it lists 150 households as either members or indirect beneficiaries. With the completion of the project in December 2017, the BHPC was functioning independently with the help of the community members. One of the major outcomes of the project was the establishment of a new brand, Kailash—Truly Sacred, to promote local products. SABAH-Nepal has introduced a buyback guarantee scheme in the community through which the members of the BHPC can directly sell their entire produce to SABAH Nepal. Overall, the BHPC has reduced the cost of logistics, because members do not have to travel to sell their products; instead, the private sector entity buys them from the BHPC. The direct business link has also increased local residents' share in profits from the sale of *allo* and *allo* products by reducing the third-party involvement.

As a semiautonomous body of SABAH, the BHPC now serves as a key functional link in the *allo* value chain (Figure 2). Details of the approach used and the improvements introduced at different points along the chain are provided in ICIMOD (2015).

Impact assessment

The KSLCDI team evaluated the impact of the BHPC on the *allo* value chain, and particularly on women's lives,

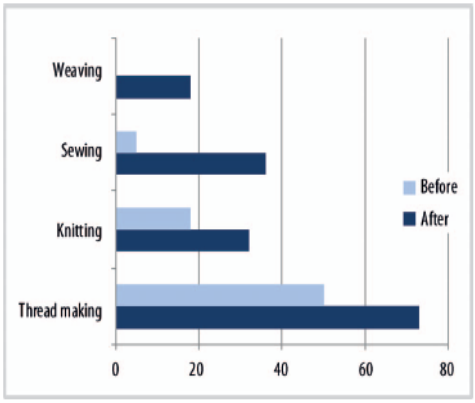
FIGURE 3 Participation in skills training by gender.



using primary and secondary data collected as follows. Primary data were collected using an open-ended questionnaire survey targeting the female members of the BHPC in November 2016. Of the 82 BHPC members, 24 (30%) were randomly selected for interviews. Key topics in the survey included traditional uses of *allo* in Naugad, benefits of the enterprise setup, types of skills training the women had received and their impact on income generation, degree of involvement of women and other marginalized groups, and market linkages. The survey data were supplemented by key informant interviews and focus group discussions. For key informant interviews, factors such as age, sex, caste, and ethnicity were taken as important parameters for capturing the views and experiences of diverse social groups and deriving information from a social perspective. The interviews and discussions focused on the status of *allo* as an alternative source of income before and after the *allo* value chain project.

The findings from the primary data collection were strengthened using secondary data, mostly from a review of journal articles, reports (published and unpublished), working papers, and conference proceedings. Microsoft Excel was used to carry out descriptive analysis, like deriving the percentages of women benefiting in different forms from the *allo* enterprise setup.

FIGURE 4 Women's participation in production activities before and after the project.



Results and discussion

Skills and trust development

A total of 15 training sessions were organized by SABAH-Nepal in 2016 and 2017 for BHPC members at the facility center in Naugad and in Kathmandu. The aim was to increase the number of women participating in the training for sewing, tailoring, and weaving, because these activities could be carried out at home and would help build entrepreneurial skills. Participation in the training sessions is shown by gender in Figure 3. Following the training, the number of women in Naugad who participated in thread-making skills increased by 23%. An additional 14% of women in Naugad developed their skills in knitting, and 31% developed sewing skills; 18% of women members were engaged in weaving *allo* fabrics for the first time in the community center (Figure 4). The buyback guarantee scheme encouraged women and marginalized groups to spend more time on *allo* activities, because it ensured their economic security. To increase understanding of the potential markets and demand–supply processes, 9 women and 4 men from the BHPC participated in 3 exposure trips—visits to trade fairs in the national capitals of India and Nepal. These trips helped to increase their knowledge of how they could add value to their products and increase their earnings.

TABLE 1 Improvements in *allo* production and their impacts.

	Before the project (Oct–Nov 2014)	After the project (Nov 2015 onwards)	Impact
Harvesting technique	Uprooting	Cutting 8 cm above the ground	Ensured regrowth and sustainability of *allo* plants
Type of bark removed	Dry bark	Fresh green bark	Better yarn quality
Substance used to extract fiber	Caustic soda	Ash	Reduced water pollution; reduced health issues; organic product
Type of equipment used	Simple (eg hand spindles)	Modern (eg sewing machines and handlooms)	Increased efficiency; improved designs
Boiling technique	Traditional stoves	Energy-efficient rocket stoves	Reduction in fuelwood use; less tree felling; less smoke; reduced work time
Thread quality	Thick and uneven with much lint; wastage of around 200 g/kg	Thin, fine, even; wastage of less than 50 g/kg	Reduced wastage; thread compatible with handlooms and suitable for finer designs
Thread price[a)]	NPR 250 per kg	NPR 1100 per kg	Increased income
Product design	Simple knits without refined design; limited range of items	Expanded product range in various styles, including river, brick, and fish-cut design	Increased demand for products; better prices

[a)] US$ 1 = NPR 103, June 2017.

A similar approach was taken in Bangladesh in 2012 by the Swedish fashion company KappAhl (BSR 2016). KappAhl worked in partnership with a local development nongovernmental organization (NGO) to open a training center in the town of Tongi. The aim was to provide economic opportunities in global value chains for poor women in the community. Women age 18–25 from poor households participated in a 3-month training program that included basic education (reading, writing, and arithmetic), as well as technical skills such as sewing. Around 500 women were trained and guaranteed a job at one of KappAhl's suppliers. Many of them went to work in factories and were able to earn an independent living for the first time. After the Rana Plaza Factory collapse in 2013, families worried about the safety of workers in such factories; because of traditional cultural norms, many fathers and husbands did not want female household members to work outside the home. However, the NGO adopted culturally sensitive awareness campaigns in communities and visited families in person to build trust. This in-person engagement proved important to gaining the support of family members, which is considered a key strategy for the program's success.

In value chain development, building trust is of utmost importance for the sustainability of the chain. This was addressed by KappAhl in Bangladesh; similarly, trust was maintained among the partners in the *allo* value chain development project in Naugad. In both cases, because of the training given to women, the mindset of the community has changed, and men have slowly started allowing women to work outside their homes and earn money for themselves, even if it is just enough to fulfill limited household needs. One of the unexpected benefits was the creation of an informal network for some of the women, who made friends within the group and used the platform to share thoughts on common issues and support each other both inside and outside work.

Improved processing techniques and value addition

A number of improvements were introduced in the techniques for *allo* harvesting, processing, and value addition (Table 1). Among others, respondents noted that the new practice of making yarn from fresh green bark rather than dried bark improved yarn quality considerably, while the use of ash in place of caustic soda for extraction reduced negative health impacts like allergies, coughing, and gastritis and was more environmentally friendly. Previously, large amounts of caustic soda were used in processing to reduce the extraction time and generate finer fiber (Singh and Shrestha 1988), and the chemicals were washed into nearby rivers, polluting the water. The ash method enables production of chemical-free fiber, an organic product with a niche value in the international market.

