ECOLOGICAL, MORPHOLOGICAL AND MOLECULAR STUDY OF SWERTIA CHIRAYITA (ROXB.EX FLEM) KARST OF NEPAL

A dissertation submitted as a partial fulfillment for the requirement of Master's Degree of Science in Botany (Plant Systematics)

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RECOMMENDAION

This is to certify that the dissertation entitled **"Ecological, Morphological and Molecular Study of *Swertia chirayita* (Roxb.Ex Flem) Karst of Nepal"** Submitted by Ms. Upashana Regmi has been carried out under our supervision. The entire work is based on the results of her research work and has not been submitted for any other degrees. Hence, We recommend this dissertation work to be accepted for the partial fulfillment of Master of Science in Botany Tribhuvan University, Kirtipur, Kathmandu, Nepal.

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LETTER OF APPROVAL

The dissertation entitled "**"Ecological, Morphological and Molecular Study of *Swertia chirayita* (Roxb.Ex Flem) Karst of Nepal"** Submitted by Ms. Upashana Regmi has been accepted for the partial fulfillment of the requirement of M.Sc. degree in Botany (Plant systematics).

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DECLARATION

I hereby declare the dissertation entitled **"Ecological, Morphological and Molecular Study of *Swertia chirayita* (Roxb.Ex Flem) Karst of Nepal"** submitted as the partial fulfillment of Masters Degree (M.Sc) in Botany (Plant Systematics) is based on the originally done work. Any literature , data, or works done by others and cited within this thesis has been given due acknowledgment and listed in the reference section.

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Upashana Regmi

Date: March 25, 2013

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ABSTRACT

Swertia. chirayita is the most valuable and highly demanded species in national and International trade. In the present study, attempts have been made to conduct morphological, ecological and molecular study on *S. chirayita*. Morphological and ecological studies were conducted in Pharche VDC of Kaski district while for the molecular study, samples from four districts namely Sankhuwasabha, Terhathum, Kaski and Lalitpur were considered. The objectives of ecological and morphological studies were to explore quantitative information on the ecological status of *S. chirayita* in the study sites of (Parche VDC, Kaski) as well as the morphological variation of the plant population with respect to various altitudinal and geographical gradients. *S. chirayita* was found to occur between 1650m to 2700m but highest number of plants were found between 1900-2650m. The species was mostly concentrated on moist slopes than in dry and rocky places with density value ranging from 1.7-3.2 plant/ m^2. *Swertia chirayita* was observed in south east, north east and south west face moist open habitats and under canopy of Acer and *Quercus* mixed forest. *Swertia chirayita* was also observed beyond the study sites at different altitudinal range but the number was very low. The regular grazing and movement of livestock were observed through out the study area but the higher pressure was seen in field site there i.e. Gorejure danda. The highest biomass content in the fresh sample of the plant of *Swertia chirayita* was found to be 69% while for air dry sample was found only 15.06% in the site at 2650m altitude in Gorujure danda..Correlation between morphological parameters shows that all morphological parameters except numbers of branches are significant correlated with altitude ($p=0.01$) within the range of studied area.

The overall objective of the molecular study was to detect DNA sequence polymorphism in the nrDNA ITS sequences of *Swertia chirayita* samples collected from various geographical regions of eastern, central and western Nepal using Polymerase Chain Reaction (PCR)-Restriction Fragments Length Polymorphism (RFLP) and DNA sequencing based molecular marker tools. Preliminary studies employing two main marker systems *viz.* 1) PCR-RFLP on the PCR amplified ITS sequences and 2) DNA sequencing of nuclear ribosomal DNA ITS region for the generation of DNA barcodes for *S. chirayita* were performed. As an initial step, Polymerase Chain Reaction (PCR) amplification of Nuclear Ribosomal DNA Internal

Transcribed Spacers (ITS) sequences for the samples collected from Kaski, Sankhuwasabha, Terhathum, and Lalitpur districts were amplified using specific primers. Restriction analysis patterns on amplified ITS sequences were generated using three different Restriction Endonucleases (REs) *viz*. *Bst* UI, *Eco*RI and *Xho*I. Of the three REs used in the study, enzyme *Eco*RI was revealed to be a non-cutter. Whereas with *Xho*I, single cut in the ITS sequences of all the *S. chirayita* genotypes resulted into two fragments in the agarose gels. With *Bst*UI, all genotypes (except three genotypes from Sankhuwasabha) of *S. chirayita* were restricted into two fragments indicating single site in the ITS sequence.

CONTENTS

ACRONYMS

dNTPs- Deoxyribonucleoside triphosphate

AC- PCR- Arbitrarily Primed PCR

AFLP- Amplified Fragment Length Polymorphism

CTAB-Cetyl Trimethyl Ammonium Brimide

CTVT-Citrus Tristeza Virus

dATPs-Deoxyriboadenosine Triphosphates

dCTP- Deoyrobothymidine Triphosphate

dGTP- Deoxyribocytocin Triphosphate

DNA- Deoxyribonucleic Acid

dTTP- Deoxyriboguanosine Triphosphate

EDTA- Ethylene Diamine Tetra Acetic Acid

ELISA-Enzyme-Linked Immunosorbent Assay

ETS-External transcribed sequence

GLB- Gel loading buffer

HCL- Hydrochloric Acid

HIV- Human Immune Deficency

IGS- Intergenic Spacer

ISSR- Inter Simple Sequence Repeats

ITS- Internal Transcribed Spacer

$MgCl_2$- Magnesium Chloride

NaCl- Sodium Chloride

NaOH-Sodium Hydroxide

NTS- Non- Transcribed Spacer

PVDF- Polyvinylidene Fluoride

RAPD- Random Amplification of Polymorphic DNA

rDNA- Ribosomal DNA

RFLP- Restriction Fragment Length Polymorphism.

SCARs- Sequence Characterized Amplified Region

SDS-PAGE-Sodium Dodecyl Sulphate- Poliyacrylamide Gel Electrophoresis

SNPs- Single Nucleotide Polymorphism

SSCR- Single Standard Conformation Polymorphism

t DNA- Template DNA

TBE- Tris Borate EDTA

UV-Ultraviolet

VNTR- Variable Numbers of Tandem Repeats

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INTRODUCTION

1.Introduction

Nepal is a land locked country which lies in the lap of The Himalayas . It lies between the latitude of 26° 22’ to 30° 27’N and longitude of 80° 4’ to 88° 12’E. Nepal falls in the Indo-Malayan and Paleoartic region which contributes to its rich biodiversity. In addition to this, the high range of variation in altitude, temperature, moisture and rainfall within a small area, it presents a multifarious and inimitable biodiversity in the world. Nepal is rich in bio-diversity due to availability of various agro-ecological zones prevailing in different parts of the country.

Of the non-timber forest products (NTFPs), Over 1900 species of medicinal and aromatic plants have been reported in Nepal out of which 250 species are endemic (Kunwar, 2006). Many of these plants are in high demand in national and international markets. Among them one of the most important medicinal plant is *Swertia chirayita* (Roxb.ex Flem.) Karst (Ghimire,1999). Humankind has explored the importance of this plant from ancient time. "Vaidyas" (Traditional healers) have been extensively using it in various treatments. *S. chirayita* is mentioned in Classical Ayurvedic books as Kirata, Kirataka, Bhnimba, Kiratiktata (Kiratatakta, 2001). *S. chirayita* is commonly known Chiraito and locally known as Tite or Lekhtite, Pothi chiraito, Kalo chiraito, Dakhle chiraito in various parts of Nepal.

Nepal comprises around 30 species of *Swertia* including varieties, nine species of *Swertia* are being traded in Nepal among them *Swertia chirayita* is the most valuable and high demanding medicinal plant of Nepal (Preass *et al.,* 2000). These are *Swertia chirayita, S. angustifolia, S. tetragona, S. racemosa, S. ciliata, S. multicaulis, S. alata, S. nervosa*. Among these *Swertia chirayita* plays a dominant role in trade covering about 80% of the traded volume of *Swertia* sp. The demand is adversely triggering the illegal exploitation of *S.chirayita* and its related species which has been a national problem of Nepal. *Swertia chirayita* falls under IUCN threatened category "Vulnerable plant” (Press *et al.,* 2000).

There is an increasing pressure on the natural population due to illegal collection, stealing and over grazing (Bhattarai, 1991). Unscientific collection and over-

harvesting of *S. chirayita* from its natural habitat are taking place in Nepal which has contributed to rapid depletion of *S. chirayita* from its natural habitats, . Due to its high price, collectors have high competition for its collection and it is collected before maturation. Thus, this practice of unsustainable harvesting of *S. chirayita* has resulted into the depletion in its natural habitats (Bhattari and Acharya 1998). It has become one of the threatened species of the Nepal Himalaya. This has probably resulted in decrease in the regeneration potential and its vulnerability can be observed in the field, since this plant grows only in specific habitats and at a specific altitude (1500-3000 m). In recent years, more interests have been focused on evaluation of the ecological status and conservation aspects of *Swertia chirayita*. Due to harvesting of the whole plant before maturity, limited information is available on availability and population status of *Swertia chirayita*. No quantitative information on availability in the population of *Swertia chirayita* is available in Nepal and there has been no research regarding the ecology of this species.

Studies on quantitative assessment of population help in determining the performance of populations under different sets of conditions and provide desired information about the specialized ecological requirements of a taxon (Kaul, 1997). Variation in response to environmental stresses is species- specific and therefore must be considered while developing strategies for sustainable harvest and conservation (Airi *et al*.,2000). Even if a particular variety of a plant is put under several millions hectors of cultivation, the species can still go extinct in the wild if its wild population with all their inherent intra-specific diversity is not conserved (Bhatt *et a*l.,2007). It will also help in the management, recommendation and monitoring of the locals to promote the cultivation in private as well as community forest areas. The present study was undertaken in Parche VDC, Kaski, to document the status, availability and morphological variatio of S. *chirayita* found in in different altitudinal range of temperate belt of the Himalaya,

1.2 Objectives

The present study attempts to explore the quantitative information on the ecological status of *Swertia chirayita* in the study sites as well as the variation of the plant population in respect of the various altitudinal and geographical gradent.

Specific objectives of the present study are to:

- To find out the quantitative information on the ecological status and availability of *S. Chirayita* population in Parche VDC, Kaski district
- To find out the variation on morphology of *S. chitayita* with respect to different altitudinal gradient.

1.3 Limitation of the study

This study had followings limitations.

- A detailed phenological work on the plant could not be studied due to the inaccessibility of the sites monthly.
- More localities and elevation higher than 2700 m or the whole VDC could not be surveyed due to the difficulty in location in geography and the time constraints.
- Biomass allocation pattern in different plant parts and within the population could not be studied in the study sites due the threatened status of the plant.
- Due to limitation of time and budget, more results could not be employed to phylogeny.

2 LITERATURE REVIEW

2.1 The Family Gentianaceae

Family Gencianaceae includes 84 genera and 970 species. It includes herbs, sometimes mycoparasites (with reduced leaves and lacking chlorophyll), to shrubs or small trees. Stems are often winged usually with internal phloem. Leaves are usually opposite, simple, entire, sessile, with pinnate venation. Stipule is lacking. Inflorescesnces are determinate, sometimes reduced to a single flower, axillary or terminal. Flower are usually bisexual and radial. Sepals are usually four or five and connat. Petals are usually 4 or 5 connate, forming a wheel-shaped, funnel-shaped or bell-shaped corolla. The lobes are sometimes fringed, often with nectar glands or/and scales on adaxial surface of the tube, usually convolute, sometimes plicate. Stamens are usually 4 or 5 in number where filaments adnate to corlla. Anthers are occasionally opened at pores and the pollen grains usually tricolporate or triporate. The Carpels-2 in numbers in connate. Ovules are usually numerous, on each placenta, with one integument are a thin-walled mega sporangium. Nectar-producing disk or glands are present. Fruit is usually a capsule (Judd *et. al.*,2002).

2.2 The genus

The genus *Swertia* L (Gentianaceae – Gentianeae – Swertiinae) is a morphologically diverse but taxonomically distinct genus. The genus in its present circumscription comprises 150 species globally and are annual, biennial or perennial herbs ranging from 2-4 cm to over 1.5 m in height with 4-5 merous flowers, having 1 or 2 nectaries at the base of characteristically rotate corolla lobes.

Several members of the species of the genus mostly occurs in alpine or temperate habitats in Asia, Africa and N. America. The plant is native to temperate Himalayas, found at an altitude of 1200-3000m (4000 to 5000ft), from Kashmir to Bhutan and in the Khasi hills at 1200-1500 m (Joshi ,2008). In Nepal, *Swertia* species are widely distributed in hills and mountains of the Eastern, Central and Western regions . The meadow, open forest and scrub ecosystems of the mountainous regions of the country are the suitable habitats of *Swertia* species. The altitudinal range for the species varies from 600 m to 5600 m while most of the species are recorded from 2000-4000 m of altitude (Press *et al.*, 2000).

2.3 Taxonomic Description of *Swertia chirayita*

Swertia chirayita is an erect herb, 50-170 cm tall, light brown, roots small 5 cm to 10 cm long, somewhat twisted and gradually tapering with few rootlets or their remnants. Stem robust, branching, cylindrical below, 4-angled upwards containing large pith. Leaves cauline, opposite, united at the base, sub-sessile, broadly lanceolate, 5-nerved 3-13×1-5 cm, entire, acute. Inflorescence densely flowered, panicle. Flowers small, lurid, greenish yellow near the base of each lobe, two glandular depression each terminated by long hairs, tinged with purple in panicles; calyx 4, deeply partite; corolla 4, ovate acuminate; stamens 4, opposite the corolla, united, style sessile or sub-sessile; stigma capitate, 3 lobed, capsules egg shaped, many sided sharply pointed, seed smooth and many angled (Stainton,1988). Fruiting of *S. chirayita* occurs in the month of Kartik - Mangsir . The peak time of harvesting is Kartik - Mangsir.

2.4 Ecological study of *Swertia chirayita* and its allied species

Bhatt *et al.*(2007) studied the assessment of status and biomass of *Swertia angustifolia* in the selected population of Kumaon Himalaya, India. They found low population density across the surveyed population. Species showed random distribution and higher frequency of occurrence in most of the population, density was positively correlated with biomass. The biomass showed the peak value at the senescence phase.

Joshi and Dhawan (2005) reviewed and highlighted a found that there is a wide scope for exploring different aspects of *S. chirayita.* There is the strong need to screen the different chemo-types of *S.chirayita* growing at different phyto-geographical locations. They suggested biodiversity studies at morphological, biochemical and genetic levels will enable the research community to realize the extent of variability within the existing germplasm of *S. chirayita* and hence help in conservation of the plant.

Bhatta *et al*. (2005) studied the ecological features of a critically rare medicinal plant, *Swertia chirayita*, in the Himalaya. They studied the selected four populations. They found that the density of *S. chirayita* was low in all populations and ranged between 1.65 to 2.35 individuals/m². The frequency of occurrence was high (90–95%) in all

populations and either matched or exceeded the frequency of the other dominant species in the plot. Patterns of Abundance/ Frequency (A/F) ratio revealed random distributions in some populations and regular distributions in the other populations . When comparing biomass during different stages, the maximum value of above-ground biomass was observed in the flowering stage. The maximum value of below-ground biomass was, however, obtained in the senescence stage. The variation in mean belowground biomass of the flowering stage and senescience stages was significant ($P < 0.05$) in all populations. However, total biomass of the flowering and S enescence stages did not show significant variation except in the one population.

Out of 75 districts of the country, *Swertia chirayita* have been documented from 54 districts so far (Joshi 2008). But the main districts where the commercial cultivation is established are namely: Sankhuwasabha, Tehrathum, Dhankuta, Illam, Panchthar, Rasuwa, Solukhumbu, Taplejung, Ramechap, Dolakha, Sindhupalchok, Gorkha, Dolpa, Rolpa, Salyan, Sinduli, Accham, Doti and Makwanpur (Bhattarai & Acharya 1998). Distribution of *S. chirayita* is not uniform; it depends upon the altitude and slope and are found in patches, preferring in the north facing slope where it is found even below 1500m, while it grows in south facing slope between 1500m to 3000m. In general, 2000 -2500m altitude is most preferable range (Bhattarai and Acharya1998). *S. chirayita* prefers to grow in acidic soil condition with pH of 4.7 to 5.5 (Bhattarai & Shrestha, 1996). *S. chirayita* is found being mixed with other species. The most common associated species are: *Anaphalis* spp., *Bidens* spp., *Eupatorium adenophorum*, *Centella asiatica*, *Viola* spp., *Bistorta amplexicaulis*, *Rhododendron arboreum*, *Quercus* spp. and *Acer* spp. (Phoboo *et. al.*, 2010*)*

2.5 Ethnobotanical study on *Swertia chirayita*

Swertia chirayita is an important medicinal herb used for curing various diseases. This plant is known by different names in different community Nepali name is Tite, Rai name as Khuple Sherpa as Tigta and Tamang as Tamadaa. Locally this plant is given for malarial fever. It is also used in common ailments like cough, cold, and fever. *Swertia chirayita* is much praised in India as a tonic and bitter without aroma or astringency (Joshi & Dhawan,2005).

