
# GENETIC DIVERSITY ANALYSIS OF *SWERTIA CHIRAYITA* [ROXB. EX FLEM] KARST POPULATIONS OF NEPAL USING POLYMERASE CHAIN REACTION BASED RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD-PCR) TECHNIQUE

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## *Certificate of Evaluation*

This is to certify that this thesis entitled **"GENETIC DIVERSITY ANALYSIS OF *SWERTIA CHIRAYITA* [ROXB. EX FLEM] KARST POPULATIONS OF NEPAL USING POLYMERASE CHAIN REACTION BASED RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD-PCR) TECHNIQUE"** presented to evaluation committee by Mr. Jagat Krishna Chhipi Shrestha is found satisfactory for the partial fulfillment of Master of Science in Biotechnology.

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## GLOSSARY ACRONYMS

| | |
|---|---|
| ABS | Access and Benefit Sharing |
| AFLP | Amplified Fragment Length Polymorphism |
| AMOVA | Analysis of Molecular Variance |
| AP-PCR | Arbitrarily primed PCR |
| a.s.l | Above Sea Level |
| CAPS | Cleaved Amplified Polymorphic Sequence |
| CBD | Convention on Biological Diversity |
| CBOL | Consortium for the *Barcode* of Life |
| cDNAs | Complementary DNA |
| $CI_C$ | Consensus fork Index |
| CITES | Convention on International Trade of Endangered Species of Wild Fauna and Flora |
| CNS | Central Nervous System |
| CONSEN | Consensus trees and indices |
| COPH | Cophenetic value |
| cpDNA | Chloroplast DNA |
| CTAB | Hexadecyltrimethylammonium Bromide |
| DAF | DNA Amplification Fingerprinting |
| d.f. | Degree of Freedom |
| dNTPs | Deoxynucleotide phosphates |
| DOF | Department of Forest |
| DNA | Deoxyribonucleic Acid |
| EDTA | Ethylene-Diamietetraacetic acid |
| EST | Expressed Sequence Tag |
| EtBr | Ethidium Bromide |
| GLB | Gel Loading Buffer |
| GM | Genetically Modified |
| GON | Government of Nepal |
| $G_{ST}$ | Degree of genetic differentiation among populations |
| H | Nei's gene diversity |
| HPLC-DAD | High Performance Liquid Chromatography – Diode Array Detection |
| Hs | Mean heterozygosity within population |
| $H_T$ | Total heterozygosity (in overall population) |
| I | Shannon's diversity Index |
| ICIMOD | International Centre for Integrated Mountain Development |
| $I_B$ | Band Informativeness |
| IFN | Interferon |

| | |
|---|---|
| IGS | Intergenic spacer |
| IL | Interleukin |
| IPR | Intellectual Property right |
| IR | Infra- Red |
| ISH | *In situ* Hybridization |
| ISSR | Inter Simple Sequence Repeat |
| ITS | Internal Transcribed Spacers of the Nuclear rDNA Repeat |
| IUCN | International Union for Conservation of Nature |
| Kb | Kilobase, Unit of Length used for nucleic acids and polynucleotide, corresponding to 1000 base pairs |
| L | Litre |
| LNA | Locked Nucleic Acid |
| MAAP | Multiple Arbitrary Amplicon Profiling |
| MAPs | Medicinal and Aromatic plants |
| MAS | Marker Assisted Selection |
| MatK | MaturaseK gene of chloroplast |
| mL | milliliter |
| mM | millimolar |
| MOEST | Ministry of Environment, Science and Technology |
| MP-PCR | Microsatellite primed PCR |
| MS | Mean Square |
| mtDNA | Mitochondrial DNA |
| MVSP | Multi – Variate Statistical Package |
| MXCOMP | Matrix comparision |
| $N_a$ | Observed number of alleles |
| NAST | Nepal Academy of Science and Technology |
| NBS | Nepal Biodiversity Strategy |
| $N_e$ | Effective number of alleles |
| NILs | Near-Isogenic Lines |
| Nm | Gene Flow |
| NPB | Number of polymorphic band |
| NRTU | Nuclear Ribosomal Transcriptional Unit |
| NTFPs | Non-Timber Forest Products |
| NTSYS | Numerical Taxonomical System (Statistical Package) |
| OTUs | Operational Taxonomic Units |
| PCO | Principle Coordinate Analysis |
| PCR | Polymerase Chain Reaction |
| PGR | Plant Genetic Resources |
| PIC | Polymorphic Information Content |