As a part of developing and strengthening the *allo* value chain, considerable effort was put into technology transfer. In addition to training, modern equipment was provided to BHPC members to improve efficiency (Shah et al 2017). Survey respondents indicated that replacement of the traditional *katuwa* (hand spindle) with

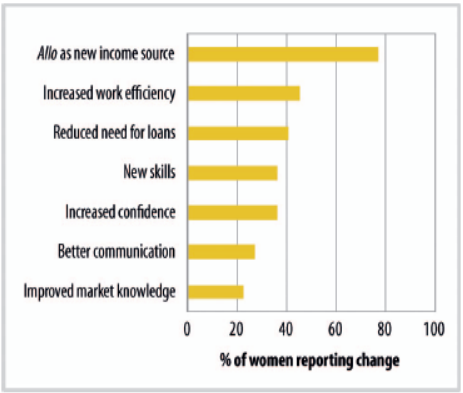
modern equipment had improved thread quality and increased income from *allo* by 27%. The introduction of energy-efficient rocket stoves (hot-burning stove using small-diameter fuelwood) to replace conventional stoves had reduced the amount of firewood needed to boil 30 kg of nettle bark by two-thirds, from 240 to 80 kg (ICIMOD 2016). Although a detailed analysis has not been conducted, according to almost all respondents, the fuelwood requirement for a rocket stove is easily met with twigs and dried bark from nearby fields, and the frequency of visits to forests to cut trees has decreased, in contrast with conventional stoves that require more and thicker fuelwood from the forest. Hence, the use of energy-efficient stoves has helped to reduce tree felling, excessive smoke emission, and indoor air pollution. Cooking time per batch was reduced by 45 minutes, providing relief to women from household chores and allowing them to spend more time on childcare and income-generating activities.

Socioeconomic transformation, especially for women

Small and medium enterprises are particularly important in developing countries because of their potential for entrepreneurship development and employment generation (Cook 2000), and Nepal's government policies have recognized the potential of such enterprises to contribute to poverty reduction (Kunar et al 2009). However, in traditional societies, normalized gender discrimination and attitudes about behavior in the public arena often limit women's ability to take advantage of economic opportunities (Kabeer 2012). The lives of women are generally more precarious than those of men in terms of access to resources and income-earning opportunities, as well as quality of life and wellbeing (Seddon and Hussein 2002; Acharya et al 2007). Although lack of income affects all members of a family, the human impact of poverty tends to affect women and children disproportionately (Sawhill 1988; Wood 2003; Acharya et al 2007). Research conducted in countries like Bangladesh, Brazil, Kenya, and South Africa has indicated that globally, financially independent women have more control over income expenditure, which results in improved health and education for children and has a positive impact on development (United Nations 2009). Thus, there is growing recognition of the importance of promoting women as business leaders, which is also true of the *allo* value chain development in Naugad, as discussed later.

Allo collectors in Nepal are mostly women from poor families with little access to financial resources, and their contribution to the value chain is rarely recognized (Gurung et al 2012; ICIMOD 2015) in terms of remuneration by the actors involved in the chain. In Naugad, a focused effort was made to include women and other people from marginalized groups as important

FIGURE 5 Socioeconomic impact of improvements in the *allo* value chain.



actors in the value chain. The training and exposure visits helped them build their capacity and take a leading role in the value chain. Women noted that they had considerably increased their skills in thread-making, sewing, and weaving following the training (Figure 4) and could now produce diversified products such as bags, purses, suits, cushion covers, scarves, shawls, pot holders, and runners. This had led to an increased demand for their products in the market.

One of the most important impacts of the *allo* project was the perception (by 77% of survey respondents) of *allo* as a new source of income (Figure 5). BHPC members reported earning NPR 3000–4000 per month, ie NPR 130 per day on average from sewing, which compares favorably with the average daily labor wage in the community of NPR 100. In the past, social and cultural norms in Naugad limited the participation of women in mainstream development activities. However, there has been increasing acceptance of women's leadership since *allo* value chain development began in Naugad in 2015, and women are now considered important actors in the chain. People in Naugad have received various skills and leadership training irrespective of their age, caste, religion, or ethnicity and are producing a variety of nettle fiber products. Women who were previously dependent on their husbands for cash resources now contribute to the family income. The cost of general household items like soap, butter, and sugar (NPR 3000–5000 per month) is covered by the income generated through *allo*. The BHPC also serves as an enabling workspace where women and members of other marginalized groups work together to address issues related to personal and community wellbeing. Enterprise-related decision-making is now entrusted to women.

The success of the BHPC has encouraged entrepreneurs to develop small businesses at all points along the *allo* value chain. More than 25 women entrepreneurs were identified in Naugad with *allo*-based

microbusinesses, from home production of thread to tailor shops using *allo* and other fabrics and even exploring cross-border sales options in India. As of December 2017, 36 women were employed at BHPC making value-added products.

A study conducted by ICIMOD (2015) reported an annual income of NPR 35,000 for a woman who had been working on *allo* for the last 15 years in Naugad. It reported that she collected 500–800 kg of *allo* every year from the forest and sold semiprocessed bark at the district headquarters. She also produced about 200 thick threads for use with livestock and for the production of *damlo* (rope to carry loads) and sold them at a rate of NPR 200 each until 2016. The same woman in the survey reported that with improved skills at BHPC, she is now able to sell fine *allo* thread instead of semiprocessed bark, thus earning more.

Another woman member of BHPC reported that her increased skills in producing finer *allo* threads and in sewing and weaving *allo* products (ICIMOD 2016), after receiving the training in 2016, had established her as an entrepreneur in Naugad, making her financially stronger and more independent than in 2014. The income generated by these women entrepreneurs has helped them cover household expenses and invest more in the education and welfare of their children (Subedee et al 2017).

A study conducted by Action Aid in 2014 in Palestine (Morioka and Nicholas 2014) showed similar results. Before participation in Action Aid's savings and loans groups, women did sewing, knitting, and weaving in isolation and mostly for friends and acquaintances. They had limited capacity to negotiate prices with suppliers for raw materials, were inhibited by expectations about appropriate behavior for women interacting with men outside the family, and operated only as processors, depending on others for sales and with little incentive to improve or upgrade. The women selected embroidery, vine leaf packaging, and loofah production as livelihood activities, and the program gave them the knowledge, skills, and confidence to add value to their work. The project eased their financial situation and helped them meet other women and overcome their isolation, as well as providing a much-valued opportunity to learn skills for production and managing an economic venture.