In Nepal this plant is used by different community differentially, in west Nepal plant is boiled with water for 1-2 hours and the water is drunk to get relief from fever at

Jumla district. In Central Nepal plant is used for stomach disorder at Chauba village. Decoction of aerial parts is taken by the Sherpas of Helambu as an antipyretic and to treat bodyache. Similarly in East Nepal decoction of plant is given by the Limbus of Morng district to treat fever. Leaf of this plant together with young stem of *Achyranthes asperata* and bark of Phyllanthus emblica are pounded and the decoction is given by the Saters of Morang and Jhapa districts to cure fever and cholera (Rajbhandari, 2001).

2.6 Trade on *Swertia chirayita*

Swertia chirayita, as an important medicinal plant,contributing significantly in Nepal's economy. Every year, tones of *S. chirayita* are exported to the neighboring countries contributing to the country's foreign currency earning. Nepal is reported to trade more than 45% of the world's total volume of *S. chirayita* (Barakoti 2004). Only about 1% of the *S. chirayita* is collected for local use. Most of the *S. chirayita* is exported to India, while some are exported to China, Malaysia, Singapore, Germany, Italy, France, Switzerland, Sri Lanka, Bangladesh, Pakistan, and USA (Phoboo *et al.* 2010). *S. chirayita* has high demand due to its multi-purpose medicinal values in different medical system such a Ayurvede, herbal, allopathy etc

The trade and sustainable conservation of *Swertia chirayita* in Nepal was studied by Phoboo and Jha (2010). They suggested that the large scale export of this plant has made it vulnerable in Nepal and there is an immediate need for its conservation. They reported that *S. chirayita* is traded in 61of the 75 districts of Nepal and constituted about 3% in the total medicinal plant traded in the fiscal year 2008-2009. Due to extensive collection of *S. chirayita* from the wild, there is a need for exploring alternative conservation options in order to preserve the remaining wild population. Thus, sustainable harvesting and cultivation can help in its conservation.

According to the rules and regulation of the government of Nepal , no collection sell or purchase of *Swertia chirayita* is allowed . A permit from District Forest Office is needed to collect *S. chirayita* from forest and Rs 3 /kg of royalty is to be paid . For the expert of *S. chirayita* , half percent of custom has to be paid. Annapurna Conservation Area Project (ACAP) has been playing a leading role in the field of conservation, people's awareness and development. ACAP allows small collection of the plant only for their household purpose they smuggle some *S. chirayita* through

various trade channels. Now, surveying is done for sustainable harvesting of medicinal plants for the benefit of local people by Annapurna Conservation Area Project (ACAP) .

Local has named as Tite: Pothi Tite and Vale Tite for the species of *Swertia chirayita* and *Swertia angustifolia* respectively as mentioned (Joshi and Joshi 2008). *S. chirayita* is also known as Lekali Tite due to its occurrence in high altitude (Leka). Present study showed that the local people used the technique common in other parts of Himalaya After collection of plant or plant parts , they are washed , if necessary dried either in sunlight or in shade . Mostly the plant is dried in shade . Then the dried plant are packed in jute bags (Bora) Mostly it is harvested in its fruiting season. Because at this time the yield is maximum for trade and the constituents of chemicals are concentrated at this time . Local amchis have more knowledge about the medicinal value of many species and they suggest that efficiency of extract is high in those parts which are harvested during their fruiting season .

2.7 Phytochemical study and Bioactivity of *Swertia chirayita*

Quantification of major phytochemicals of *Swertia chirayita*, was studied by Phoboo *et al.* (2011). They prepared crude aqueous and ethanolic extracts of main phytochemicals from different plant parts of *Swertia chirayita* collected from nine different districts of Nepal representing west, central and east Nepal amarogentin, mangiferin, swertiamarin were quantified. They also quantified these photochemical between wild and cultivated plant parts of *Swertia chirayita*. The highest quantity of all the three phytochemicals was found in IL (inflorescence and leaf mixture) of all the collected plants samples. They found no significant difference in the amounts of these three photochemical extracts from wild and cultivated plants. Neurological studies of novel compounds from *Swertia chitayita* were carried out by Srivastava *et al* (2010). They extracted, isolated and characterized the active principles mainly novel compounds like xanthones, seco-irridoid glycosides etc and their biological activity was performed. Isolation resulted in the elution of different compounds

Several pharmaceutical studies have been conducted in *Swertia chirayita*. Bhargava *et. al.,* (2008) studied antipyretic potential of *Swertia chirayita* Buch Ham. root extracts. They evaluated for its antipyretic potential on Brewer's yeast induced pyrexia in albino rats and Typhoid-Paratyphoid A, B vaccine induced Hyperexia in

rabbits from aqueous extract of *Swertia chirayita* Buch Ham. They found, in both models, the extract, at dose of 200 mg kg−1 body wt. and 400 mg kg−1 body weight, produced significant ($p<0.001$) reduction in elevated body temperature in a dose dependent manner. The antipyretic effect of the extract was comparable to that of paracetamol (150 mg kg−1 body weight, p.o.).

Antiviral activity of the plant extract, *Swertia chirayita* against herpes simplex viruses was studied by Verma *et al.* (2008). They tested antiviral activity of plant extract of *Swertia chirayita* against Herpes simplex virus (HSV) type-1, using multiple approaches both at cellular and molecular level. They found, *Swertia* plant crude extract (1gm/mL) at 1:64 dilution inhibited HSV 1, plaque formation by more than 70%. The Infected cell cultures treated with *Swertia* extract at various time intervals, tested by PCR, failed to show amplification at 12, 24-72 hours. HSV-1 infected cells treated with Acyclovir (antiviral drug) did not show any amplification by PCR. The result concluded that *Swertia chirayita* showed antiviral properties against Herpes simplex virus type-1.

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3 STUDY AREA

3.1 Background

The study was carried out in Sikles, Parche VDC of Kaski district. The study is based on mainly on primary data. Primary data were collected through transect survey method, semi-structured interview and herbarium collection. The first visit was conducted during November 2008 and was for 15 days in senescence stage of *Swertia chirayita*. Second field visit was conducted during August 2009 for 7 days during which ecological study was carried out.

source: **DNPWC**

Figure 1. Map showing the study area

3.2 Geographical location

The Kaski district is located in Gandaki zone of the western development region of Nepal. Geographically, it extends from 28° 6' to 28° 38' N latitude and 83° 40' E to 84° 12' E longitude. The district is bounded on the west by Myagdi district on the east by Lamjung and Tanahu districts, on the north by Manang District and the south by Syangja and Tanahu District. This district covers an area of 2017 sq. km. The climate of this district is sub – tropical, temperate and alpine (Tundra). The temperature varies from an average minimum of 15.9°C in the winter and to an average maximum of 26.1°C in the summer with an annual rainfall of 388.3 ml (District profile 1997).

Sikles belongs to Parche VDC, which is one of the largest VDC of Kaski district situated in the North Eastern corner of the district at the mountainside. Geographically it is located 28° 19' to28° 29' north latitude and 84° 03' to 84° 08' east longitude. Namarjung VDC and Madi River are situated to the east, Machhapuchhre, Sardikhola VDCs to the west, and Sildujure and Namarjung VDCs to the South West, and Sildujure and Namarjung VDCs to the South of the Parche VDC. There are three villages in this VDC known as Khilang, Parche and Sikles.

Among these villages, Sikles village is located in the North Eastern corner of Pokhara at mountainside perched on a hillock at an elevation of about 2000 m and is one of the largest and most beautiful Gurung village in the district with Gurung occupying almost 60% of the total population of the VDC. The study area is about 32 km. away form Pokhara valley. The study area is located at an elevation of 1200 m to 6100m from the sea level on the southern slopes of Annapurna Himalayan range. The climate of this area is sub tropical to alpine and remains rather cool throughout the year with heavy snowfall once or twice in a year in the winter season with mean monthly precipitation ranges from only 34.25mm in December to 899.63mm in July. The mean annual precipitation in the area is a 3752mm. Mean monthly precipitation recorded in Sikles and estimated temperature data based on recorded data at Pokhara are presented in Table 1.

3.3 Climate

Based on the data of the nearest weather station (Pokhara) highest average rainfall was recorded in july and minimum rainfall was recorded in December. The highest average maximum temperature was recorded in june and lowest average temperature during January.

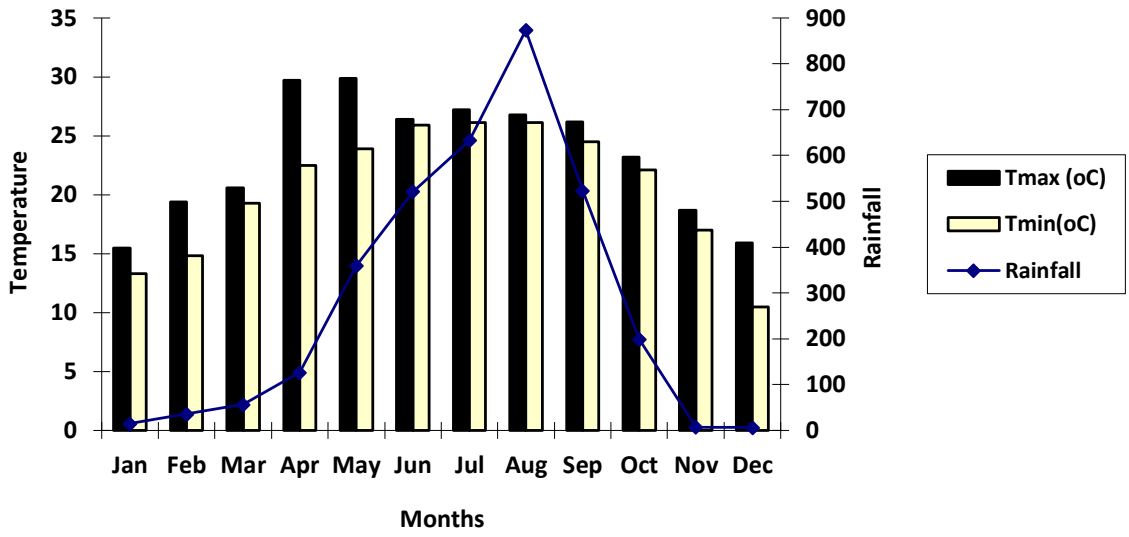


Figure 2. Average monthly Temperature (°C) and Precipitation (mm) recorded in Pokhara weather station between2005 and 2008. (Source: Department of Hydrology and Meterology,Kathmandu).

3.4 Vegetation and flora of Sikles

Sikles area consists of vegetation types ranging from subtropical to alpine. There are mainly three specific forest areas, i.e. Ban Pala (Community owned forest areas) Nasa ban (Whole village owned forest or natural forest) and Nizi ban (Private forest areas). The distribution of forest areas also varies into different places. According to the attitudinal zone and climatic variations, mixed forests, both evergreen coniferous as well as bushes and shrubs are found in the study area. The main flora include *Schima-Castonopsis*, *Alnus nepalensis*, Lower temperate mixed broadleaved, Oak, Upper temperate mixed broadleaved, *Rhododendron*, *Betula utilis*, *Pinus wallichiana* and Juniper forest types and arid bushes.

In the high Himalaya the predominant vegetation comprises mostly grasses and valuable medicinal plants i.e. *Dactylorhiza hatagirea* (Panch awale), *Nardostachys grandiflora* (Jatamansi), *Cordyceps sinensis* (Yarshagumba), *Neopicrorhiza scrophulariifolia* (Kutki), *Aconitum* sp. etc. Sufficient wild nettle and bamboo (*Nigalo*) are also available in both high hill forest areas and low altitude forest areas, which has been widely used by the villagers to prepare many local handicrafts. The predominant vegetation of the study area comprises *Alnus nepalensis* (Utis), *Rhododendron* species, *Arundinaria sp., Schima wallichii* (Chilauni), *Berberis aristata* (Chutro), *Betula utilis* (Bhoj Patra), etc.

3.5 People, Population and socio-economic status

In the study area there are three major ethnic groups i.e. Gurung, Kami and Damai. Sikles is one of the largest Gurung village in Nepal. There are three different major ethnic groups in Sikles area viz. Gurung (84.78%), Kami (11%) and Damai (3.59%) (Gurung . 1999). Among these the most dominant groups is Gurung having their own tradition and culture. There is close relationship between their culture and plants. On the contrary Kamis and Damais are present as occupational caste group and are least in number

4 MATERIALS AND METHOD

4.1 Ecological study

Selection of sampling design was applied in which stands were selected by either a random or stratified random plan then locating the starting point and direction of a transect within the stand, Samples were taken according to the systematic plan (Barbour *et.al,* 1999). The total of four population were studied in four different sites. The sampling sites were selected in different altitudes, aspects, habitat in different localities so as to cover the whole VDC.

Sampling sites in different localities:

Four sites chosen for the morphological and ecological study are shown in table 1.

Table 1

S .N	Sampling site	Elevation	Latitude	Longitude	aspect
1	Below Sikles village	1950m	28 º21'34.4"	84º06'34.5"	South east
2	Way to Ghalekharka	2360m	28 º21'30.2"	84º 4 '50.1"	South east
3	Ghorujure Dada	2650m	28 º22 '4.9"	84 º5 ' 47.4"	North east
4	Avalanche Viewpoint (Top)	2670m	28 º21'58.6"	84 º 5 '34.9"	South west

Transect were laid in the selected populations within Parche VDC, Sikles Kaski

In each population three transect lines were set up at an interval of 30-50 m in *Swertia chirayita* available sites. In each transect lines five quadrats of 1m×1m were laid down at an interval of 5- 7 m. Hence, altogether 15 quadrats were studied from each locality making total of 60 such quadrats in the area of four populations within the study area. Herbs associated were also recorded within the sampling plot in each quadrat. Coverage, frequency, density were recorded in the field.

Soil samples were collected from each quadrat by digging up the soil from 25 cm depth. Every one plant of *S. chirayita* from each quadrat was dug and taken as sample specimen for the study of biomass.

Disturbance factors in the selected sites like grazing, distance from nearby village, collection of plants, fire were also observed. Specific details of localities (altitude, latitude and longitude of different sites were recorded with the help of Global Positioning System (GPS) and the aspect and slopes were measured by using clinometers.

Quantitative analysis

For each locality, data were analyzed to assess compositional features such as frequency, density and coverage of the plant and abundance and Abundance/ Frequency (A/F) ratio (Zobel *et al*., 1987).

Frequency and Relative Frequency

The frequency of occurrence of *Swertia chirayita* was determined to assess the distribution pattern of the species.

$$\text{Frequency (F)} = \frac{\text{Number of quadrats in which species occur}}{\text{Total number of quadrats}}$$

$$\text{Relative Frequency (RF)} = \frac{\text{Frequency of individual species}}{\text{Sum of all frequencies}} \times 100$$

Density and Relative Density

Density is the number of individual per unit area, which gives the numerical strength of species. In general, density is the total number of individual of a species relative to total area studied and is calculated by following formula.

$$\text{Density (Individuals/ m}^2\text{)} = \frac{\text{Number of individuals of species 'A' in all quadrats}}{\text{Total number of quadrats} \times \text{size of the quadrat}}$$

$$\text{Relative Density (RD)} = \frac{\text{Density of species 'A'}}{\text{Total density of all species}} \times 100$$

Cover and Relative Cover

Cover (also called coverage) is the percentage of quadrates area covered by a given species. Cover will be estimated visually with the help of following scale.

Table 2. Scale Value, Range and Mid-point Value of the Estimate of Plant Cover.

Scale value	Range of coverage (%)	Mid point value
1	0-5	2.5
2	5-25	15.0
3	25-50	37.5
4	50-75	62.5
5	75-95	85.5
6	95-100	97.5

Average cover of a species will be calculated by taking average of the value for all quadrates. Relative cover is the cover of a particular species as a percentage of total plant cover. It is calculated by the following formula.

$$\text{Relative Cover (RC)} = \frac{\text{cover of particular species}}{\text{Total cover of all species}} \times 100$$

4.2 Estimation of Dry Biomass and Moisture

Dry biomass and moisture content of *Swertia chirayita* was estimated. Both above ground and underground parts of *S. chirayita* was harvested from the sampling sites. Individuals were harvested from each sites. A pointed digger was used to dig out the roots and after removing the soil particles of each plant from quadrat were packed separately in a polythene bag for weighing. After taking the fresh weight, the samples were kept on open air and again weighed . Then the samples were packed in paper bag separately and brought to the laboratory and kept inside the hot air oven at 75° C for 48 hrs. Thereafter,the samples were weighed, again. The oven dry weight was taken as dry biomass. The percentage of dry biomass and moisture were calculated by using the following formula (Zobel *et al*., 1987)

% Moisture (In Market Going Sample) =Air dry weight.– Oven dry weight .

Air dry weight.