Another successful project took place in Sri Lanka: the 3-year Dairy Enhancement in Eastern Province program, launched in 2009 by Land O'Lakes International Development, the US Agency for International Development, and CIC Agri Business, a Sri Lankan dairy company (Steensland 2014). The program was designed to introduce improved technologies and link smallholder farmers to commercial markets, with the goal of increasing farmers' incomes by 75%. It also helped women move beyond irregular informal sales by developing a market-driven link to a private-sector processor willing to provide a higher farm-gate price. Today, CIC Agri Business is selling 50,000 cups of yogurt and 15,000 small packets of milk for children per day around Sri Lanka, providing a good example of successful scaling up.

Scalability and sustainability of the value chain

In value chain development, scaling up aims to extend long-term benefits to more beneficiaries by ensuring sustainability and providing additional resources and expertise (IFAD 2015), locally or over a larger area. Scaling up generally requires an organized group to ensure that integration in the value chain is viable and sustainable and that there is a regular supply of produce in sufficient quantity, which further improves bargaining power when interacting with private-sector actors along the chain (IFAD 2015; IFPRI 2016).

In Naugad, after the project intervention, BHPC members continued working together to maintain the supply of *allo* items required by the private sector. Using their improved skills, community members have started to produce high-quality items. The involvement of actors in the *allo* value chain in Naugad starts with the collection of *allo* bark from forests and ends with the export of value-added products by SABAH Nepal. Value addition occurs at 3 levels: first, by BHPC members in Naugad; second, at the district capital of Darchula by the traders from Naugad who travel there; and finally, at SABAH Nepal's facility in Kathmandu. After that, the products are either sold in SABAH Nepal's outlets in Kathmandu or exported. The income generated by selling *allo* products is shared by rural communities and urban entrepreneurs to ensure equitable benefit sharing among actors in the chain and to ensure the sustainability of the chain.

SABAH Nepal has expanded the idea of branding and collective production to other mountain commodities beyond *allo*. The Kailash–Truly Sacred brand now includes kidney beans, vegetables, dairy products, and medicinal plants from Nepal, as well as *churia* honey from India. This has led to the adoption of a basket approach that considers a variety of other products in the value chain besides *allo*, which has contributed to business diversification of the people of Naugad. The Naugad community has been able to make a barter agreement with SABAH Nepal to minimize the risks of a shortage of *allo* and maintain the business through the supply of other products available in the area. The project has helped community members improve their bargaining skills and enabled them to negotiate equitable prices for their products and thus sustain their businesses. In total, 6 national and international market linkages have been established by the *allo* producers, including a 3-star hotel in Kathmandu and buyers in India, Japan, and Norway. *Allo* is supplied in different forms depending on demand. These networks were established when community

members participated in the trade fairs in Kathmandu and New Delhi.

As mentioned in the introduction, the textile industry worldwide is looking for alternative sources of natural fiber to reduce dependence on cotton and silk (Radhakrishnan and Preeti 2015). In Uttarakhand in India, *van panchayat* user groups (community-based forest management groups), started cultivating nettle fiber after realizing its potential to ensure secure alternative livelihood options (Radhakrishnan and Preeti 2015; Debnath et al 2017). *Allo* is widely distributed in subtropical to temperate areas across the Himalayan region, including Bhutan, China, India, Myanmar, and Nepal (Singh and Shrestha 1985, 1988; Radhakrishnan and Preeti 2015; Debnath et al 2017). Considering the resource availability and growing interest of local communities, the community-owned enterprise model introduced in far west Nepal has the potential for extension not only to other parts of Nepal but also to neighboring areas in Bhutan, China, and India. However, before promoting the approach, it is important to conduct a preliminary study of local conditions, as was the case before the project described here (ICIMOD 2017). This analysis found that although *allo* is a high-value product with substantial scope for value addition within the community, there is a risk of inadequate quality control, which may lead to difficulties in competing in international markets. In addition, the production of *allo* fiber at the local level is not cost effective, and because of the limited technology for and tedious nature of *allo* extraction, processing, and thread-making, *allo*-based entrepreneurs may lose interest, even though nettle products are emerging as one of the most important souvenir items in the tourist trade. Such findings need to be taken into account in developing a value chain project.

Conclusion

The *allo* value chain development in Naugad demonstrated a successful model for women's empowerment through collective action. Local women have become active members of the *allo* enterprise and are increasing financial security at the household level by selling *allo* products in different forms. The project identified good practices using environmentally friendly and energy-efficient technologies and methods that minimize the use of external inputs such as chemicals and fuelwood. The project produced the following lessons:

1. An orchestrated effort targeting training, skills development, exposure visits, and women's active participation is important for promoting inclusive development of a value chain.
2. Enterprises based on natural resources like *allo* require sound ecosystem management to ensure sustainability.
3. Partnership with a private-sector entity that considers social and environmental values, as well as economic gains, is key for value chain promotion in a remote landscape.

ACKNOWLEDGMENTS

This study was conducted under the KSLCDI, supported by the Deutsche Gesellschaft für Internationale Zusammenarbeit, and commissioned by the Bundesministerium fur wirtschaftliche Zusammenarbeit (GIZ) GmbH und Entwicklung and the United Kingdom Department for International Development. It was also partly supported by core funds of the ICIMOD, which were contributed by the governments of Afghanistan, Australia, Austria, Bangladesh, Bhutan, China, India, Myanmar, Nepal, Norway, Pakistan, Switzerland, and the United Kingdom. The authors also gratefully acknowledge the expertise and support provided by the Ministry of Forests and Soil Conservation of the Government of Nepal, the Research Centre for Applied Science and Technology, SABAH Nepal, and the community of Naugad.

REFERENCES

Acharya S, Yoshino E, Jimba M, Wakai, S. 2007. Empowering rural women through a community development approach in Nepal. *Community Development Journal* 42(1):34–46. doi:10.1093/cdj/bsi064.

Barakoti TP, Shrestha KP. 2009. Commercial utilization of *allo (Girardinia diversifolia)* by the Rais of Sankhuwasabha for income generation. *Banko Janakari* 18(1):18–24. doi:10.3126/banko.v18i1.2162.

Bhattarai B, Leduc B, Choudhary D, Pandit BH. 2010. *Engendering the Value Chain: The Case of Community Based Bay Leaf Enterprise in Nepal.* Kathmandu, Nepal: International Centre for Integrated Mountain Development.

BSR [Business for a Better World]. 2016. *Women's Empowerment in Global Value Chains: A Framework for Business Action to Advance Women's Health, Rights, and Wellbeing.* Copenhagen, Denmark: BSR. https://www.bsr.org/reports/BSR-Report-Womens-Empowerment-Supply-Chains.pdf; accessed on 9 October 2017.

Clarke RC. 2007. Traditional Nepali hemp textiles. *Journal of Industrial Hemp* 12(2):97–113. doi:10.1300/J237v12n02_07.

Cook P. 2000. *Finance and Small and Medium Sized Enterprise Development.* Finance and Development Research Programme Working Paper Series. Manchester, United Kingdom: Institute for Development Policy and Management, University of Manchester.