4.2.1 Biomass allocation pattern

Biomass allocation pattern was assessed in all four different locations at varied altitudes to know the relative contribution of above ground and below ground biomass. In each site one mature plant of *Swertia chirayita* was selected for harvesting different plant parts ie. above ground and below ground biomass. For this the plant was air dried again by kept inside the hot air oven for 48 hrs maintaining the temperature of 75° C. After the oven dry weight of each parts were measured for biomass allocation.

4.3 Morphological study

Along with the ecological data that were collected in the field morphological feature were also noted by averaging all plants found from every quadrate in each plot of the studied area. In each site 15 quadrats were selected, altogether 60 individual plants samples were used for morphological study. And 7 morphological traits have been observed in representative plants as follows:

Height of plant (HP), Length of internode in main stem (LI), Number of nodes in main stem (NN), Leaves length (LL), Leaf width (LW), Branch number in main stem (BN) and pigmentation of stem

Height of plant, length of internodes, leave length and width were measured by means of measuring tape or scale in metric system. Number of nodes, pigmentation of stem, branches number were directly observed and recorded. The largest central branches considered as main stem.

4.4 Laboratory analysis of soil

Soil samples were air dried in shade and analyzed for pH, total nitrogen (%), total organic matter (OM)%, phosphorus (P_2O5), and potassium (K_2O) at National Agriculture Research Centre (NARC), Soil Sience Laboratoy, Khumaltar, Lalitpur.

4.4.1 Total organic matter content (OM) %

Soil organic matter (%) was determined by Walkely and Black rapid titration method (as described in Gupta (2000).

In this method, 0.5 g air dried fine soil was taken in a clean and dry 500 ml conical flask. Then, 5ml of 1N $K_2Cr_2O_7$ (Potassium dichromate) and 10ml of conc. $H_2SO_4$was added successively. The mixture was shaken well and then allowed to cool down for 30 minutes. To this mixture 100 ml of distilled water and 5ml of orthophosphoric acid were added. Then 0.5 ml of diphenylamine indicator was added in conical flask containing the mixture of soil and reagents. Lastly, the content was titrated with 0.5 N ferrous ammonium sulphate solution till the color changes from blue-violet to green. A blank solution without soil sample was also run simultaneously.

Now, the organic carbon (%) of the soil sample was determined by using following formula:

Soil organic carbon estimated (%) = $\frac{0.003\times100(blank\ reading\ titration\ reading)}{blank\ reading\ \times weight\ of\ soil\ (g)}\times100$

The organic carbon (%) obtained by above formula was multiplied by a factor 1.3 (based on the assumption that there is incomplete oxidation of the organic matter in this procedure and only 77% recovery occurs through this method).

Hence, organic carbon (%) = organic carbon estimated (%) ×1.3

Now, to determine organic matter content (%) of soil, this value of organic carbon was multiplied by Van Bemmelen factor of 1.724 (because organic matter is assumed to contain 58% organic carbon).

Hence, organic matter content (%) =organic carbon (%) ×1.724

4.4.2 Total Nitrogen

The total nitrogen (%) of soil was determined by Microkjedahl method (as described by Gupta 2000). This method includes the following three steps:

Digestion

One gram air dried fine soil was taken in a clean and dry Kjeldahl digestion flask containing soil and then placed on the preheated heating mantle for digestion. Temperature was raised to about 310 degree C (after the bubbles started disappearing on the content of the flask). The end of digestion process was known as the color changed from black to brownish and ultimately greenish. Then the flask was removed from the mantle and allowed to cool for 30 minutes. 50ml of distilled water was added

to the digest and the mixture was shaken well. A blank without soil sample was also run simultaneously through this process.

Distillation

The diluted digest of Kjeldahl digestion flask was transferred to Kjeldahl distillation flask. A 100 ml beaker with 10 ml of boric acid indicator was placed below the nozzle or the condenser in such a way that the end of the nozzle dipped into the indicator. After the digest become warm, 30ml of 40% NaOH solution was added in distillation flask and its mouth was closed with cork making the system air tight. The temperature of the mantle was raised to about 310^0C. The distillation was continued until the volume of distillate in beaker reached to about 50ml.

Titration

The distillate was then titrated with 0.1N HCl. The volume of HCl consumed in titrating distillate was recorded on the basis of which the total nitrogen content (N%) of the soil sample was calculated by using the following formula.

Soil N (%)= $\frac{1.4\times N\times(S-B)}{M}\times 100$

Where, N= normality of HCl

S= Volume of HCl consumed with soil sample (ml)

B= Volume of HCl consumed with blank (ml)

M= weight of soil taken (mg)

4.4.3 Phosphorus

Available phosphorus was determined by Olsen's method (Gupta, 2000). Soil is shaken with neutral normal ammonium acetate. During the extraction, ammonium ions replace potassium ions adsorbed on the soil colloids. In addition the use of ammonium acetate as the extractant eliminates the error due to inclusion of non-exchangeable potassium.

The extract was filtered and potassium in the filtrate was determined with a flame photometer.

Method

One gram sieved soil sample was taken in polythene bottle. $NaHCO_3$ 20 ml of 0.5 M solution was added and shaked for 30 minutes. The solution was filtered. Algnote 5ml was taken in 25 ml volumetric flask and 0.5 ml of M H_2SO_4 was added. 4ml of ammonium molybdate was added in the solution. It was made up to 25 ml. Absorbance reading was taken in spectrophotometer of 760 nm.

Preparation of standard curve of phosphorus

1, 2, 3, 4 and 5 ml of 5 ppm Phosphorus solution was taken in 50 ml volumetric flasks separately. 5 ml of the extracting solution ($NaHCO_3$) was added to each of these solution. Then 10 ml of distilled water and one drop of *p*-nitrophenol indicator were added. Then 2.5M H_2SO_4 was added to it drop wise until the solution becomes clear. At the point where indicator's yellow color disappeared, the correct pH (5.0) for the color development was attained.After that 8 ml of the Murphy Riley solution was added to each flask. The volume was made to 50 ml with distilled water and was mixed thoroughly. Thus, those standards had Phosphorus concentration 0.1, 0.2, 0.3, 0.4 and 0.5 µg P/ml. A blank was also prepared with NaHCO3 solution, distilled water and Murphy-Riley reagent. After 15 minutes, the intensity of the blue color was read on spectrophotometer at 730 nm. Absorbance values for the standards having 0, 0.1, 0.2, 0.3, 0.4 and 0.5 µg P/ml were used to construct a standard curve between absorbance values and the concentration of P in standards.

4.4.5 Available Potassium

Available potassium was determined by ammonium acetate extraction method (Gupta.2000) 2 gm of soil was taken in a 150 ml conical flask. 20 ml of N ammonium acetate solution was shaken for five minutes on an electric shaker. The content was filtered through Whatman no-1 filter paper. 5 ml filtrate was pipette in a 25 ml volumetric flask and the volume was made up to mark. This extract was feed to the flame photometer. The 100 reading of which has been set with a solution having 10ppm potassium and the reading obtained was noted.

4.5 Statistical analysis

Statistical analysis was conducted for ecological and morphological traits by following ways:

To determine correlation between morphological and ecological parameters with altitude correlation and regression analysis were performed using SPSS (Version 11.5).

Grand mean, mean, standard deviation and coefficient of correlation were also computed through descriptive statistical analysis by using SPSS for every individuals from each quadrat for all morphological and ecological parameters.

5 Results

5.1 Altitudinal occurrence of *Swertia chirayita*

In the study area, the altitudinal occurrence of *Swertia chirayita* was between 1650m to 2700 m, good number of *Swertia chirayita* was found between 1900-2650 m. The range of distribution of the *Swertia chirayita* was not common to all the areas and was restricted only to certain areas in all the studied populations. *Swertia chirayita* was observed in south east, north east and south west face moist open habitats and under canopy of Acer and *Quercus* mixed forest. *Swertia chirayita* was also observed beyond the study sites at different altitudinal range but the number was very low.

5.2 Ecological occurrence of *Swertia chirayita*

The population of *Swertia chirayita* was observed in the small localized populations in patches. The regular grazing and movement of livestock were observed through out the study area but the higher pressure was seen in field site there i.e. Gorejure danda.

Table 3. Ecological status of *Swertia chirayita*

S.N	Site	Population	Average frequenc	Average density	A/F	Average plant	Distribution
1	Below Sikles	1	60	1.73	0.036	87	Regular
2	Way to Ghalekharka	2	70	2.4	0.036	110.53	Regular
3	Gorujure danda	3	70	2.13	0.031	113.06	Regular
4	Top	4	70	2.66	0.052	121.46	Regular

Abundance/ Frequency=A/F

The species was mostly concentrated on moist slope than in dry and rocky slope with density value ranging from 1.7-3.2 plant/m^2 . considering species performance across the population, the top population appears to be the best performed with its high density ie 3.2. among the four population, south east facing population of below

Sikles show least density ie 1.7 plant/m^2. The density of north east facing slope of Gorujure danda was 2.13. The average density of the species in the study area was 2.23 plant m^2. The lowest frequency was found to be that of the below Sikles and other three population were having same frequency.

5.3 Moisture content and biomass

Table 4 . Showing Dry Biomass and Moisture Percentage Content

S.N	Sampling Site	Average dry mass (gm/m²)	Average moisture content % in wet sample	Moisture % in Air Dry Sample
1	Below sikles	24.65	61	12.15
2	Way to Ghalekharka	38.52	63	13.58
3	Gorujure danda	34.65	69	15.06
4	Top	53.50	66	13.12

The highest biomass content in the fresh sample of the plant of *Swertia chirayita* was found to be 69% while for air dry sample was found only 15.06% in the site at 2650 m altitude in Gorujure danda. Below Sikles at 1950 m altitude has least moisture content in wet sample and air dry to be 61 and 12.15 respectively. Similarly the highest biomass was at 2670 m altitude 53.50 g/m² in the south west facing slope and lowest was 24.65 g/m² at the site Below Sikles at 1950m of altitude in south east facing slope.

5.3.1 Biomass allocation pattern

When comparing biomass during different developmental stage the higher value of above ground biomass was observed in the flowering stage. Significantly higher

($P<0.05$) above ground biomass (FL 28.15g/m^2, S 25.35 g/m^2) was observed in the Top site (Table 17).

Table 5. Biomass allocation pattern

S.N	Site	Growth stage	Above ground biomass (g/m^2)	Below ground biomass (g/m^2)	Total biomass (g/m^2)
1	Below sikles	FL	11.12	1.41	12.53
		S	8.61	3.92	11.30
2	Way to Ghalekharka	FL	7.34	2.56	19.9
		S	10.87	7.75	18.62
3	Gorujure danda	FL	14.57	3.19	17.51
		S	12.18	4.72	16.90
4	Top	FL	24.67	3.48	28.15
		S	17.53	7.82	25.35

FL, flowering; S, senescence

The higher value of below ground biomass was however obtained in the senescence stage at all sites. The variation in mean below ground biomass of FL and S stage was significant ($P<0.05$) in all populations. Total biomass of the FL and S stage did not show significant variation. However, in all cases, total biomass was greater at the flowering stage.

5.4 Soil characters in the sampling sites

Soil of *Swertia chirayita* occurring region was found acidic in all sites. The mean pH value of all sites was not significantly differing. The highest pH value was found in Way to Ghalekharka and the lowest value was found in the site below Sikles the average pH value of the area was 4.3 which is acidic in nature.

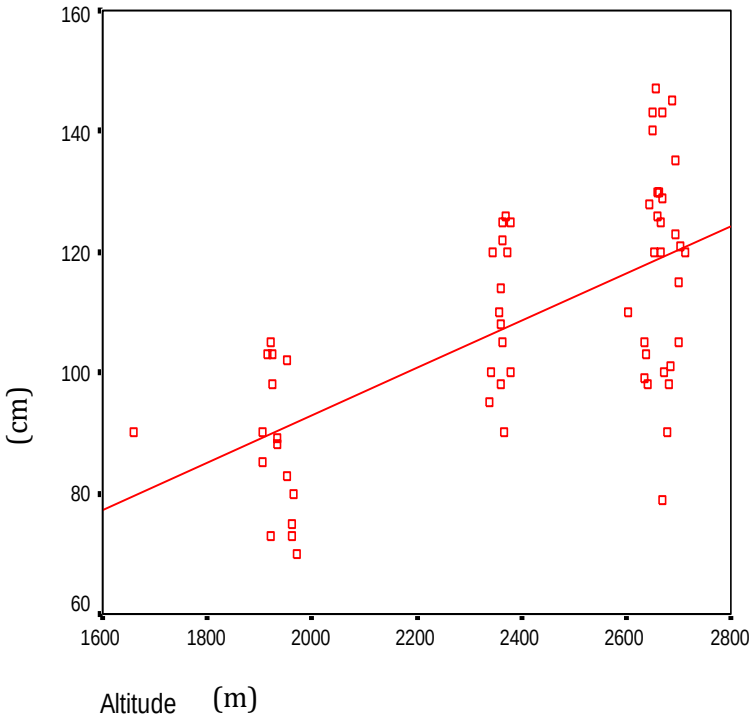
Table 6. Result of different soil parameters studied

S. N	Site	Organic matter content (%)	Nitrogen (%)	Phosphorus (kg/h)	Potassium (kg/h)	Organic carbon(kg/h)	Soil p^H
1	Below sikles	7.1215	0.35	182.45	413.33	4.13	4.68
2	Way to Ghalekharka	14.04	0.60	138.46	745.93	8.14	5.03
3	Gorujure danda	13.98	0.56	142.93	635.46	7.55	4.89
4	Top	15.72	1.12	114.86	519.46	19.12	4.72

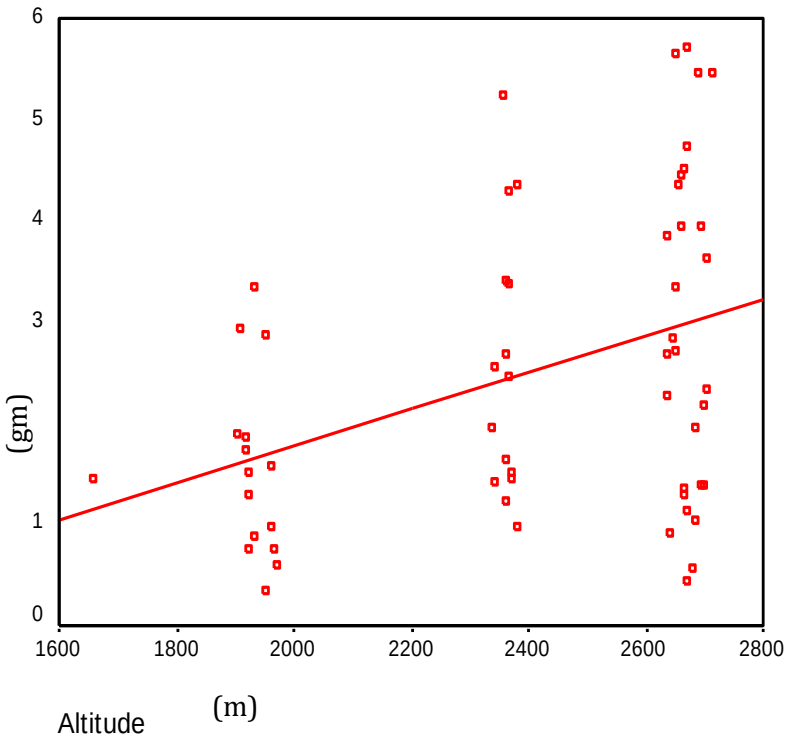
The average organic content (%) of the study area was 12.7% among the studied sites. The organic matter content (%) found in top (15.72%) as highest value. The lowest value is found that of site 1 below Sikles. The way to Ghalekharka and Top lies in intermediate position. The average nitrogen content (%) in soil of below Sikles was 0.35 % which is the lowest value. And that of Top is maximum (0.78). the average nitrogen content (%) , across the study site was 0.57. Similarly, the highest phosphorus content of soil was in below Sikles 182.46kg/ha while the lowest phosphorus content was found in Top 114.86 kg/ha. The average phosphorus content of the area was 144.67 kg/ha.

Likewise, the mean soil Potassium content (kg/ha) of way to Ghalekharka 745.93 is the highest and lowest is Below Sikles 413.33. The average Potassium content (kg/ha) of soil of *Swertia chirayita* of the study area was 134.89.

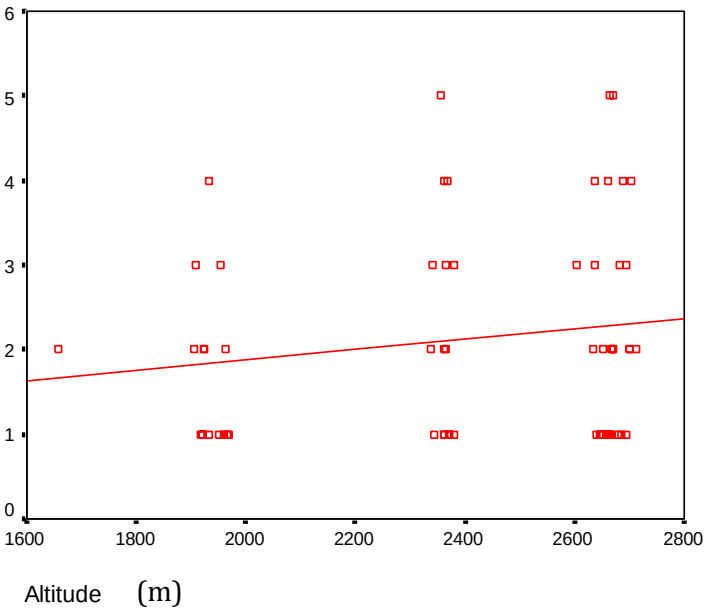
Fig 4. Linear regression line between plant parameters with altitude.



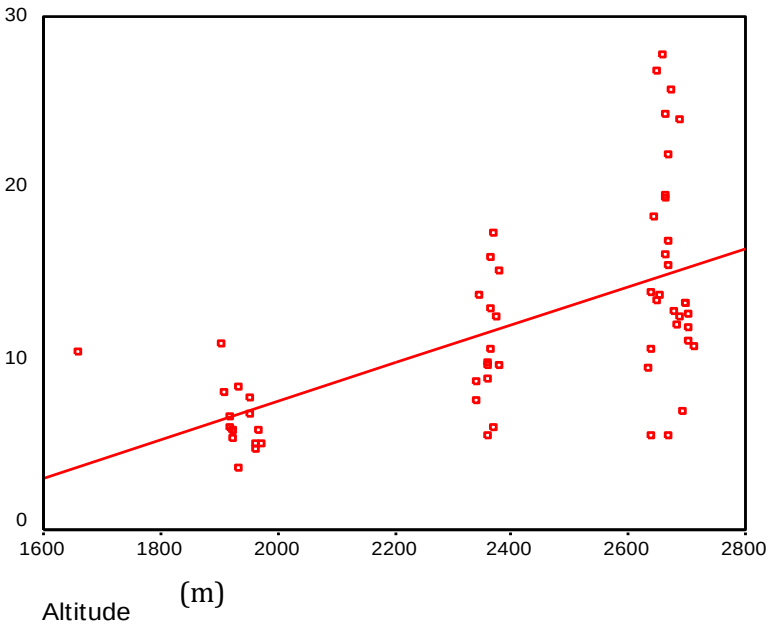
a) Relationship between plant height and altitude



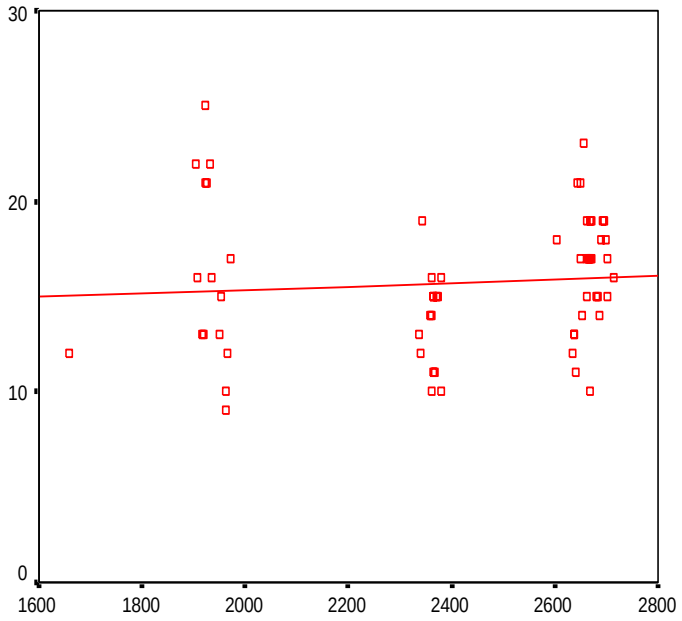
b) Relationship between biomass and altitude



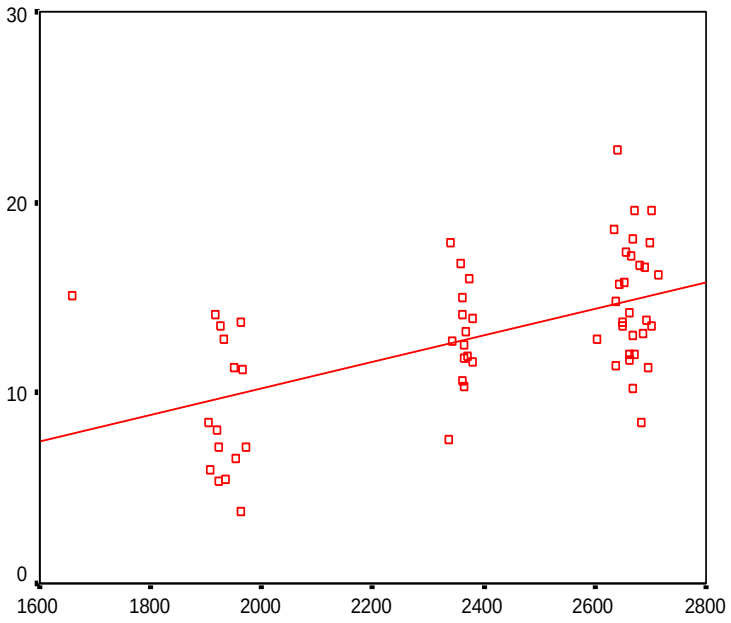
c) Relationship between density and altitude



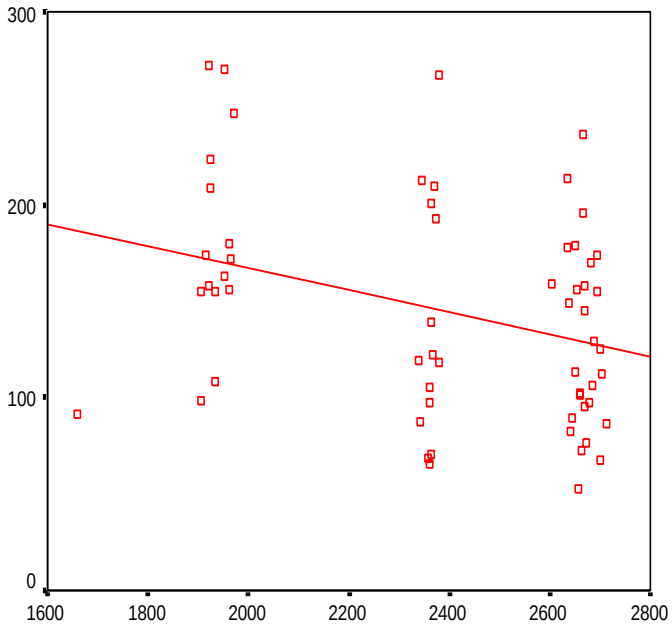
d) Relationship between leaf area andaltitude



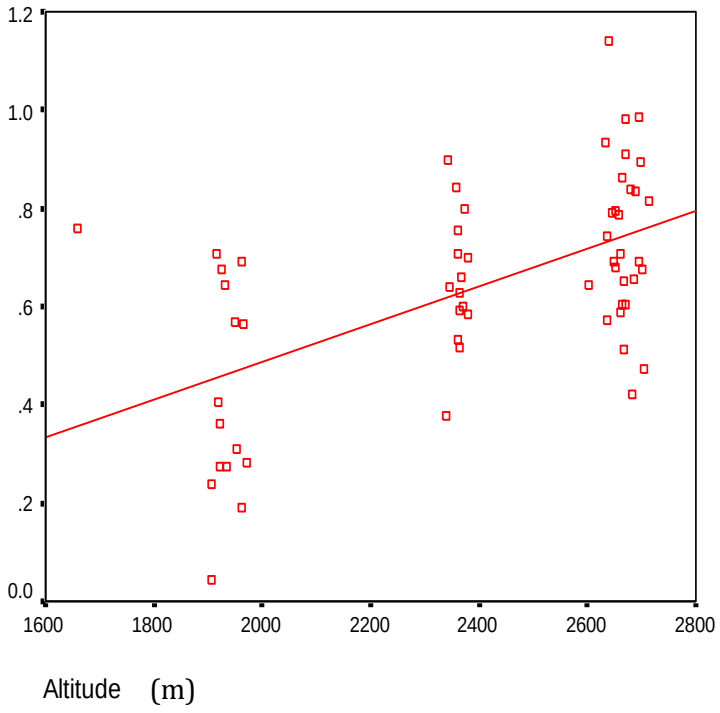
e) Relationship between branch number and altitude



f) Relationship between organic matters and altitude



g) Relationship between phosphorous and altitude



h) Relationship between nitrogen and altitude

5.5 Morphology

The investigated sites have varied elevation, latitude, longitude and climatic conditions belonging to different location in the Kaski district of Nepal. Each of these four sites have been considered different populations. *Swertia chirayita* usually associated with *Anaphalis* species but some where they were also associated with other species of plants.

Analysis of morphological traits of *S. chirayita* showed variation within and among populations. Standard deviation and coefficient of variation are two important tools of statistical measurements to know about the dispersion of individuals around mean value and to know the measurements of relative variability of given plant within population.

Statistical analysis among population were carried out and tabulated which reflects the mean of each character, their ranges, standard deviation plant to plants. Variance indicates the dispersion of individuals around mean while coefficient of variation expressed in percentage represents the measure of relative variability of a given traits in different sites:

5.5.1 Observation in Height of Plant (HP)

The mean height of plant was observed highest in site 4, followed by site 3 and site 2, Site 1, observed lowest mean 87.80.

Table 7. Height of Plant (HP)

S. N	Site	Mean(cm)	Min.(cm)	Max.(cm)	Range	Variance	S.D	C.V%
1	Below sikles	87.80	70	105	35	148.60	12.19	13.88
2	Way to Ghalekharka	110.53	90	126	36	147.12	12.12	10.97
3	Gorujure danda	113.06	79	143	64	382.35	19.55	17.29
4	Top	121.46	98	147	49	275.83	16.60	13.67

5.5.2 Observation in Branch No (BN)

Highest mean of branch number 15 was observed in site 2 followed by site 4. The lowest mean 12.6 was found in site 1. Range of variation in the mean of branches number is similar in site 3 and site 4. The minimum branch number was observed in site 1. Maximum, branches no. is 21 in site 4 (Table 8).

Table 8. Branch No (BN)

S.N	Site	Mean (cm)	Min (cm)	Max (cm)	Range	Variance	S.D	C.V%
1	Below sikles	12.6	9	16	7	4.4	2.0976	16.64
2	Way to Ghalekharka	15	10	19	9	7.143	2.6726	17.81
3	Gorujure danda	13.73	9	19	10	9.210	3.0347	22.10
4	Top	14.93	11	21	10	11.495	3.3904	22.70

5.5.3 Observation in Numbers of Nodes (NN)

Highest mean value of 15.26 was observed in site 4 having maximum numbers of nodes 22 among whole population studied with range . Lowest mean was observed 12.5 in site 2 . The minimum numbers of node among populations was found 7 in site 1(Table 9).

Table 9. Numbers of Nodes (NN)

S.N	Site	Mean (cm)	Min. (cm)	Max. (cm)	Range	Variance	S.D	C.V %
1	Below sikles	11.46	7	19	12	12.26	3.50	30.54
2	Way to Ghalekharka	12	9	19	10	7.63	2.76	23.03
3	Gorujure danda	12	8	17	9	11.17	3.34	27.85
4	Top	15.26	11	22	11	11.92	3.45	27.85

5.5.4 Observation in length of Inter Node (LL)

Range of variation in mean was observed 4.29 to 6.82 .The highest mean was seen at site 4, followed by site 3 and 2 with values 6.82, 6.2 and 6.0. Where as minimum mean was found in site 1 that was 4.29 (Table 10).

Table 10. Length of Inter Node

S. N	Site	Mean (cm)	Min. (cm)	Max. (cm)	Range	Variance	S.D	C.V %
1	Below sikles	4.29	3.25	6.42	3.17	1.02	1	23.44
2	Way to Ghalekharka	6.03	4.25	8	3.75	1.20	1.09	18.15
3	Gorujure danda	6.26	4.45	8.40	3.95	1.34	1.16	18.52
4	Top	6.82	4.1	7.90	3.80	0.86	0.93	13.64

5.5.5 Observation in Leaf Length (LL)

The mean of LL among population ranged from 3.90 cm to 6.22 cm. Maximum leaf length mean was seen in site 4 followed by site 3. Lowest mean was found in site 1

with range 3.32 to 4.76 cm to 5.69cm . Percentage of C.V for LL was observed highest at site 4 and lowest was observed at site 2 (Table 11).

Table 11. leaf length (LL)

S.N	Site	Mean (cm)	Min. (cm)	Max. (cm)	Range	Variance	S.D	C.V%
1	Below sikles	3.9087	3.20	5.69	2.49	0.52	0.72	18.44
2	Way to Ghalekharka	5.4807	4.23	6.50	2.27	0.44	0.66	12.10
3	Gorujure danda	6.0493	4.51	7.53	3.02	0.66	0.81	13.48
4	Top	6.222	4.50	7.90	3.40	1.58	1.25	20.22

5.5.6 Observation in Leaf With (LW)

Similarly of leaf width highest mean was observed in site 4 , followed by site 3. Lowest mean observed in site 1, 1.65 cm. Variance in leaf width were observed more or less similar in all sites (Table 12).

Table12. Leaf Width (LW)

S.N	Site	Mean (cm)	Min. (cm)	Max. (cm)	Range	Variance	S.D	C.V %
1	Below sikles	1.61	0.95	1.93	0.98	0.051	0.22	13.98
2	Way to Ghalekharka	1.94	1.25	2.96	1.65	0.207	0.45	23.24
3	Gorujure danda	2.16	1.15	3	1.85	0.278	0.52	24.32
4	Top	2.62	1.20	3.51	2.31	0.459	0.67	25.77

5.5.7 Observation in Leaf area

Similarly, of leaf area highest mean was observed in site 4 followed by site 3 and 2. Lowest mean was observed in 1. Likewise highest range, variance and coefficient of variance were found in site 4 and lowest in site 1 (Table 13).

Table13. Leaf area (LA)

S. N	Site	Mean (cm)	Min. (cm)	Max. (cm)	Range	Variance	S.D	C.V%
1	Below sikles	6.38	3.71	10.82	7.11	3.15	1.77	27.79
2	Way to Ghalekharka	10.91	5.49	17.40	11.91	12.87	3.58	32.86
3	Gorujure danda	13.41	5.54	19.50	13.96	20	4.47	33.35
4	Top	16.91	6.94	27.72	20.78	53.42	7.30	43.22

5.6 Statistical analysis between altitude and morphological parameters

The relation between different morphological parameters which are supposed to be linked with altitude are determined by using Pearson Cofficient (Table 13). The test shows that the all morphological parameters except numbers of branches are significant positively correlated with altitude (p=0.01).

Table 14. Pearson's Correlation Coefficient (r) between altitude and morphological parameters

	Altitude	Plant height	No. of branches	No. of nodes	Length of internode	Leaf length	Leaf width	area of leaf
Altitude	1 . 60	. .	.			.		.
Plant height	.630(**) .000 60	1 . 60						
No. of branches	.302(*) .019 60	.763(**) .000 60	1 . 60					
No. of nodes	.354(**) .006 60	.644(**) .000 60	.559(**) .000 60	1 . 60	.			
Length of internode	.635(**) .000 60	.658(**) .000 60	.529(**) .000 60	.539(**) .000 60	1 . 60			
Leaf length	.703(**) .000 60	.773(**) .000 60	.621(**) .000 60	.553(**) .000 60	.789(**) .000 60	1 . 60		.
Leaf width	.502(**) .000 60	.680(**) .000 60	.641(**) .000 60	.585(**) .000 60	.634(**) .000 60	.772(**) .000 60	1 . 60	
area of leaf	.594(**) .000 60	.744(**) .000 60	.671(**) .000 60	.618(**) .000 60	.713(**) .000 60	.906(**) .000 60	.956(**) .000 60	1 . 60

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

5.7 Statistical analysis between soil properties and morphological parameters

The relation between different soil properties with morphological parameters was determined by using Pearson's Correlation Coefficient (Table 15). This test shows that the biomass of *Swertia chirayita* is positively correlated with density, plant height, numbers of branches and leaf area (p=0.01). Similarly, soil properties like organic matter (%) and organic carbon (%) are positively significant with plant height and leaf area (p=0.01). Likewise, plant height is positively with soil nitrogen content (%) and leaf area (i.e r=0.002, p=0.01).