Debnath K, Singh I, Dvivedi A. 2017. On the analysis of force during secondary processing of natural fiber-reinforced composite laminates. *Polymer Composites* 38(1):164–174. doi:10.1002/pc.23572.

Duthie JF. 1960. *Flora of the Upper Gangetic Plain and of the Adjacent Siwalik and Sub-Himalayan Tracts.* Calcutta, India: Superintendent of Government Printing.

GIZ [German Corporation for International Cooperation]. 2013. *Value Chain Development by the Private Sector in Africa: Lessons Learnt and Guidance Notes.* Bonn, Germany: GIZ. http://www.pluginthewold.com/mobisol/wp-content/uploads/2014/01/Value-chain-development-by-the-private-sector-in-Africa.pdf; accessed on 10 October 2017.

Gurung A, Flanigan H, Ghimeray AK, Karki R, Bista R, Gurung OM. 2012. Traditional knowledge of processing and use of the Himalayan giant nettle (*Girardinia diversifolia* [Link] Friis) among the Gurungs of Sikles, Nepal. *Ethnobotany Research and Applications* 10:167–174. doi:10.17348/era.10.0.167-174.

Hoermann B, Choudhary D, Kollmair M. 2010. *Integrated Value Chain Development as a Tool for Poverty Alleviation in Rural Mountain Areas: An Analytical and Strategic Framework.* Kathmandu, Nepal: International Centre for Integrated Mountain Development. http://lib.icimod.org/record/8058/files/attachment_703.pdf; accessed on 10 October 2017.

ICIMOD [International Centre for Integrated Mountain Development]. 2015. *Promotion of the* Allo *(Himalayan Nettle) Value Chain in Nepal: Sustainable Livelihoods in the Kailash Sacred Landscape*. Kathmandu, Nepal: ICIMOD. http://lib.icimod.org/record/31164/files/Allo_15.pdf; accessed on 10 August 2017.

ICIMOD [International Centre for Integrated Mountain Development]. 2016. *Fabric for Future: Women Entrepreneurs Building Better Livelihoods Through* Allo *Production in Far-western Nepal*. Kathmandu, Nepal: ICIMOD.

ICIMOD [International Centre for Integrated Mountain Development]. 2017. *Greening* Allo *Value-Chain in Darchula, Nepal: A Process Documentation*. Kathmandu, Nepal: ICIMOD. Unpublished report. Available from corresponding author of this article.

IFAD [International Fund for Agricultural Development]. 2015. *Sustainable Inclusion of Smallholders in Agricultural Value Chains: Scaling Up Note*. http://www.ifad.org/documents/10180/77b9f80c-038e-4596-a6a1-10596555975b; accessed on 12 October 2017.

IFPRI [International Food Policy Research Institute]. 2016. *Innovations for Inclusive Value Chain Development: Successes and Challenges*. Washington, DC: International Food Policy Research Institute. http://dx.doi.org/10.2499/9780896292130; accessed 10 October 2017.

Joshi SR, Rasul G, Shrestha Joshi A. 2016. *Pro-poor and Climate Resilient Value Chain Development: An Operational Guidelines for the Hindu Kush Himalayas*. ICIMOD Working paper 2016/1 Kathmandu, Nepal: International Centre for Integrated Mountain Development.

Kabeer N. 2012. *Women's Economic Empowerment and Inclusive Growth: Labour Markets and Enterprise Development*. Discussion Paper 29. London, United Kingdom: Centre for Development Policy & Research, School of Oriental & African Studies, University of London.

Kaplinsky R, Morris M. 2001. *A Handbook for Value-Chain Research*. Gapresearch.org, IDS, United Kingdom. http://www.ids.ac.uk/ids/global/pdfs/VchNov01.pdf; accessed on 1 October 2017.

Kunar SC, Ansari AS, Luintel H. 2009. Non-timber forest products enterprise development: Regulatory challenges in the Koshi Hills of Nepal. *Journal of Forest and Livelihood* 8(2):39–50. doi:10.3126/jfl.v8i2.2307.

Lanzilao G, Goswami P, Blackburn RS. 2016. Study of the morphological characteristics and physical properties of Himalayan giant nettle (*Girardinia diversifolia* L.) fibre in comparison with European nettle (*Urtica dioica* L.) fibre. *Materials Letters* 181:200–203. doi:10.1016/j.matlet.2016.06.044.

MEDEP [Micro-Enterprise Development Programme]. 2010. *Value Chain Based Approach to Micro-enterprise Development. Value Chain Analysis:* Allo. Value Chain Series 1. Bakhundole, Lalitpur: MEDEP. http://medep.org.np/epanel/publication/docs/Value%20Chain%20Analysis.pdf; accessed on 28 September 2017.

Mitchell I, Keane J, Coles C. 2009. *Trading Up: How a Value Chain Approach Can Benefit the Rural Poor*. London, United Kingdom: Overseas Development Institute. https://www.odi.org/sites/odi.org.uk/files/odi-assets/publications-opinion-files/5656.pdf; accessed on 17 January 2018.

Morioka K, Nicholas G. 2014. *Women Empowerment and Value Chains: Experiences of Women in Cambodia, Palestine and Uganda*. Camperdown, Australia: Action Aid. https://acfid.asn.au/sites/site.acfid/files/resource_document/WEVC%20report%20_%20fnl.pdf; accessed on 12 October 2017.

Negi CS. 2007. Changing face of polyculture in the Darma and Johaar valleys, Pithoragarh, Kumaun Himalayas. *International Journal of Sustainable Development and World Ecology* 14(4):428–436. doi:10.1080/13504500709469743.

NHPC [Nepal Health Professional Council]. 2011. *Nepal Population*. Kathmandu, Nepal: NHPC.

OECD [Organization for Economic Co-operation and Development]. 2009. *Promoting Pro-poor Growth: Private Sector Development*. Paris, France: OECD. http://www.oecd.org/development/povertyreduction/43514563.pdf; accessed on 2 September 2017.

OECD/WTO [Organization for Economic Co-operation and Development and World Trade Organization]. 2015. *Aid for Trade at a Glance 2015: Reducing Trade Costs for Inclusive, Sustainable Growth*. Paris, France: OECD. https://www.wto.org/english/res_e/booksp_e/aid4trade15_chap8_e.pdf; accessed on 8 January 2018.

Pant B, Rai RK, Wallrapp C, Ghate R, Shrestha UB, Ram A. 2017. Horizontal integration of multiple institutions: Solutions for *yarshagumba* related conflict in the Himalayan region of Nepal? *International Journal of the Commons* 11(1):1–23. doi:10.18352/ijc.717.

Pyakurel D, Baniya A. 2011. *NTFPs: Impetus for Conservation and Livelihood Support in Nepal. A Reference Book on Ecology, Conservation, Product Development, and Economic Analysis of Selected NTFPs of Langtang Area in the Sacred Himalayan Landscape*. Kathmandu, Nepal: World Wide Fund For Nature (WWF).