Table 15. Pearson's Correlation Coefficient (r) between soil properties & morphological parameters

	Plant height	No. of branches	area of leaf	Density	Biomass	Organic matter (%)	Organic carbon (%)	Nitrogen content(%)	Phosphorous (Kg/Ha)	Potassium (Kg/Ha)
Plant height	1 60									
No. of branches	.763(**) .000 60	1 . 60								
area of leaf	.747(**) .000 60	.676(**) .000 60	1 . 60							
Density	.183 .161 60	.005 .969 60	.268(*) .038 60	1 . 60						
Biomass	.558(**) .000 60	.433(**) .001 60	.581(**) .000 60	.790(**) .000 60	1 . 60					
Organic matter (%)	.386(**) .002 60	.043 .743 60	.283(*) .029 60	.083 .531 60	.144 .272 60	1 . 60				
Organic carbon (%)	.390(**) .002 60	.042 .748 60	.284(*) .028 60	.079 .549 60	.138 .293 60	.998(**) .000 60	1 . 60			
Nitrogen content(%)	.392(**) .002 60	.022 .867 60	.261(*) .044 60	.026 .846 60	.086 .512 60	.885(**) .000 60	.882(**) .000 60	1 . 60		
Phosphorous (Kg/Ha)	-.113 .388 60	.142 .279 60	-.268(*) .038 60	-.390(**) .002 60	-.354(**) .005 60	-.366(**) .004 60	-.363(**) .004 60	-.283(*) .028 60	1 . 60	
Potassium (Kg/Ha)	.254 .050 60	.027 .838 60	.049 .711 60	-.164 .210 60	-.133 .310 60	.286(*) .027 60	.300(*) .020 60	.277(*) .032 60	-.051 .700 60	1 . 60

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed)

6 DISCUSSION

6.1 Availability and Status of the *Swertia chirayita*

The low density (1.73-2.66 individuals/m²) accompanied by high (>60) frequency of *Swertia chirayita* occurrence in the surveyed population indicates that the species has wide tendency to cover a wide space in the given field, but is unable to cover thick stocking. One potential reason for this could be its extensive harvesting from its wild population. As the entire plant is used for its medicinal properties, individuals are uprooted indiscriminately and, therefore, low density in each population is expected. Furthermore, the removal of the entire plant before seed maturation reduces the possibility of seed development for future regeneration (Sheldon *et al*. 1997). Low numbers of *S. Chirayita* across the populations and localized distribution in specific pockets (habitats) reflects its rare status. This has conservation implications because species with specific habitat requirements have greater possibilities of extinction than species with a broad habitat range (Samant *et al*. 1996b). In addition, a minimum population size is required for the long-term viability of rare and endangered species (Cunningham 2001). Although, It is commonly recognized that phenotypic variation along altitudinal gradients is due to increasingly harsher conditions with altitudinal gain (Körner 2007). Density and frequency had significant positive (p>0.5) relationship with altitude. The density pattern of *S. chirayita* among different altitude zone exhibited a significant (p>0.5) difference in the mean density of the sites. The mean density (2.66 indivifual/m²) at 2670 m altitude zone on south west facing slope (Top) was significantly high compared to all other sites. This suggests favourableness of the altitude (>2500 m) and aspect (south west) under the conifer mixed broad-leaved forests for the species proliferation, which probably offer a preferred microsites for the species. This is in the general agreement that altitude, to a large extent determines the vegetation pattern in the altitude (Airi *et al*2000). As the entire *S. chirayita* plant is used for its medicinal properties , individuals are uprooted indiscriminately and, therefore, low densities in each population are to be expected and the removal of the entire plant before seed maturation reduces the possibility of seed development for future regeneration (Sheldon *et al* . 1997).

The similar result is found on several medicinal plants such as *Huttuynia cordata* (Bhattascharya and Sarma,2008), *Nardostachys jatamansi* (Airi *et al*.,2000). *Swertia aungistifolia* (Bhatta *et al*.2007). In this study, greater densities of *S. Chirayita* at

higher altitudes suggests that it performs better at higher elevations. This result in this study can be concluded in the fact that the actual distribution pattern may be found beyond studied altitude so far the distribution of *S.chirayita* is recorded (1500-3000 m) in literatures, in this study limitation is the incapability to reach above this height due to difficult geographical location. In support of this *S. chirayita* correspond well with geographical distribution of the species heterogeneous with the density attaining a peak near the center of its distribution range and lowering towards the margins.

6.2 Biomass Allocation Pattern

Considering species performance across the populations, population of the higher altitude appears to be the best performed with its high biomass (above ground, belowground and total biomass) and maximum density. In the case of medicinal plants, the time of harvesting is considered critical for the availability of optimum biomass/active constituents. The site facing south west at 2670 m altitude has highest biomass (53.50g/m^2) and the site facing south east at 1950 m altitude has lowest biomass (24.65g/m^2) .

When comparing biomass during different stages, the maximum value of above-ground biomass was observed in the flowering stage. Across sites, a considerable variation in mean biomass of *Swertia chirayita* was found maximum in the site at 2670 m was FL (28.5 g/m^2) at Flowering and Senescense S (25.35 g/m^2). The biomass variation is significant with increase in altitude. No significant variation in total biomass during the FL and S stages of *S. Chirayita* indicates that both stages are equally good for optimum biomass harvest (on a fresh and dry weight basis). Similar result was found in another high altitude medicinal plant *Houttuynia cordata*,which showed the biomass production 214.12gm in flowering stage which was very different from in senescence stage 82.82gm. Similar result was found in another species of *Swertia* (*Swertia aungistifolia)* the result showed no significant difference in biomass in the senescence phase and maturation phase.

For the long-term survival of populations it is recommended that plants are harvested in the senescence stage when most of the reproductive stages are over. However, finer details of the active compound availability in the two growth stages need to be determined using detailed photochemical analyses. The present data on species availability status in different populations will help understand the distribution and

status of *S. chirayita*, which will consequently help in designing conservation strategies for this species. But the biomass variation in this study area may be due to the less human encroachment, since the population occurred in inaccessible area.

Moisture content on the basis of both fresh weight and air dry weight was high in both sites Gorujure danda and at Top. It may be due to the moist shady area under the canopy of mixed deciduous forest. While the lowest moisture was found in the lower site below sikles which may be due to the open area in grassland.

6.3 Soil Paramters

Soil nutrients like organic matters, organic carbon, Nitrogen content and Potassium found higher in site Gorujure danda, Way to Ghalekharka and at the Top of Ghrujure danda But the Phosphorus was found just opposite.

Result of such high soil nutrients like organic matter, organic Carbon, Nitrogen content may be due to high litter accumulation in the deciduous Oak forest in higher elevation. As litter is the nutrient reserve that becomes available for the plant to use as they are decomposed by the microorganism.

Comparatively lower soil nutrients including Organic matters, organic Carbon and Nitrogen content in the lower elevation in the site below Sikles area is supported by the comparatively lesser litter accumulation as it is marginal area of abandoned agricultural land. Among three sites Gorujure danda, Way to Ghalekharka and Top of Gorujure danda soil nutrients llike organic matter, organic Carbon, and Nitrogen content are slightly higher in Top of Gorujure danda due to north-west facing slope of this site possesses more soil moisture than south-east facing slope soil moisture is another factor to decompose the litter accumulation. Aspect of the hill has made the northern part to become more dense with more canopy, providing less exposure to either wind or light which ultimately facilitate to retain more moisture and nutrients in the south part of the site, in contrary to the north has more steep slope and highly exposed to light and high run off due to which the area has become sparsely covered with ground vegetation. All these factors are responsible for less surplus of litter to substrate of the soil. More dense the forest more will be the soil nutrient.

The correlatiion analysis has pointed out that there is a significant correlation between the plant height, leaf area, numbers of branches with Nitrogen, Organic Carbon, Organic Matters and Potassium in the soil.

6.4 Morphological Analysis

We studied various morphological traits like plant height , numbers of branches, numbers of nodes, length of internode, leaf width, leaf length and area of leaf. Height of plant shows significant positive correlation with traits like numbers of branches, leaf length, leaf width and area of leaf. This shows that height of plant has strong correlation with almost all traits of the plant. Bhatta (2000) found Similar result that leaf area positively correlated with all traits of *Euphorbia hirta*. Similarly, numbers of branches shows positively correlation with length of internode, leaf width, leaf length and leaf area. Leaf area shows significant positive correlation with leaf width and leaf length. In the same way leaf area shows significant positive correlation with all most all traits.

Pearson's correlation coefficient (r) between altitude and morphological parameters like plant height , numbers of branches, numbers of nodes, length of internode, leaf width, leaf length and area of leaf has significant positively correlated with altitude . Which is found to be just opposite from normal distribution of high altitude medicinal plant. This may be due to limitation of the study area upto only 2650m altitude.

This inaccessibility of study area above this altitude is due to the difficult geographical location which prevail over the study area. Which concludes that above this altitudinal area population may be decreasing again having maximum population at 2560m altitude K.C (2009). Similar result is found that plant population increasing upto certain height where maximum population was found again beyond that altitude population decreasing. Another reason behind this result may be that in lower altitude there is more collection and illegal treading by local people.

Both phenotypic variation and genetic determination can be important for understanding how plants respond to environmental change. However, This study highlights the role of phenotypic variation and altitude relationships.

Along altitudinal gradients, morphological differentiation in *Swertia chirayita* was detected for all seven traits. These results are consistent with other studies showing morphological differentiation in plant parameters, height and reproductive traits along altitudinal gradients (Heloise Gonaldo-Turnip and Laurent Hazard,2009) . Biomass and density found to be positive correlation with altitude.

7 CONCLUSION AND RECOMMENDATION

7.1 Conclusion

Swertia chiryita is a threatened medicinal plant in the temperate Himalaya. In present study the plant is found to be distributed from 1900m-2700m altitude although its distribution in literature is recorded in 1500-3000 m. The plant grows in the acidic habitat with high organic matters. Mostly its distribution is recorded in the marginal lands in the either in the canopy of mixed broad leaved temperate forest or in the cultivated area. In the studied area density and biomass is found very low, but the frequency is high. This may be due to the rare occurrence of the plant species which again may be due to the over-collection of the plant for various medicinal purpose.

Among the studied sites in the different altitudinal location the highest density, frequency and availability is found in the south facing slope of 267 0m height. This result may be due to the preferable climatic conditions and all soil parameters, favorable microhabitat coexisted in that site, and also can be the reason of less human disturbance in that site. Low plant preference is found in site at the altitude (1950 m) below Sikles, where all the morphological parameters is low. This study concludes the altitudinal occurrence of the plant species in the high altitudes of about 2600 m in the temperate Himalaya, and show the several significant positive correlation with altitude. It conclude that the ecological condition is best suitable for the plant in this area but its population is at highly risk which needs to be conserved in time.

7.2 Recommendation

- Awareness programs on the concervation and importance of natural resource, sustainable utilization, management and proper harvesting of the plant should be enhanced among the local people and tribal communities through common meetings.
- Phenotypically superior plants population from wild population is recommended to be cultivated.
- Domestication and cultivation are widely accepted means for the decrease of harvesting pressure of wild population of MAPs (Chhetri 2005;Rawat *et al*.1992.). So, the cultivation techniques should be promoted.
- The present study also recommends the collection of plant material in the senescence stage to ensure sustainable utilization of this species.

PART - 2

Use of PCR-RFLP tool for the Study of Genetic Polymorphism in Internal Transcribed Spacers sequences of *Swertia chirayita* (Roxb. ex. Flem.) H. Karst Polymorphism of Nepal

1. Introduction

Taxonomy is based upon the similarities and dissimilarities between organisms. Historically, taxonomy is a descriptive science based on the variation and forms of morphological characters The classification scheme of the taxonomist of 1700s and 1800s placed similar appearing organism together in species and comparable species in genera. With the advent of Darwinism in the late 1800s the concept of species relatedness and evolution were incorporated into classification . Today most taxonomic treatments are implicitly phylogenetic. They attempt to recognize and bring together the related groups of plants. For over a century taxonomy has used comparative plant anatomy to aid in classification and several principals concerning the use of anatomical data have been established. Information concerning the chromosomes ie. chromosome number, shape or pairing at meiosis is also used for classification propose (Stebbin,1971). Cytotaxonomy refers to the use of chromosomes numbers and morphology as a data source of classification. In recent times, phytochemical studies have attained a significant role in aiding the solution of systematic problems. There is need for various kinds of classification of phytochemical compounds (Mentzer 1966). His biogenetics classification aims at reflecting the natural relationship among various compounds. After the development of chromatography and electrophoresis, systematist were able to separate more than 300 free amino acids

A numbers of PCR based and DNA sequence based molecular marker systems such as Random Amplified Polymorphic DNA (RAPD), Microsatelite, Amplified Fragment Length Polymorphism (AFLPs), PCR-RFLPs or various nuclear and cpDNA-based sequence can be employed to generate information in genetic diversity, phylogeny, kinship determination and diagnostic development (Graham *et al.*,1994;Golembielwski *et al.*,2007) Molecular data have revolutionized our view of phylogenetic relationship. Early proponents of molecular systematics claimed that molecular data were more likely than morphological data to reflect the true phylogeny because molecular data reflect gene-level change, which were thought to be less subject to convergence and parallelism than were morphological traits (Judd *et al*,1999). A big difference between morphological and nuclear approach is that there are simply many more molecular characters available, and interpretation is generally

easier. As a result, molecular data are now widely used for generating phylogenetic relationship.

A diverse molecular approaches to address various systematic problems are now available to plant systematics. Chloroplast genome was the molecular tool of choice for the decades (Soltis *et., al* 1995). Four main approaches employ the chloroplast to infer relationship: (1) Restriction sites analysis (2) Structural change in the chloroplast genome, including inversion, large deletion and the loss of specific introns and gene (3) comparative DNA sequencing and (4) Polymerase Chain Reaction (PCR) based techniques.The advantage of the chloroplast genome in phylogeny reconstruction include the fact that the chloroplast genome is of small size (typically between 120-200kb) and uniperantally inherited gonome. While the conservative evolution of the chloroplast genome is considered as disadvantage to study closely related species on the populations. Mitodhondrial genomic study either in restriction analysis or in DNA sequencing has been focused on the phylogenetic study of animals. During past few decades use of Chloroplast genome for the study of genetic diversity, phylogeny has been gradually replaced by Nuclear gene sequences.

Swertia chirayita is reported to be distributed in various districts of Eastern, Central and Western of Nepal. There might have been introduced in their localities by humans at various time in the past. Depending upon their spatial distribution in various localities, thus have different genetic make up. Therefore present study is aimed at studying genetic polymorphism in *S. chirayita* population of Nepal using nuclear ribosomal (rDNA), Internal Transcribed Spacer (ITS) Restriction Fragment Length Polymorphism (RFLP) based molecular marker.

1.1 OBJECTIVES

The overall objective of this project is to study DNA sequence polymorphism in the nrDNA ITS sequences of *Swertia chirayita* genotypes collected from various geographical regions of eastern, central and western Nepal using Polymerase Chain Reaction (PCR)-Restriction Fragments Length Polymorphism (RFLP) based molecular marker tool.

Specific objectives of this study are to:

- Collect *S. Chirayita* harbarum and DNA samples from various geographical locoations of Nepal.
- DNA extraction from the collected samples using CTAB method (Graham *et. al*,1994).
- PCR amplification of nuclar ribosomal DNA (nr DNA) Internal Transcribed Spacer (ITS) sequence of various *Swertia chirayita* genotypes found in different districts of Nepal (Kaski, Lalitpur, Sankhuwasabha and Terhathum).
- Study of DNA sequence polymorphism in ITS sequences of *S. chirayita* genotypes using three different Restriction Endonucleases (REs; *Eco*RI, *Bsh*UI and *Xho* I).

2. LITERATURE REVIEW

2.1 Overview of Molecular Markers Used in systemetics/Diagnostics

Molecular markers are specific macromolecule (proteins and nucleic acids) found in the location of the genome and can be efficiently employed to address various problems pertinent to different biological disclipines eg. Kinship analysis, genetic diversity diagnosis, forensics, molecular breeding, phylogeny etc (Lamboy *et. al.,* 1994). Molecular markers provide additional tools for germplasm characterization and assessement of genetic relationships. Molecular markers includes biochemical and DNA based markers. Biochemical markers were introduced in 1960s and involves protein and enzymes electeophoresis.Enzymes are differently charged and can be separated by electeophoresis. DNA markers are generally based either on the Restriction Fragmant Length Polymorphism (RFLP) or on Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Internal Trinscriber Spacer (ITS) region and Simple Sequence Repeats (SSR) Microsatillites (Soltis *et. al., 1*995).

Molecular techniques can be broadly classified into protein based and enzyme based techniques.

2.1.1 Protein-Based Molecular Techniques

This technique unvolves the method which depends on the distinguishing the variability using different proteins and enzymes. Most widely used techniques used are, Isozyme analysis, Sodium Dodecyl Sulphate- Polyacryamide Gel Electrophoresis (SDS-PAGE), Enzyme Linked Immunosorbent Assey (ELISA), Western Blotting etc.

2.1.1.1 Isozymes/Allozymes

Isozymes (Isoenzymes) are those enzymes that convert the same chemical substances but are not necessarily products of the same gene (Weising *et al.,* 1995). Isozymes vary in their amino acid sequences. Due to this sequence dissimilarity the net charge carried by amino acid also differs. And this can be observed clearly in gel electrophoresis (Spooner et al.,1995). Isozymes have been proven useful for varietal identification (Ashari *et. al.*, 2005). Allozymes are isozymes which are produced by the orthologous genes but which differ in composition by one or more amino acid

due to allelic differences (Wesing et al.,1005). Allozymes are allelic variation of enzymes encoded by structural genes (Spooner *et. al*.,2005).

Usually two, or sometimes even more loci can be distinguished for an enzymes and these are termed as iso-loci. Therefore, there are three advantages of the allozymes studies, the cost is low and many individuals can be scored for the several allozymes loci within short time span (Kephart 1990). Three, allozymes markers are codominant so homogygous can be discriminated from heterogygous (Weising *et al*.,1995).

However there are also limitations of allozymes studies. The main limitation is the low level of polymorphism which can occur when the proteins might have the same electrophoretic mobility.(Berry and Kreightman.,1993; Hudson *et al* 1994;Kreiger and Ross 2002). Another problem is that many plant species are polyploids, and analysis of allozymes patterns of polyploids can be extremely difficult (Weising *et al.*,1995). In some cases it has also been shown that allozymes differ in one or more physiological respects and therefore, may be evolutionarily neutral (Brown *et al.*,1976)

Allozyme studies have been applied in various fields. They have been applied in population genetic studies, including measurement of outcrossing rate (Erskine and Muehlenbauer.,1991), population structure and population diversity studies (Lamboy *et al*., 1994), interspecific relationship (Garvin and Weeden,1994) and identification of parents in hybrids (Parani *et al* 19997) etc.

2.1.1.2 Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (**ELISA**), also known as an **enzyme immunoassay** (**EIA**), is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. The ELISA has been used as a diagnostic tool in medicine and plant pathology, as well as a quality control check in various industries. In simple terms, in ELISA, an unknown amount of antigen is affixed to a surface, and then a specific antibody is applied over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, and in the final step a substance is added that the enzyme can convert to some detectable signal. This is a serology assay in which antibodies used to detect a particular substance is labeled by the linkage to the enzyme. The test substance is immobilized on a plastic surface and a positive reaction i.e. antibody binding to the surface is detected by the action of

enzyme on a colorless substrate to produce a colored product (Lawrence, 1996). This technique is used as diagnostic tool for the plant and animals diseases and in food industries to detect the harmful bacteria. Comparison between tolerant and non-tolerant wheat cultivar in the Barly Yellow Dwarf virus contents has also been performed (Herrera, 1985). In Nepal, this technique has been used for the detection of plant pathogen like citrus tristeza virus (CTV) in the plant (*Ranjit et al*.,1999).

2.1.1.3 Western blotting(WB)

The **Western blot** (alternatively, **protein immunoblot**) is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract from Polyacrylamide Gel Electrophoresis (PAGE). It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (native/ non-denaturing conditions). The proteins are then transferred to a membrane typically nitrocellulose or PVDF (Polyvinyliden Floride), where they are probed (detected) using antibodies specific to the target protein (Towbin *et.al* ,1979). The name Western blot was given to the technique by W. Neal Burnette (April 1981).

There are different detection methods for proteins besides the use of colored reaction by antibody. In colored reaction, a secondary antibody conjugated to the enzyme that can catalyze the color reaction is bound to primary antibody that binds to specific protein. The other detection methods includes using radioactive compound attached to the secondary antibody(Net visit). Another method of secondary antibody detection utilizes a near- infrared(NIR) fluorophore- linked antibody. Light produced from the excitation of a florescent dye is static, making fluorescet detection more precise and accurate measure of the differences in signal produced by labeled antibodies bound on a west blot (Ambroz,2006) West blotting has wider application in medicinal field. They include testing HIV, Lyme disease, Hetapitis B infection, Bovine Spongiform Encephalopathy, etc (Internet visit 2)

2.1.2 Nucleic Acid (DNA/RNA) Based Molecular Techniques

Nucleic acid (DNA/RNA) based molecular technique can be carried out either by hybridization-based(RFLPs/Southern blotting/Northern blotting/) or Polymerase Chain Reaction (PCR) based. Hybridization based molecular technique includes

restriction fragment length polymorphism (RFLP) Southern blotting (SB) Northen blotting (NB), Variable Number Tandom Repeats (VNTR) etc.

2.1.2.1 Hybridization Based Technique

Hybridizatioin-based method includes restriction fragment length polymorphism (RFLP) and variable tandem repeats (VNTP). Labeled probes such as random genetic clone, cDNA clones,probes for microsatelite and minisatellite sequence are hybridizes to filters containing DNA, which has been digested with enzymes. Polymorphisms are detected by presence or absence of bands upon hybridization.

2.1.2.1.1 Northern Blotting

The northern blot technique was developed in 1977 by James Alwine, David Kemp, and George Stark at Stanford University (Alwine *et al.*,1977). In this technique , RNA sample are separated according to size by gel electrophoresis and detected with a hybridization probe complementary to the part of or the entire target sequence. The term northen blot actually refers specifically to the capillary transfer of RNA from the electrophoresis gel to the blotting membranes, however the entire process is commonly referred to as Northern Blotting (NB).

The technique is used to study gene expression by detection of RNA (or isolated m RNA) in a sample. One of the application of this technique is to observe a particular gene' s expression pattern between tissue, organs, developmental stages, environmental stress levels, pathogen infection, and over the course of treatment (Mori *et al.*,1991;Liang *et al.*,1999,Baldwin *et al.*,1999).Upregulated and down regulated genes can be analyzed by observing over the RNA expression and the RNA can be separated to know whether it is the transcribed product of novel gene or not (Utans *et al.*,1994). The variation in size of gene product (using single probe) indicates deletion or errors in transcript processing (Internet visit 4).

2.1.2.1.2 Southern Blot

The technique southern blot was developed by British biologist, Edwin Southern. In this technique, DNA samples are separated according to size through gel electrophoresis and then transferred to the filter membrane subsequently allowed to hybridize with labeled probe (Southern,1975). Radiolabeled oligonucleotide probe are found very advantageous for in –gel hybridization (Thien and Wallace,1986) it is also

claims that gel hybridization is also convenient with non- radiolabled probe (Yavachev,1991;Pena *et al*.,1991).

Southern blotting has major application in the position of a cloned gene and is often used in gene cloning. Southern blots of cloned genomic DNA fragmentation can be probed with complentary (cDNA) molecule to find which parts of the genomic clone correspond to the cDNA fragment(Brown,2001). Southern blot along with RFLP can be used for identification of particular stain of organism. Southern blots from different organism can show how conserved a gene is between species (Turner, 2001). This method has been adapted for the differentiation of bacterial strains on the basis of observation that the locations of various restriction enzymes recognition sites within a particular genetic locus of interest can be polymorphic from strain, resulting in gel bands that differ in size between unlike strains (Olive and Bean 1999).

2.1.2.1.3 Restriction Fragment length Polymorphism(RFLP)

Restriction Fragment length Polymorphism(RFLP) was the first technology that enabled the detection of polymorphism at the DNA sequence level (Chawala,2003). In RFLP DNA is digested with restriction enzymes, which cuts the DNA at specific fragments within the range of 2-10 kb due to the presence of different restriction sites called restriction Fragment Length Polymorphism(RFLP). The RFLPs are simply inherited naturally occurring Mandelian characters and are codominant in nature. The polymorphism occur due to evolutionary process, point mutations within restriction sites, insertion or deletion within fragments are unequal crossing over (Weising,1995).

In RFLP analysis, restriction enzyme-digested genomic DNA is resolved by gel electrophoresis and then blotted onto nitro-celluleose membreane . Then it is visualized by labeled probe (single locus) obtained from genomic library or cDNA library (Joshi *et al*.,1999). RFLP markers were used for the first time in the construction of genetic map of what by Botstein *et al*.,1998. They are vary reliable markers in linkage analysis and breeding and can easily determine if a linked trait is present in a homozygous or heterozygous state in a individuals , information highly desirable in recessive traits (Winter and Kahl, 1995). However this marker type has inability to detect single base change that restricts their use in detecting point mutations. RFLPs, have also been used to investigate relationships of closely related

taxa (Miller and Tanksley, 1990; Lanner *et al*.,1997), as fingerprinting tools (Fang *et al*.,1997) and for diversity studies (Debreuil *et al*.,1998)

2.1.2.2 PCR- based Techniques

The discovery of Polymerase Chain Reaction(PCR) by Kary Mullis and co-workers has revolutionized the field of molecular biology. PCR is a versatile technique, based on enzymatic amplification of DNA, *in vitro*. It is an extremely powerful technique that allows to make millions of copies of a selected DNA sequence and has become an indispensable tool of molecular biology. In the PCR technique. DNA is amplified *in vitro* by a series of enzymetic polymerization cycles consisting of three temperature dependent steps: DNA denaturation, primer template annealing and DNA synthesis by a thermostable DNA polymerase (Rychlik *et al*.,1990).

2.1.2.2.1 Random PCR based Techniques

2.1.2.2.1.1 Random Amplified Polymorphic DNA (RAPD)

Random Amplified Polymorphic DNA(RAPD) is an arbitrarily-primed PCR developed by Williams and his co-workers (Williams *et al*.,1990). Primer used in PCR is generally10bp in case of RAPD and to 20-34bp in case of AP-PCR (Welsh and McClelland,1990). Being a fast and simple method, requiring low quantities of templet DNA, this is efficiently applied to identify useful polymorphism (Waugh and Powell,1992). In RAPD no prior sequence information of organism is required for primer designing. This procedure detects nucleotide sequence polymorphism using a single primer of arbitrary nucleotide sequences. These oligonucleotide serves as both forward and reverse primer, and are able to amplify fragments from 1-10 genomic sites simultaneously (Spooner *et al*.,2005) RAPD is a powerful and popular technique for the investigation of genetic variation (Williams *et al*.,1990 and Welsh and McClelland 1990). RAPD has been extensively used for plant species and varietal identification (Graham *et al*.,1994; Golembielwski *et al*.,2007). RAPD is a powerful multilocus technique for the detection of polymorphism in organism.. A RAPD-PCR based technique was used to create a series of genetic markers that distinguished/identify the five major weed species (Sporobolus pyramidalis, S.fertilis, S.fricans and S.jacqeumontii) found in Sporobolus species (Shrestha *et al*.,2005)

RAPD procedure works with anonymous genome, required only small amount of DNA markers and is simple, less costly and less labor intensive than other DNA

markers methodology (Caetano-Anolles *et al*.,1991a, b, Hadrys *et al.*,1992). The main drawbacks of RAPD is their low reproducibility and high sensitivity to reaction and cycling parameters (Schierwater and Ender 1993).Other drawback is that it is difficultly in scoring bands, which leads to inappropriate inferences (Joshi *et al.*,1999).

2.1.2.2.1.2 Arbitrarily Primed PCR (AP-PCR) and DNA Amplification Fingerprinting (DAF)

Arbitrarily Primed PCR (AP-PCR) is similar to RAPD technique in which discrete amplification pattern are generated by employing single primers of 10-50 base in length in PCR of genomic DNA(Welsh, J. And McClland, 1991). In the first two cycle, annealing is under non-stringent conditions. Compared DNA Amplified Fingerprinting (DAF), this variant of arbitrarily primed PCR is not vary popular as it involves autoradiography. However, it has been simplified by separating the fragments on agarose gels and using ethidium staining for visualization (Joshi *et al*.,1999). However , for DNA Amplification Fingerprinting (DAF), are greater than 4 nucleotides in length, may be single or multiple primers are used, separation is by polyacrylamide gel electrophoresis and detection by silver staining (Cateno-Anolles *et al*.,1991),in contrast to agarose gel electrophoresis and ethidium bromide staining in RAPD. Arbitrarily Primed PCR (AP-PCR) technique has been applied in parentage determination in maize hybrids using the arbitrarily primed polymerase chain reaction (Welsh *et al.*,1991), to stain identification and genetic mapping in mouse (McClelland) and genomic fingerprinting Elsh and McClland M, 1991).

2.1.2.2.1.3 Amplified Fragment Length Polymorphism (AFLP)

This technique was developed by Zabeau *et al*.,known as AFLP, is based on the detection of genomic restriction fragments by PCR amplification and can be used for DNAs of any origin or complexity(Zabeu *et al*.,1993). The technique involves three steps: (i) Restriction digestion of the DNA and ligation of oligonudleotide adapters, (ii) selective amplification of sets of restriction tragments, and (iii) gel analysis of the amplified fragments (Pieter *et al*.,199) . In this technique, first a genetic primer is used during PCRA to obtain DNA fragments. Then the numbers of fragments can be turned by the use of specific primer during the next PCR cycle (Joshi *et al*.,1999). This technique thus shows an indgenious combination of RFLP and PCR technique and is

extremely useful in detection of polymorphism between closely related genotype (Saiki *et al*.,1988).

There are various applications of this technique. AFLP analysis depicts unique fingerprints regardless of the origin and complexity of the genome. Most AFLP fragments corresponds to unique position on the genome and hence ca be used in genetic and physical mapping (Vos et al.,1999). AFLPs are extremely useful tools for DNA fingerprinting (Hongtrakul *et al*.,1997) and also for cloning and mapping of variety-specific genomic DNA sequence(Yong *et al*.,1996; Paglia *et al*.,1998). Similarly to RAPDs, the bands of interest obtained by AFLP can be converted into SCARs (Sequence Characterized Amplified Region).Thus AFLP can provide a newly developed, important tool for a variety of applications.

2.1.2.2.2 Specific PCR Based Techniques

2.1.2.2.2.1 Simple Sequence Repeats(SSRs)/Microsatellite

Simple Sequence Repeat (SSR) is a specifically primed PCR-based assay. Simple Sequence Repeats(SSRs) is also known as Microsatellite (Jacob *et al*.,19910,are polymorphic loci present in organnellar DNA that consist of repeating unit of 1-6 base pairs in length. They are widely dispersed throughout eukaryote genome and are often highly polymorphic due to variation I the number of repeats units(Karp *et al*.,1998). They are typically neutral, co-dominant and are used as molecular markers which have wide range applications in the field of genetics. Microsatellite can be amplified for identification by PCR, using the unique sequence of flanking region as primer (Beyemann *et al.*,1992).

One of the main drawbacks of microsatellites is that high development costs are involved if adequate primer sequence for the species of interest are unavailable, making them difficult to apply to unstudied groups. Although, microsatellite is a principal co-dominant marker, mutation in the primer annealing sites may result in the occurrence of null alleles (no amplification of the intended PCR products), which may lead to errors in genotype scoring (internet visit 6)

2.1.2.2.2.2 Inter Simple Sequence Repeats (ISSR)

ISSRs are DNA fragments of about 100-300bp located adjacent, oppositely oriented microsatellite regions. It is arbitrarily primed PCR technique. The complementary sequence to two neighbouring microsatellite are used as PCR primers (Spooner *et al.*,2005). In this technique, promers based on the microsatellites are utilized to amplify inter- SSR DNA sequence (Zietkiewicz *et al.*,1994). Here, various microsatellites anchored at the 3 prime end are used for amplifying genomic DNA which increase their specificity. An unlimited numbers of primers can be synthesized for various combination of di-,tri-,tetra- and penta-nucleotides etc. With an anchor made up of a few bases can be exploited for a broad range of applications in plant species(Joshi *et al.*, 1999). The main advantage of this technique is that sequence data is not needed for primer construction and low amount of sample DNA is sufficient as this process involves PCR method. However, like RAPD, ISSR also has reproducibility problems(Spooner *et al.*,2005).

This technique has been applied in studies involving genetic identity, clone and strain identification, taxonomic studies of closely related species. They are also useful in gene mapping studies (Zietkiewicz *et al.*,1004).

2.1.2.2.2.3 PCR-RFLP

This is a technique in which PCR is performed first followed by restriction analysis of the PCR amplified bands. Simply restriction analysis is performed after the amplification of the DNA fragments. Restriction analysis involves the treatment of the amplified DNA bands with restriction enzymes and then running under the gel. The obtained DNA bands pattern is called PCR-RFLP pattern. The restriction analysis has various application like species identification , phylogenetic tree construction, etc.

To overcome the cumbersome morphological identification of the species, a PCR-RFLP test can be performed (Lempereur *et al.*,2010,Shretha *et al.*,2010) . PCR-RFLP is also used for the phylogenetic analysis of the plants species by using chloroplast (cp)-DNA, mitochondrial DNA and nuclear DNA sequence. Each fragment was scored as present(1) or absent (0) and then similarity matrix was estimated by pairwise comparison based on the percentage of common fragments. Distance values were calculated and cluster analysis based on distance matrix was performed by applying (UPGMA) (Isshiki *et al* 2004)

3. MATERIALS AND METHOD

3.1 STUDY AREA

Swertia chirayita collected from four districts namely (eastern- Shankhuwasaba, Therathum), western (Kaski), and central (Lalitpur) in Nepal were selected for the study (fig 3.1), Among these four districts, the field survey was carried out in Parche VDC Sikles of Kaski district. The field visit was made during August 2009 for 7 days and required herbarium and DNA samples were collected. For the DNA sample young apical leaves were collected and put inside the small pouches of lens paper, then stored in silica gel which was then prevented from air contact. The samples thus collected were brought to the NAST laboratory for subsequent DNA extraction and PCR study.