Radhakrishnan S, Preeti A. 2015. Development of fabric from *Girardina diversifolia* stem fibres and its blends. *International Journal of Innovative Research in Science, Engineering and Technology* 4(11):10,499–10,506. doi:10.15680/IJIRSET.2015.0411023.

Rasul G, Hussain A, Sutter A, Dangol N, Sharma E. 2016. *Towards an Integrated Approach to Nutrition Security in the Hindu Kush Himalayan Region*. ICIMOD Working Paper 2016/7. Kathmandu, Nepal: International Centre for Integrated Mountain Development.

Sawhill IV. 1988. Poverty in the US: Why is it so persistent? *Journal of Economic Literature* 26(3):1073–1119.

Seddon D, Hussein K. 2002. *The Consequences of Conflict: Livelihoods and Development in Nepal*. Overseas Development Institute Working Paper 185. London, United Kingdom: Overseas Development Institute. http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.509.2760&rep=rep1&type=pdf; accessed on 10 October 2017.

Shah G, Khadka M, Ahmad F, Budhathoki N, Shrestha A. 2017. Assessment of Himalayan nettle *(Girardinia diversifolia)* value chain development interventions: Evidence from rural households in the far western Nepal. *Journal of Agriculture Science* 9(5):19–32.

Sharma JR. 2011. Culture of migration in the middle hills of Nepal. *In*: ICIMOD [International Centre for Integrated Mountain Development], editor. *Labour Migration. Opportunities and Challenges for Mountain Livelihoods*. Kathmandu, Nepal: ICIMOD, pp 18–20.

Shrestha R. 1997. Cytological studies in *Girardinia diversifolia* (Link) Friis. *Pakistan Journal of Botany* 29(2):263–269.

Shrestha UB, Bawa KS. 2013. Trade, harvest, and conservation of caterpillar fungus *(Ophiocordyceps sinensis)* in the Himalayas. *Biological Conservation* 159:514–520.

Singh SC, Shrestha RR. 1985. *A Study of Himalayan Nettle*. Kathmandu, Nepal: Research Center for Applied Science and Technology, Tribhuvan University.

Singh SC, Shrestha RR. 1988. *Girardinia diversifolia* (Urticaceae), a non-conventional fiber resource in Nepal. *Economic Botany* 42(3):445–447.

Singh SC, Shrestha RR. 1989. Observations on ecodemes in *Girardinia diversifolia* (Link.) Friis, Urticaceae in Nepal. *Pakistan Journal of Botany* 21(1):185–190.

Steensland S. 2014. Productive dairy value chain improves lives of women in Sri Lanka. Global Harvest Initiative. http://www.globalharvestinitiative.org/index.php/2014/03/productive-dairy-value-chain-improves-lives-of-women-in-sri-lanka/; accessed on 12 October 2017.

Subedee BR, Chaudhary RP, Dorji T, Shrestha AJ. 2017. Indigenous and local knowledge of conservation and sustainable use of Himalayan Giant Nettle (*Girardinia diversifolia* [Link] Friis) in eastern and far-western regions of Nepal. *In*: Roué M, Molnár Z, editors. *Knowing Our Lands and Resources: Indigenous and Local Knowledge and Practices Related to Biodiversity and Ecosystem Services in Asia*. Paris, France: UNESCO [United Nations Educational, Scientific and Cultural Organization], pp 191–198.

TEPC [Trade and Export Promotion Centre]. 2014. Foreign trade statistics of Nepal [First eleven months (Shrawan-Jestha) of Fiscal Year 2070–71]. Kathmandu, Nepal.

United Nations. 2009. The global financial crisis: Assessing vulnerability for women and children, identifying policy responses. Paper Presented at the *53rd Session of the Commission on the Status of Women*. New York, NY, 2–13 March. http://www.un.org/womenwatch/daw/csw/csw53/panels/financial_crisis/Buvinic.formatted.pdf; accessed on 8 October 2017.

USAID [United States Agency for International Development]. n.d. Gender and Pro-poor Value Chain Analysis: Insights From the Gate Project Methodology and Case Studies. http://www.microlinks.org/sites/microlinks/files/group/resource/files/GATE_Gender_Pro-Poor_Value_Chain_Analysis_05-09.pdf; accessed on 8 October 2017.

Wood D. 2003. Effect of child and family poverty on child health in the United States. *Pediatrics* 112(3):707–711.

World Bank. 2014. *Expanding Women's Access to Financial Services*. http://www.worldbank.org/en/results/2013/04/01/banking-on-women-extending-womens-access-to-financial-services; accessed on 12 December 2017.

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▶ **Edited by:**

Madhav Karki, Rosemary Hill, Dayuan Xue, William Alangui, Kaoru Ichikawa and Peter Bridgewater

▶ **Organized by the:**

Task Force on Indigenous and Local Knowledge Systems
Intergovernmental Platform on Biodiversity and Ecosystem Services (IPBES)

▶ **in collaboration with the:**

IPBES Expert Group for the Asia and Pacific Regional Assessment

▶ **with support from**

Intergovernmental Platform on Biodiversity and Ecosystem Services (IPBES)
Institute for Global Environmental Strategies (IGES)
United Nations Educational, Scientific and Cultural Organization (UNESCO)

▶ **26–28 June 2016 • Chiang Mai • Thailand**

▶ **29 November – 2 December 2016 • Dhulikhel (Kathmandu) • Nepal**

Published in 2017 by the United Nations Educational, Scientific and Cultural Organization, 7, place de Fontenoy, 75352 Paris 07 SP, France

ISBN: 978-92-3

To be cited as:
Madhav Karki, Rosemary Hill, Dayuan Xue, William Alangui, Kaoru Ichikawa and Peter Bridgewater (eds.). 2017. *Knowing our Lands and Resources: Indigenous and Local Knowledge and Practices related to Biodiversity and Ecosystem Services in Asia*. Knowledges of Nature 10. UNESCO: Paris. pp. 200.