Fig 3.1 Nepal map showing study sites

3.2 Plant Materials

- Altogether 24 collected DNA samples of *S. chirayita* and one sample of *S. dilatata* were used in the present study (Table 1)

Table 1. Details of *S. chirayita* and *S. dilatata* collections

S.N	Plant Species	Sample ID	District/Locality/	W/C/E Nepal	Number of Samples Collected	Altitude(m)
1	*S.chirayita*	Lalitpur 1-7	Lalitpur/ Phulchowki, Godawari/	C	7	2150
2	*S.chirayita*	Terathum 3-10,	Terathum/ Tirikhimti to Guphapokhari/	E	8	1500-2800
3	*S.chirayita*	San 1,San 2,San 4,San 5,San 8	Sankhuwasabha/ Lampokhari, Shreemane, Manlabre and Chauki	E	5	2600-2950
4	*S.chirayita*	Kaski 501,kaski 505, Kaski 509	Kaski /Sikles	W	3	2000-2500
5	*S. dilatata*	Kaski 517	Kaski/Sikles	W	1	2000-2500
				Total	24	

3.3 Gel Electrophoresis Reagent

3.3.1 (10x) Stock buffer(Tris,Boric acid and EDTA)

Tris base(108g;Merck Co.Ltd;India) and boric acid (55g) were placed into a Schott bottle (1L), which into a separate Schott bottle, sodium EDTA (18.6g) was dissolved in double distilled water (70ml). To this solution, 5 ml of NaOH (10M) was added, the pH adjusted to 8.0 and the final volume made up to 100ml. Then 40ml of EDTA solution was added to the tris base and the boric acid contained in the previous Schott bottle and final volume was made up to 1L. This Tris, boric acid and EDTA (TBE) stock (10x) was diluted(to 1x) with double distilled water prior to being used for gel running.

3.3.2 (5x) Gel loadingbuffer (GLB)

Sucrose (2.5g) was dissolved in double distill water (7ml) in which bromophenol blue (25mg) was added and the final volume was made up to 10ml. This gel loading buffer (GLB) was added to the sample in proportion as 1(GLB) to 4(PCR product) and 1(GLB) to 1(DNA sample) by volume, during electrophoresis.

3.3.3 Agarose gel (1.5%)

Agarose gel (1.5%) was dissolved in TBE buffer (100ml,1x) in the microwave. It was then cooled to approximately 55^0C and poured onto the gel casting tray and appropriate comb was (6 to 17 toothed) fixed in place.

3.4 General use reagents and extraction buffers

3.4.1 EDTA (0.5M, pH 8.0)

Disodium ethylene-diamine-tetra-acetate.$2H_2O$ (EDTA,93.05) was added to a schott bottle containing double distilled water (400ml), mixed on a magnetic stirrer and the pH was adjusted to 8.0 by adding NaOH pellets (approximately. 10g). The volume was adjusted up to 500ml with double distilled water, autoclaved and stored at room temperature until needed.

3.4.2.DNA Extraction Buffer

Grahams CTAB extraction buffer (2% CTAB, 1.4M NaCl, 0.1 M EDTA, 0.1M Tris HCl pH 8.0)

Tris HCl (100ml; pH 8.0) was placed in 1L Schott bottle and EDTA (37.2g/L),NaCl (81.8g) and CTAB (20g) was mixed and the final volume was made up to 1L.

3.4.3 TE buffer (TRIS-EDTA;10mM Tris HCl, 1mM EDTA)

0.5 M EDTA (1mL;Ph 8.0) was added to a Schott bottle containing Tris HCl(0.78g) and final volume made up to 500ml. This was autoclaved and stored at room temperature.

3.4.4 Primer, dNTPs, Enzymes, Buffer, $MgCl_2$ and Tempelate DNA

Two types of primers were used; $ITSN_1$ and $ITSC_2$(forward and reverse primers separated by slash). Primers are available in lyophilized form and must be diluted to required concentration prior to use primer.

Deoxyribonucleoside triphosphate (dNTPs) include dATPs, dGTPs, dTTPs and dCTPs. Commercially available dNTPs (10Mm;MBI Fermentas, Lithuania) was used and store at 20^0C. Normally during PCR the combined volume of dNTPs of 0.5 µL was taken for a single reaction mixture (1x). Tag. Polymerase enzyme (0.3µL for 1x) was taken for polymerization of sample DNA . Buffer of 2.5µL for a single reaction mixture was taken.2.5µL (1x) of $MgCl_2$ is taken. Sample DNA/Template DNA of 0.3µL for 1x was taken. DNA dilution to required concentration (50ng) was carried out by estimating the concentration via Biophotometer.

3.5 METHODS

3.5.1 DNA Extraction by CTAB Method (Graham *et al.*, 1994)

Approximately 20 mg of freshly harvested young leaf samples preserved in silica gel was ground in sterilized mortar and pestle in presence of liquid nitrogen. The samples were treated with 1000µL CTAB buffer transfered to centrifuge tubes (1.5 mL) at 55^0C incubated for 15 minutes. Then the tube were centrifused at 11000rpm for 5 minutes. The supernatant was transferred to a clean sterile centrifuge tube and treated with chloroform: isoamylealchole (24:1). The solution was mixed gently by inversion. Tube were then centrifuge at 11000 rpm for 1 min at 25^0C. The upper aqueous phase centrifugation was transferred to new tube and extracted with equal amount of chloroform: isomylalcohol (24:1). The aqueous layer were transferred to new tube and treated with $1/10^{th}$ (approx 50µL) Ammonium acetate (7.5M), followed by addition of 500µL absolute ice-cold 70% ethanol (-20^0C). The tubes were placed overnight at -20 ^{0}C to allow precipitation of DNA. The tube were centrifuged at 11,000 rpm for 10-15 minutes at 4^0 C, after overnight precipitation. The supernatant were discarded and the pellet washed twice with 500µL ice-cold ethanol (-20^0C). In order to get rid of unwanted salts, pellets were dried in air for 5 minutes after centrifugation at 11000 rpm for 1 minute. Finally pellets were suspended in 1x TE and stored at -20^0C.

3.5.2 DNA quantification

DNA is quantified by using UV Biophotometer (Eppondorf-AG 22331, Germany). A cuvette was taken and added with 100µL of sterile distilled water as blank to calibrate the machine at zero DNA concentration. Then, a new cuevate was taken and added with 90 µL of water and 10 µL of sample DNA. This was then placed inside the the machine and the concentration of DNA (ng/µL), absorbance and the ratio of absorbance can be directly observed on the display of the screen. The purity was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm and the value should be between 1.8 to 1.85. The purity was also observed at 230 nm and 260 nm as the proteins are strongly absorbed at 230 nm due to presenc of peptide bonds and the value should be between 2.2 to 2.25. DNA concentration and purity was also determined by runnig thr sample at 1.5% agarose gel based on the intensity of bands when compared with Lamda DNA markers. DNA quantification of four different districts (Ilam, Terhathum, Sankhuwasabha and Kaski) was done before running ITS-

PCR. DNA quantification is performed before PCR so that later the quantified DNA can be diluted to get the exact concentration that is required for the master mix preparation later in the PCR.

3.5.3 ITS-PCR Amplification

Different sample were amplified by ITS-PCR. PCR amplification of the ITS1and ITS2 region of the nuclear rDNA was performed with the ITSN1 (5'-CGCGAGAAGTTCATTGAACC-3') and $ITSC_2$

(5'TGGTTAGTTTCTTTTCCTCCG-3') (Shrestha *etal*.,2002) or ITSA(5' GGAAGTAAAAGTCGTAACAAGG-3') and ITSB

(5'-TCCTCCGCTTATTGATATGC-3') (White et al.,1990). A reaction volume of 25 µL contained 50ng of template DNA, Tag. Polymerage (1.5units), 200 µM each of dATPs, dGTPs, dCTPs, dTTPs, 2.0 µL of PCR buffer (1x; 75Mm, Tris- HCL, 1.5Mm $MgCl_2$, 50mM KCl, 20Mm $(NH4)_2SO4$,pH8.7) (MBI Fermentas Co) and primers (0.8) µM of either ITS1 and ITSC2 or ITSA and ITSB. The final concentration of $MgCl_2$ was maintained at 2.5Mm in each tube. The PCR amplification of ITS region (ITS1,5.8S gene and ITS2) was carried out in a thermal cycle with PCR program (denaturation programmed as 3 min at 94°C, annealing programmed as 1 min at 50°C and extension programmed as 2 min at 72°C) (Eppondeof, Germany). This single initial cycle was repeated by 39 cycles of 30s at 94°C, 30s at 50°C and 75s at 72°C . A final extension cycle consisted of 5 min at 72 °C and 2 min at 25°C.

3.5.4 Gel Electrophoresis

This method was used to analyze PCR amplified and restriction digested DNA. The DNA bands were visualized on the agarose gel. Electrophoresis was performed in agarose gel of 1.5% in TBE buffer (1x) 50V (8.47V/cm) for half an hour and 25V (4.2V/cm) for one and half hour respectively using EMBI TEC (Santiago, CA), gel tank. Total volume volume loaded in the well was 15µL(12µL PCR product+3µL GLB for PCR product and 10µL marker and 5µL TBE buffer for DNA ladder). After running the gel, it was stained in TBE buffer containing 35µL Ethidium bromide (10mg/ml) for 45 minutes and then de-stained for 15 minutes in water. The gels were then visualized in UV trans-illuminator (UVITE, Japan) and photographed using a digital camera.

3.5.5 Restriction Analysis of ITS-PCR Products

ITS-PCR products amplified under standard condition before treated with restriction analysis. 8 µL of PCR amplified products were subjeced to PCR- RFLP analysis using three different restriction was carried out in 28 µL containing 8 µL of PCR product 2 µL of RE buffer and 16 µL of nuclear free water.The reaction mixture were prepares in ice and after adding all reagents, PCR tubes were incubeated at 37° for 2 hours.

3.5.6 ITS sequencing

Amplified ITS PCR products of Kaski samples of s. Chirayita were sent to Macrogen, Korea for double stranded sequencing. Following sequencing, sequence data were retrieved from Macrogen website and sequence analyses were performed using 'Codon Code Aligner' and 'Bioedit' softwares prior to submission to NCBi genbank.

4 RESULTS

4.1 PCR Amplification

All the samples under study produced a single PCR fragment representing ITS region of nuclear ribosomal DNA. This includes ITS1, 5.8s gene and the ITS2 sequence of the rDNA repeat unit. Amplified fragment of ITS region of all *S. chirayita* genotypes under study was approximately 750 bp long (Plate 1 and 2).

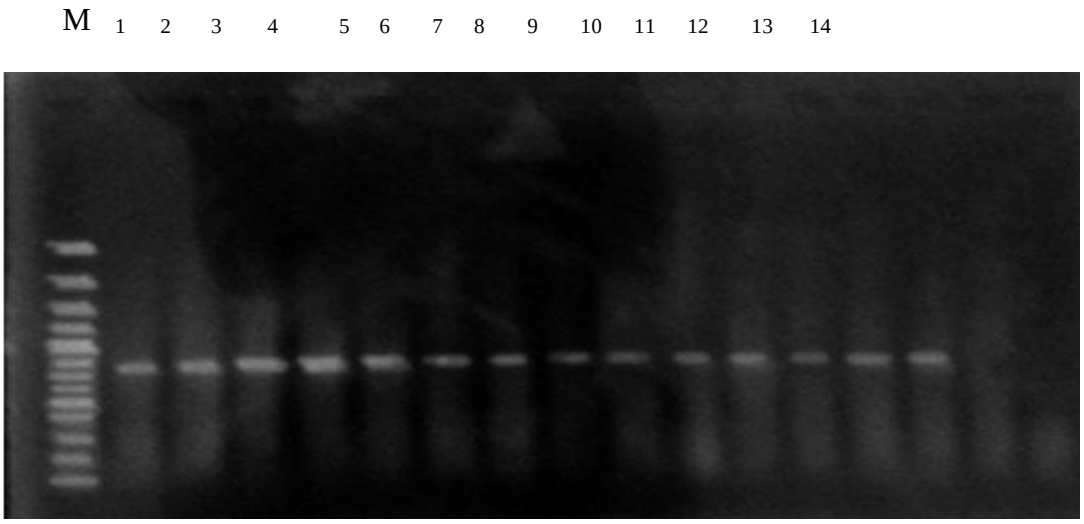


Plate 1. Gel pictures showing PCR amplified DNA fragment of ITS region; lane marked M is 100 bp ladder molecular weight marker ; Lanes 1- 5 represents *S. chirayita* samples from Shankhuwasabha; Lanes 6-13 represents samples from Terhathum; Lane14-*Swertia dilatata* sample.

M 1 2 3 4 5 6 7 8 9 10

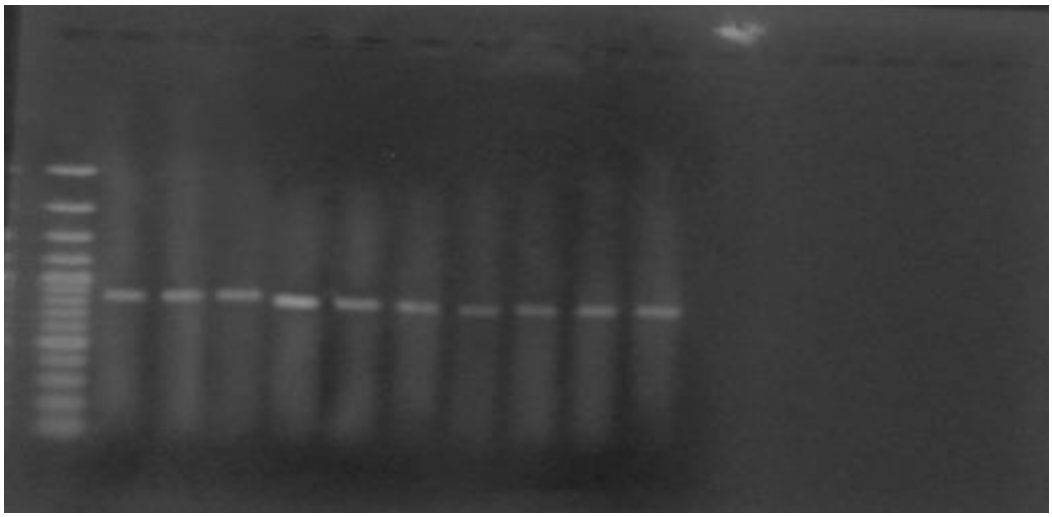
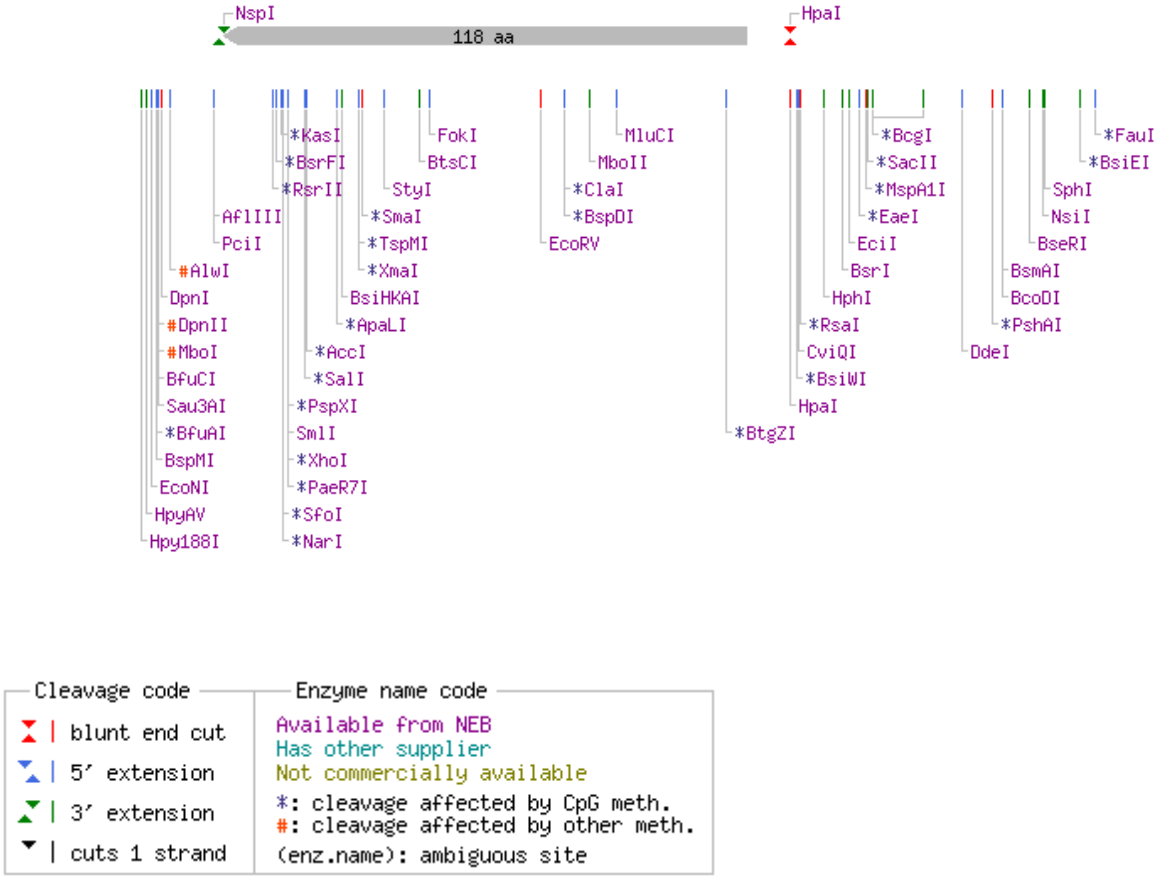


Plate 2. Gel pictures showing PCR amplified DNA fragment of ITS region; lane marked M is 100 bp ladder molecular weight marker ; Lanes 1-7 represents samples from Lalitpur, Lanes 8-10 represents samples from Kaski district.