With contributions from members of the IPBES Task Force on Indigenous and Local Knowledge (ILK) Systems
In collaboration with members of the IPBES Expert Group for the Asia–Pacific Regional Assessment

With support from UNESCO as the Technical Support Unit for the IPBES Task Force on ILK:
Douglas Nakashima, Trupthi Narayan, Tanara Renard--Truong Van Nga, Alejandro Rodriguez, Jennifer Rubis and Sungkuk Kang

Funded by:
Intergovernmental Platform on Biodiversity and Ecosystem Services (IPBES)
Institute for Global Environmental Strategies (IGES) and the Japan Biodiversity Fund
United Nations Educational, Scientific and Cultural Organization (UNESCO)

English and Copy Editor: Trupthi Narayan

Cover photo: UNDP

Graphic and cover design, typeset: Julia Cheftel

Hard copies are made available compliments of UNESCO

16. Indigenous and local knowledge of conservation and sustainable use of Himalayan Giant Nettle (*Girardinia diversifolia* (Link) Friis) in Eastern and Far-Western Regions of Nepal

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Abstract

Indigenous knowledge and practices of indigenous peoples and local communities (IPLCs) play an important role in the conservation and sustainable use of biodiversity. Himalayan giant nettle (Girardinia diversifolia (Link) Friis), locally known as 'allo', has economic and cultural values for IPLCs living in the Kailash Sacred Landscape (KSL-Nepal) (comprising Humla, Darchula, Baitadi, Bajhang Districts) of Far-Western Nepal; and Makalu Barun National Park (comprising Shankhuwasabha and Solukhumbu Districts) of Eastern Nepal. This research discusses indigenous and local knowledge of the traditional use and practice of 'allo' linked with the sustainability of resources. The study investigated the cultural linkage among 'allo' harvesting and processing techniques, traditional medicinal practice as well as conservation practice adopted on 'allo' by IPLCs of Far-Western and Eastern regions of Nepal. Different parts of the 'allo' plant species are traditionally being used by local healers (Vaidhya) and local communities such as Bohora, Dhami, Thagunna of Darchula District use 'allo' as medicine for treating gastritis, joint pain, headache, tuberculosis and asthma. The Kulung Rai people of Sankhuwasabha district use clothes made of 'allo' fibre in their rituals. The study revealed that IPLCs use the fibre of 'allo' as primary material to make ropes, fishing nets, coats, pants, bags, shawls, purses and many more items to sustain their livelihoods. The traditional harvesting techniques; use of locally available materials such as wood ash, white soil; and locally made equipment like hand spindle, wooden hammer, wooden handloom help in sustainable use and conservation of 'allo'. Increasing market demand had led to a higher supply of 'allo' products, hence, people started to harvest it extensively. The natural resource 'allo' has been declining due to high habitat competition with cash crops like Amomum subulatum. Therefore, this study identifies the existing status of 'allo' for management and sustainable utilisation to meet the increasing demand for resources, and attempts to share the management practices followed in two different regions of Nepal.

16.1. Introduction

Himalaya giant nettle (Girardinia diversifolia (Link) Friis) locally known as 'allo', has economic and cultural values for indigenous peoples and local communities (IPLCs) living in the Kailash Sacred Landscape (KSL-Nepal), which comprises four districts in Far-Western Nepal - Darchula, Baitadi, Bajhang and Humla Districts (Zomer and Oli 2011); and in Sankhuwasabha District of Eastern Himalaya in Nepal. G. diversifolia belongs to family Urticaceae. It is a fibre-yielding plant locally known as 'allo' in Nepali language. 'Allo' grows in the Eastern to Far-Western regions of Nepal between the altitudes of 1,200 to 3,000 metres a.s.l. Its range also extends to China, India, Bhutan, and East Africa including Madagascar (Friis 1981; Shrestha and Hoshion 1998; Chen et al. 2003). The plant is shade loving, grows to 1.5 to 3 metres tall and has a perennial root. Stem and leaves consist of stinging spikes. Fibre is present in the inner bark of the stalk and has high strength and length. Allo has cultural, economic and medicinal values for many communities like Rais, Gurungs, Tamangs, Sherpas, etc. Indigenous peoples and local communities utilise the fibre of this plant to make different articles for daily use. Kulung Rais use cloth made of 'allo' in their religious ceremonies, offer cloth to God during Nagi Puja and also present 'allo' cloth to their daughters during the wedding ceremony (Barakoti and Shrestha 2008). Different parts of the plant species are traditionally utilised as medicine. 'Allo' products have both national and international market value. Resources management, sustainable harvesting, conservation, and fair and equitable sharing of benefits enhance equity among the communities. The harvesting and processing system of 'allo' followed by the people of Bala, Sisuwa, Tamku (Village Development Committee) VDCs of Sankhuwasabha district and medicinal use of different parts of 'allo' followed by communities at Khar, Katae, Yerkot, Sipti VDCs of Darchula district living in KSL-Nepal are important for the sustainability of the resources.

Photo 16.1 Local people harvesting 'allo'

This research, combining cultural and economic values, investigated the indigenous knowledge on uses and practices linked with the sustainability of resources. The study investigated how local communities have established cultural linkages with harvesting practice, processing techniques and medicinal use of 'allo' in different regions.

16.2. Study area

The study sites Khar, Airkot, Katae, Septi, Sitola VDCs of Darchula District, Api-Nampa Conservation Area, Kailash Sacred landscape (KSL), Nepal are located in the Far-Western region; Bala, Sisuwa, Tamku VDCs of Sankhuwasabha District in Makalu-Barun National Park are in the Eastern region of Nepal (Figure 16.1).

Figure 16.1 Study area with KSL-Nepal in Far-Western region and Sankhuwasabha District in Eastern region of Nepal.

16.3. Materials and Methods

A field survey was conducted in the study areas following focussed group discussions, informal meetings and field observations as primary methods of data collection. Semi-structured questionnaires were followed after establishing informed consent with the communities. Information was also collected from websites and published articles in journals. Non-timber forest product (NTFPs) collectors, traders, traditional healers (Vaidhya), 'allo' processers and community members were consulted through focussed group discussions and informal meetings. Elderly people, forest guards, local traders, men and women representing different ethnic groups, castes and occupations were encouraged to participate.

16.4. Results and Discussions

16.4.1. Cultural use and practice

Indigenous and local communities like Rais, Gurungs, Sherpas, Magars and Tamangs value G. diversifolia economically and culturally. A particular community of Rai (Kulung Rai) peoples offer 'allo' cloth to god during the cultural ceremony of Nagi Puja. 'Allo' and its cloth are the most important requirements during housewarming, wedding and funeral ceremonies. It is also the source of livestock feed, bedding material and firewood (Barakoti 2008). The traditional fibre extraction technology developed by IPLCs helped them to meet the basic requirement of the people by selling their products in local markets.

16.4.2. Traditional medicinal use and practice

The present study revealed that 'allo' is a medicinal plant and has been used by the local communities in Far-Western and Eastern regions of Nepal. Local people use 'allo' as medicine for treating gastritis, joint pain, headache, tuberculosis and asthma (Table 16.1).

Table 16.1 Traditional use of G. diversifolia

Parts used	Traditional use
Root	Juice of roots is used to treat gastritis and constipation and is applied on swellings. Paste of the root is applied on aching joints. Root juice with water is used for stomach ache. For the treatment of gastritis, it is mixed with the plant Ghodtapre (Centella asiatica) and boiled for 10 minutes, strained and the liquid (about 4 teaspoons) is given twice a day. Juice of the root, about 6 teaspoons twice a day, is given for constipation (Manandhar 2002).
Bark	Fibre obtained from the bark is used to make different articles such as ropes, fishing nets, bags, sacks, clothing materials, weaving rugs, jackets (Barakoti 2008).
Leaf	Juice of leaves is used to treat headache, joint aches and tuberculosis. It is also used as a vegetable (Barakoti 2008; Gurung et al. 2012; Malla et al. 2014).
Stem	The stem is heated and wrapped around the leg or hand to treat fractures.
Inflorescence	It is used as a vegetable and soup for its high nutrient value (Malla et al. 2014).

The study revealed that IPLCs of Eastern and Far-Western regions of Nepal use 60% bark, leaves 15%, roots 10%, Stem 8%, Seeds 4%, and inflorescence 3% of 'allo' (Figure 16.2).

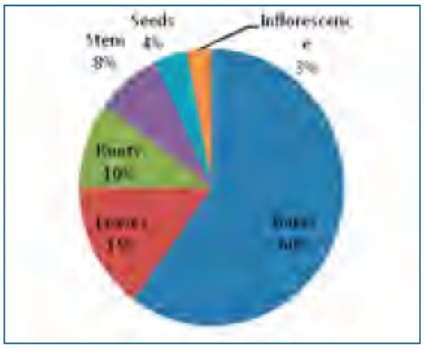

Figure 16.2 Percentage of plant parts use by IPLCs

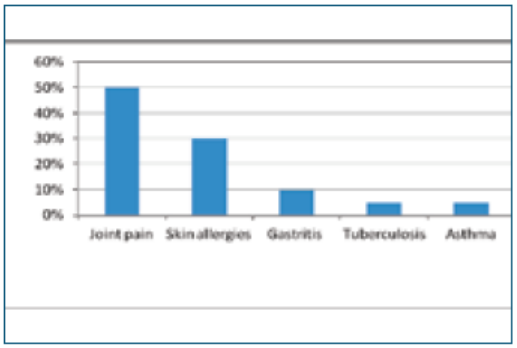

Figure 16.3 Percentage therapeutic use of G. diversifolia

The study also showed that more than 50% of people use 'allo' for joint pain; 30% in skin allergies; 10% to treat gastritis followed by tuberculosis and asthma (Figure 16.3).

16.4.3. Indigenous knowledge of allo processing held by IPLCs of study area

During October-November, local communities harvest the 'allo' shrubs. Well-developed stems are selected and the bark is removed manually by using iron sickles and hand gloves. The harvested bark is soaked in water for 24 hours.

Photo 16.2 Allo processing techniques in Far-Western and Eastern Region of Nepal: (a) Cooking of 'allo' bark in iron drum. (b) Allo bark drying with white clay (Kamero). (c) Wooden hammer (Mungro). (d) Spinning 'allo' fibre with Katuwa.

Degummed barks are dried, bundled and stored. The cooking process takes place for about 2-3 hours in an iron drum with wood ash. To cook 2.5 kg of 'allo', 5.38 kg of wood ash is needed to remove non-fibrous 'allo' bark and 7.45 kg of white soil (Kamero) is needed to soften the 'allo' fibre.

Photo 16.3 Allo cloth weaving with wooden hand loom.

Wooden hammer (Mungro) is still in use – it is a traditional method of processing of fibre. Plenty of water is required to remove the non-fibrous bark and this is usually carried out in a stream or river. The beating process is the primary traditional method for the removal of debris of 'allo' bark in both Eastern and Far-Western regions of Nepal. The clean bundles of fibre are left to dry in the sun and soaked in water with locally available white soil.

Both men and women are involved in all the stages of collection and processing. The traditional spinning method by hand spindle is still practised. Hand spindle (Katuwa) and wooden spinning machine (Charka) are used for spinning of the yarn. A hand spindle is a portable yarn spinning equipment which is used to spin fibre. The study revealed that IPLCs of Eastern and Far-Western regions use fibre of 'allo' as the primary source to prepare different types of products such as porter straps (Namlo), ropes for domesticated animals (Damlo), coats, pants, bags, shawls, purses and many more items. IPLCs have made their own groups and rules for harvesting of 'allo' from the forest. People go together to the forest and collect 'allo' bark from the mature plant, after the ripening of fruit during the months of October and November. They allow the ripened seeds to disperse around the ground for the regeneration of plants. They collect 'allo' bark in groups from different forest areas and share the resources in equal amounts. While harvesting 'allo' stem, they leave about four inches of stem above the ground so that new buds may arise easily from the root. Mostly women were involved in 'allo' processing, thus it acts as a source of income for the women, and they utilise the income generated from 'allo' for their daily use and also for the welfare of their children.

16.4.4. Loss of natural habitat

G. diversifolia generally grows from Eastern to Far-Western regions of Nepal between the altitudes of 1,000 to 3,000 m above sea level. It commonly grows in moist and shady areas. During the study, most of the respondents reported that natural habitat of 'allo' is decreasing year by year. This may be due to decrease in the traditional livestock domestication practices in barren lands which acts as one of the major sources of organic fertiliser for 'allo' germination and growth. Studies showed the community response on the present situation of natural habitat: 85% responded that they had observed a loss or decrease of natural habitat; 13% responded that there was increase in natural habitat; only 2% responded that they observed no change in the natural habitat (Figure 16.4).

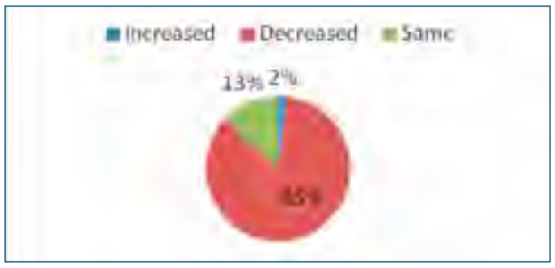

Figure 16.4 Community response on change in natural habitat of 'allo'

About 38% of the respondents reported that the loss of natural habitat of 'allo' was due to decrease of livestock domestication practices on barren land; 32% responded that the loss of habitat was due to decrease of sheep numbers and sheep herders, because sheep excreta acts as a good source of fertiliser for 'allo' plants. 'Allo' seeds also anchor on sheep wool which helps in seed dispersal in forest areas and barren lands. About 13% responded that the loss of 'allo' habitat was due to the increase in cardamom cultivation in the same lands where 'allo' grows; 8% responded that this was due to plucking out of whole plant to support fodder grass regeneration for domesticated animals; about 7% responded that there was no effect on the natural habitat and about 2% reported that the reason for loss of habitat was destruction by wild animals (Figure 16.5).