4.2 Bioinformatics Study

Restriction analysis was performed by selecting three enzymes *Bst*UI , *Xho*I *and Eco*RI. Restriction sites of different restriction enzymes were observed with help of bioinformatic study. Firstly internal transcribed spacer 1, partial sequence;5.8S ribosomal RNA gene, complete sequence;and internal transceibed spacer 2, partial sequence was draw out from gene bank then submitted into neb cutter programme as shown below.

Linear Sequence: ***unnamed sequence***



4.3 Restriction Analysis

With *Xho*I, single cut in the ITS sequences of all the *S. chirayita* genotypes resulted into two fragments in the agarose gels. In contrast, ITS sequence of *S. dilatata* remained intact with this enzyme. Enzyme *Xho*I site is C↓TCGAG and recognizes TCGA palindromes and produces TCGA overhang at 5’ end upon digestion.

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14

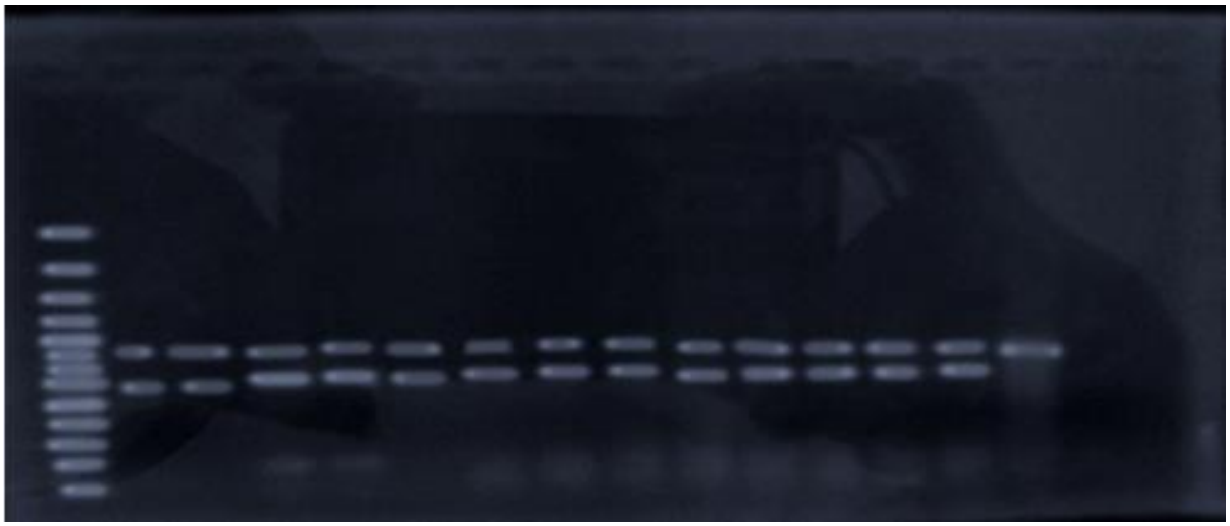


Plate 3. Restriction analysis of ITS sequence of *S. chirayita* with RE *Xho*I. Lane marked M, 100 bp ladder molecular weight marker; lanes marked 1-5, restriction digested ITS sequences with samples from Sankhuwasabha; Lanes marked 6-13, represent samples from Terhathum; Lane14, *Swertia dilatata* sample.

M 1 2 3 4 5 6 7 8 9 10

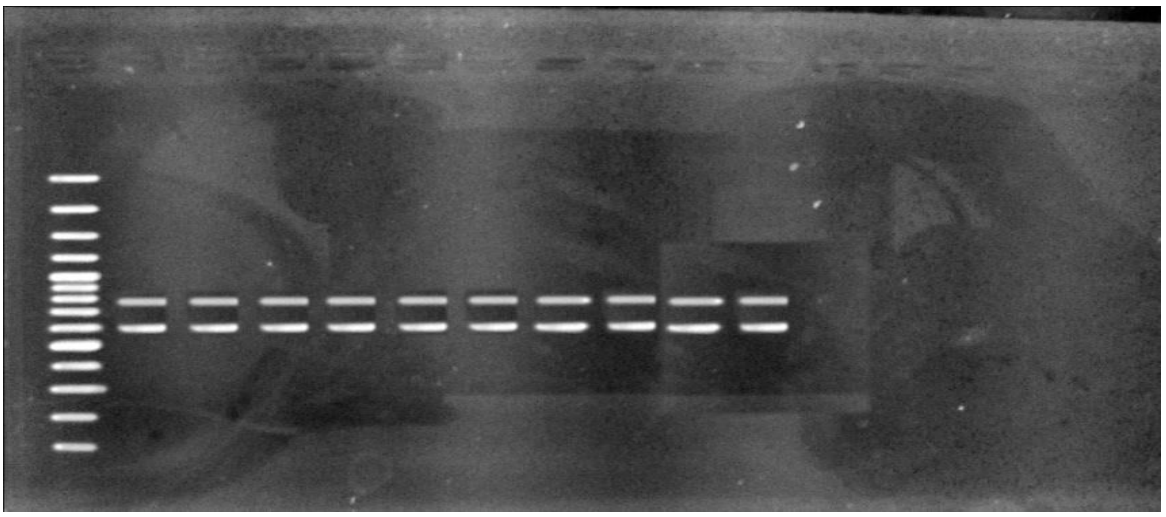


Plate 4. Restriction analysis of ITS sequence of *S. chirayita* with RE *Xho*I. Lane marked M, 100 bp ladder molecular weight marker; lanes marked 1-7, restriction digested ITS sequences of samples from Lalitpur; Lanes marked 8-10 represent samples from Kaski.

Where as with *Bst*UI, all genotypes (except three genotypes from Sankhuwasabha) of *S. chirayita* were restricted into two fragments indicating single site in the ITS sequence. Three samples from Sankhuwasabha district remained intact with this

enzyme upon digestion. It was also revealed that *S. dilatata* genotype from Kaski had multiple sites for this enzyme as multiple bands were seen in the gel picture. The recognition site for this enzyme is CG↓CG (Gel pictures not shown).

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14

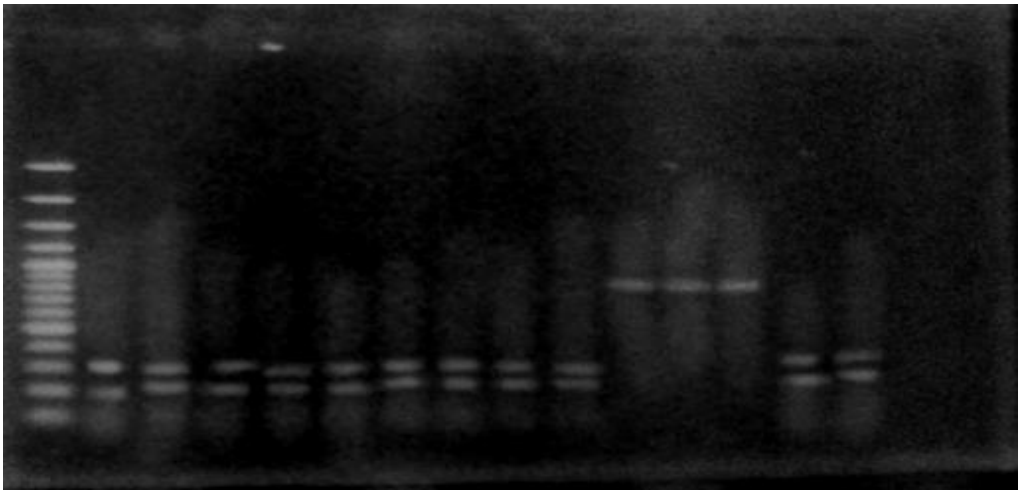


Plate 5. Restriction analysis of ITS sequence of *S. chirayita* with RE *Bst*UI. Lane marked M, 100 bp ladder molecular weight marker; lanes marked 1-6, restriction digested ITS sequences with samples from Lalitpur; Lanes marked 7-9 represent samples from Kaski; Lanes10-14 represent samples from Sankhuwasabha.

M 1 2 3 4 5 6

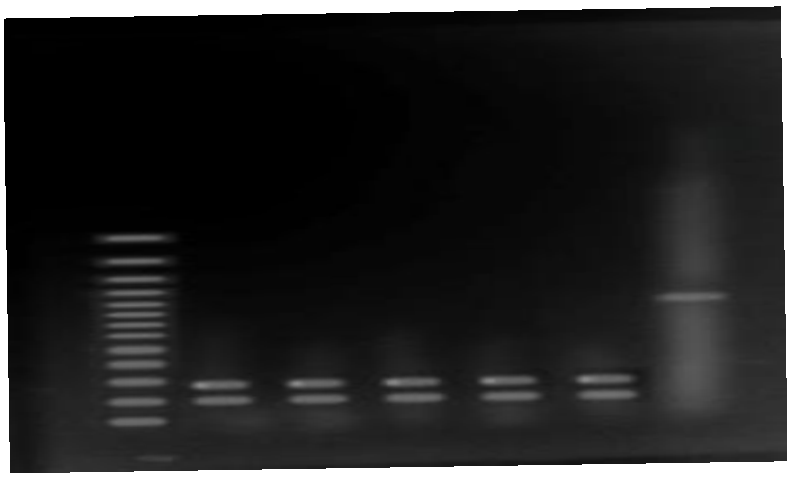


Plate 6. Restriction analysis of ITS sequence of *S. chirayita* with REs *Bst*UI. Lane marked M, 100 bp ladder molecular weight marker; lanes marked 1-5, restriction digested ITS sequences with samples from Terathum ; Lane 6 represents samples of *S. dilatata.*

Of the three REs used in the study, enzyme *Eco*RI was revealed to be a non-cutter, as ITS fragments of all the samples remained intact following restriction digestion with the enzyme. The enzyme *Eco*RI restriction site is G↓AATTC, which recognizes AATT palindromic sequences.

4.4 ITS sequencing

Sequences of two samples from Kaski have already been submitted to NCBI genbank. Details of the sequences are as follows:

Accn. No.K505

1 cgggttacaa aagaattatt ctaacctccg ctttggaatg agttcttttt accctccatc

61 attttagatg aagggtgaaa gtcggtaacg aaggtcttcg cttagactga acgttgcgga

121 aaggatcaat wgtcgaatcc tgcgaagcag acgacccgag aacatgttta ccgcacgggc

181 gtccgggacg ggggaaacca cggaccggcg cctcgagcgt ggcgtcgacc ataggtcgct

241 cgtcgtgcac ataaacaacc ccgggcgctt aaaagcgcca aggaaaacaa gaaagggatg

301 gcctgcctct cgacgctccg ttcgcggagt gcatgggagg gcaacaggca cctgaataaa

361 caaaaacgac tctcggcaac ggatatctcg gctctcgcat cgatgaagaa cgtagcgaac

421 tgcgatactt ggtgtgaatt gcagaatccc gtgaaccatc gagtctttga acgcaagttg

481 cgcccgaagc cattaggccg agggcacgtc tgcctgggcg tcacgcatcg cgtcgccccc

541 caaccccgtg tgttaactcg tacgggtgac gtgaggggggc ggaaactggc ttcccgtgct

601 tggccgcggc tggcctaaat gcgagtccct tgcgacggac gcgacgacaa gtggtggttg

661 attgcctcaa ctaaggtgct gtcgcgcgac gcccgtcgaa tgaggagact ccctgaccct

721 gatgcatgcg ttgtcacgac gcttgctacg accgcgaccc caggtcaggc gggattaccc

781 gctgagttta agcatatcaa taagcggagg aaagaaacat ttaaaaaaa

//

Accn. No. K506

1 agaacctctc gccggtttgt tatttttttt cctccggatc aaagaactcc cactcgttag

61 aaagttatta accttatcat ttagaggaag gagaagttgt aacaaggttt ccgtaggtga

121 acctgcggaa ggatcattgt cgaatcctgc gaagcagacg acccgagaac atgtttaccg

181 cacgggcgtc cgggacgggg gaaaccacgg accggcgcct cgagcgtggc gtcgaccata

241 ggtcgctcgt cgtgcacata aacaaccccg ggcgcttaaa agcgccaagg aaaacaagaa

301 agggatggcc tgcctctcga cgctccgttc gcggagtgca tgggagggca acaggcacct

361 gaataaacaa aaacgactct cggcaacgga tatctcggct ctcgcatcga tgaagaacgt

421 agcgaactgc gatacttggt gtgaattgca gaatcccgtg aaccatcgag tctttgaacg

481 caagttgcgc ccgaagccat taggccgagg gcacgtctgc ctgggcgtca cgcatcgcgt

541 cgccccccaa ccccgtgtgt taactcgtac gggtgacgtg agggggcgga aactggcttc

601 ccgtgcttgg ccgcggctgg cctaaatgcg agtcccttgc gacggacgcg acgacaagtg

661 gtggttgatt gcctcaacta aggtgctgtc gcgcgacgcc cgtcgaatga ggagactccc

721 tgaccctgat gcatgcgttg tcacgacgct tgctacgacc gcgaccccag gtcaggcgga

781 attacccgct gagtttaagc atatcaataa gcggaggaaa aaaaccttaa caaaaaagtg

841 gggtgttttt ct

//

5 DISCUSSION

Restriction analysis was carried out to check the presence of restriction sites in the ITS region of *Swertia chirayita*. Restriction analysis performed could have been furthered the variability analysis. But due to lack of enzymes, only three enzymes were used for the study viz. Eco*R1*. Xho*1* and Bst*U1*. Presence of restriction sites for. Xho*1* and Bst*U1* and no restriction site for Eco*R1* treated ITS DNA of *Swertia chirayita* is confirmed by the gel pictures (Plate3-6).The PCR amplified ITS DNA has a size of around 750 bps indicates the presence of the restriction sites.

The bands of around 550 bps is seen on the lane containing samples treated with Xho*1*(Plates 3-4). DNA bands of around 200bps and 300bps is seen on the lane containing samples treated with Bst*U1*.(5-6). DNA bands of around 750 bps were observed in the restriction only for Eco*R1*.This is due to the absence of restriction sites for the enzyme.

ITS-DNA bands of around (750) were observed in samples that has been treated with Xho*1*. While such bands were not observed in samples treated with Bst*U1* .This might be due to the incomplete digestion of the DNA sample treated with Xho*1*.While in the case of Bst*U1* ,lack of ITS-DNA shows the complete digestion of DNA samples. If we already have the sequence information on the DNA (ITS) of the species then we can choose the enzymes so that we can get variation between species by using as few enzyme as possible. For this we use the software tool named web cutter. This software allows us to view all the possible sites for a particular enzyme and also the sites for different restriction enzymes. And observing this data, one can select the enzyme neede for variability studies.

6 CONCLUSION

- *Swertia chirayita* being a highly valued medicinal plant of Nepal, its genetic diversity in different geographical gradients need to be understood for its long term conservation and sustainable utilization.
- Various PCR-based and DNA sequencing based molecular marker tools can be employed to screen intrinsic genetic diversity within and between species.
- Present study employing ITS-PCR-RFLP has revealed baseline information on DNA polymorphism at specific genomic locus of *S.chirayita* genotypes from different geographical locations under study.
- However, in order to get the complete picture more samples covering wide geographical range and more number of restriction endonucleases need to be employed.
- Intra-specific molecular phylogeography and inter-specific molecular phylogeny employing various nuclear and chloroplast gene sequences are invaluable to unravel the evolutionary dynamics within *S. chirayita* populations and among various *Swertia* species of Nepal.
- Such research endeavors would generate valuable insights for the conservation and sustainable utilization of *S. chirayita* and its allied species in Nepal.

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