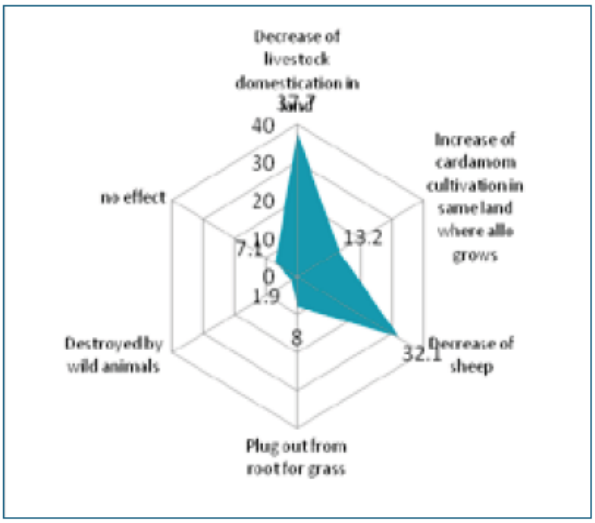

Figure 16.5 Causes of decrease of natural habitat of 'allo'.

Collection, processing, spinning and weaving of G. diversifolia is a tradition of IPLCs, and has long been used in the textile history of Nepal. Jackets, coat and, caps made of 'allo' are popular in Nepal and abroad. The IPLCs have their own traditional practice of collection, processing, spinning and weaving, where they utilise locally available materials. These days, the traditional processing practices are altered by the use of caustic soda in place of ash. Though caustic soda removes non-fibrous parts faster than the ash, it pollutes water, affects fibre quality and health of 'allo' farmers. So, the indigenous and local knowledge adopted by Kulung Rais of Sankhuwasaha, Dhami and Thangunna communities of Darchula district is environmentally safe. Generations upon generations of people are harvesting 'allo' from the forest though very few people are aware of the availability and sustainability of raw material in the natural habitat. People have to travel long distances and spend a week in the forest to collect 'allo' bark. Another risk is the massive plantation of large cardamom Amomum subulatum: people pluck out 'allo' plants and plant large cardamom in their place. Cultivation of A. subulatum has been one of the income generation options which has had adverse impacts on the habitat of G. diversifolia. So, on the one hand exploitation rate of 'allo' is high and on the other, destruction of natural habitat indicates the need for sustainable management. Thus, plantation, cultivation and conservation should be encouraged to preserve the natural habitat. Tax policy on transport of raw 'allo' bark and finished products from villages to national and international markets is unclear. Farmers/collectors have to pay taxes at several points while carrying both 'allo' bark as well as its products. Therefore, it is recommended that the Government provides a clear policy of one-door taxation for raw material and finished products.

Conclusions

Indigenous and local communities of Nepal have their own traditional way of harvesting, processing and conservation of G. diversifolia, which has cultural, economic and medicinal value among the people living in Api-Nampa Conservation Area of Kailash Sacred landscape, in Far-Western Nepal and Makulu-Barun National Park in Eastern Nepal. Economic importance has led to higher demand of 'allo' products. Hence, the residents of Darchula and Sankhuwasabha districts have started to harvest it extensively. The study emphasises the importance of sustainable harvesting, conservation of natural resources, preservation of the traditional knowledge and formulation one door taxation policy on the use of G. diversifolia.

Acknowledgements

This study has been undertaken jointly by the Ministry of Forests and Soil Conservation (MoFSC) Nepal, Research Centre for Applied Science and Technology (RECAST) under Tribhuvan University, Nepal and the International Centre for Integrated Mountain Development (ICIMOD) under the Kailash Sacred Landscape Conservation and Development Initiative (KSLCDI). KSLCDI is a joint effort to promote transboundary co-operation on landscape conservation in China, India and Nepal through the respective governments and partners which is supported by the Ministry for Economic Co-operation and Development (BMZ), Germany, the DFID/UKAid, and core funds of ICIMOD contributed by the governments of Afghanistan, Australia, Austria, Bangladesh, Bhutan, China, India, Myanmar, Nepal, Norway, Pakistan, Switzerland, and the United Kingdom. The views and interpretations expressed in this publication, however, are those of the authors and are not to be ascribed to MoFSC, ICIMOD, RECAST or their donors.

References

Barakoti, T. & Shrestha, K. (2008). Commercial utilization of allo (Girardinia diversifolia) by the Rais of Sankhuwasabha for income generation. Banko Janakari. 18(1), 18–24.

Chen, C. J., Lin, Q., Friis, I., Wilmot-Dear, C. M. & Monro, A. K. (2003). Urticaceae. Flora of China 5, 76–189. Retrieved from http://hua.huh. harvard.edu/china/mss/alphabetical_families.htm.

Dunsmore, J. (1998). Microenterprise development: Traditional skills and the reduction of poverty in highland Nepal. Himalaya, the Journal of the Association for Nepal and Himalayan Studies. 18 (2), 22–27.

Dunsmore, J. (1998). Crafts, cash and conservation in highland Nepal, Community Development Journal 33 (1), 49–56. doi:10.1093/cdj/33.1.49.

Friis, I. (1981). A Synopsis of Girardinia (Urticaceae). Kew Bulletin. 36(1), 143-157.

Gurung, A., Flanigan, H., Ghimeray, A., Karki, R., Bista, R. & Gurung, O. P. (2012). Traditional knowledge of processing and use of the himalayan giant nettle (Girardinia diversifolia (Link) Friis) among the Gurungs of Sikles, Nepal. Ethnobotany Research and Applications. 10, 167–174.

Malla, B., Gauchan, D. P. & Chhetri, R. B. (2014). Medico-ethnobotanical investigations in Parbat district of Western Nepal. Journal of Medicinal Plants Research. 8(2), 95-108.

Manandhar, N. P. (2002). Plants and people of Nepal, Oregon, USA. Timber press.

Shrestha, R . & Hoshion, T. (1998). Karyomorphological studies in Girardinia diversifolia (Link) Friis (Urticaceae) collected from Nepal. Journal of Japanese Botany 73(3), 125-127.

Singh, S. C. & Shrestha, R. (1988). Girardinia diversifolia (Urticaceae), a non-conventional fiber resource in Nepal. Economic Botany. 42(3), 445–447

Zomer, R. & Oli, K. P. (eds) (2011). Kailash sacred landscape conservation initiative – feasibility assessment report. Kathmandu: ICIMOD

ICIMOD Manual 2017

Community Training Manual

Greening of the Allo Product Value Chain

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Special acknowledgement

RECAST team, Department of Forests' team, and SABAH team for their valuable inputs

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Published by
International Centre for Integrated Mountain Development
GPO Box 3226, Kathmandu, Nepal

ISBN 978 92 9115 521 7 (printed) 978 92 9115 522 4 (electronic)
ICCN 2017-322007

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Illustrator: Asha Kaji Thaku

Printed and bound in Nepal by
Hill Side Press (P) Ltd., Kathmandu, Nepal

Note
The views and interpretations in this publication are those of the authors. They are not attributable to ICIMOD.
This publication is also available at www.icimod.org/himaldoc

Citation: ICIMOD (2017) *Community training manual: Greening of the allo product value chain.* ICIMOD Manual 2017. Kathmandu, ICIMOD