| | |
|---|---|
| POPGENE | Population Genetic Analysis (Statistical Package) |
| PP | Percent polymorphism |
| PVP | Polyvinylpyrrolidone |
| QTL | Quantitative Trait Loci |
| r | Correlation coefficient |
| RAPD-PCR | Random Amplified Polymorphic DNA-PCR technique |
| rbcL | ribulose-bisphosphate carboxylase gene of chloroplast |
| REs | Restriction Enzymes |
| RFLP | Restriction Fragment Length Polymorphism |
| RNA | Ribonucleic Acid |
| $R_P$ | Resolving Power |
| RT-PCR | Reverse Transcription – Polymerase Chain Reaction |
| SAGE | Serial Analysis of Gene Expression |
| SAHN | Sequential, Agglomerative, Hierarchial and Nested (clustering method) |
| SCAR | Sequence Characterized Amplified Regions |
| SDS- PAGE | Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis |
| SIMGEND | Similarity for genetic data |
| SIMQUAL | Similarity for Qualitative data |
| SM | Simple Matching (Coefficient of similarity) |
| SNPs | Single Nucleotide Polymorphisms |
| SS | Sum of Squares |
| SSLP | Simple Sequence Length Polymorphism |
| SSRs | Simple Sequence Repeats |
| STMs | Sequence Tagged Microsatellites |
| STRs | Short Tandem Repeats |
| STS | Sequence Tagged Sites |
| *Taq* DNA Pol. | DNA Polymerase Enzyme Isolated from *Thermus Aquaticus* bacteria |
| TAE | Tris-Acetate-EDTA Buffer |
| TE buffer | Tris-EDTA buffer |
| TIGR | The Institute for Genome Research |
| TLC | Thin Layer Chromatography |
| TNB | Total number of band |
| TOGA | TIGR Orthologous Gene Alignments |
| TRIPs | Trade Related Aspects of Intellectual property right |
| Tris | Tris [Hydroxymethyl] Aminomethane |
| UBC | University of British Columbia (random primers for RAPD-PCR) |
| UK | United Kingdom |
| UPGMA | Unweighted Pair Group Method of Arithmetic Averages |
| UPOV | International convention for the Protection of New varieties of Plants |

| | |
|---|---|
| USA | United States of America |
| UV | Ultra-Violet |
| VNTR | Variable Number of Tandem Repeats |
| WHO | World Health Organisation |
| WPGMA | Weighted Pair Group Method of Arithmetic Average |
| WTO | World Trade Organization |
| $\phi_{PT}$ | Indicator of genetic differentiation |

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# *ABSTRACT*

Among the 150 species of *Swertia* distributed globally, 30 species have been reported to be found in Nepal. Of these 30 species, nine species are reported to have the medicinal properties. *Swertia chirayita* is the most valuable species, although other species are also being traded as adulterants. Owing to its high medicinal importance and demand, *Swertia chirayita* populations in wild habitats are being overexploited beyond its regeneration capacity. Present molecular investigation was undertaken in order to 1) understand the level of genetic diversity within and between various *S. chirayita* populations collected from various districts of Nepal using Random amplified polymorphic DNA (RAPD) technique and 2) generate species specific/population specific RAPD markers for authentication purposes. Thirty four accessions of *S. chirayita* along with the 6 allied species were analyzed using 26 RAPD primers. Of the total 285 amplified bands scored for *S. chirayita*, 263 bands (92.28%) were polymorphic. Seven primers revealed nine RAPD markers that were specific to various *S. chirayita* populations. Genetic relationship among various *S. chirayita* accessions was performed by using binary data matrix created for presence or absence of RAPD bands. Cluster analysis using RAPD (qualitative) data was performed in NTSYS statistical package by pair wise similarity matrices generated by Simple Matching, Jaccard's and Dice coefficient of similarity. From comparisons of similarity matrices and phenograms, Jaccard's coefficient of similarity in constructing phenogram using UPGMA module was revealed to be the best for deducing the genetic relationship among *S. chirayita* accessions. It revealed two major clusters with distinct delineation of allied species out of the clusters reflecting rich genetic diversity among populations under study. Principal Coordinate Analysis (PCO) substantiated the results of phenograms. The genetic interrelationship between the populations were clarified by the assessment of Nei's genetic identity and distance using POPGENE 1.32 that revealed *S. chirayita* populations of Sankhuwasabha and Terathum as closest (0.9489, identity) and Kaski and Sankhhuwsabha as most distant (0.7078, identity). Assessment of genetic variation within populations estimated with Percent Polymorphism, Shannon's diversity index and Nei's gene diversity reflected the highest within diversity for the population of Nagarjun > Phulchowki> Terathum> Sankhuwasabha > Kaski. The mean genetic differentiation ($G_{ST}$) between populations over all loci was observed to be 0.5929 with low average gene flow ($N_m$) of 0.3433 reflecting genetic purity of this vulnerable species. Survival of a species depends on the maintenance of genetic variability within and among population that help to adapt and acclimatize in the new selection pressures brought about by environmental changes. It can be concluded that this genetic diversity observed according to different geographical gradient needs to be preserved and sustainably utilized based on their chemical attributes.



***Key words***: *Swertia chirayita*, RAPD, genetic diversity, population genetic, molecular marker

# INTRODUCTION

## 1.1 Background

Nepal, with an area of 147,181 sq. km, situated in the central Himalaya, has diverse physiographic zones, climatic contrasts and altitudinal variations, which provide habitats for biological species of both Indo-Malayan and Palaearctic realms including endemic Himalayan flora and fauna (Bhuju *et al.*, 2007). The country is located between latitudes of $26^0$ 22' and $30^0$ 27' N and longitudes of $80^0$ 40' and $88^0$ 12' E. Altitude varies from 67m above sea level (asl) at Kachana Kalan, Jhapa in the South-eastern Terai to 8848m at Mt. Everest, the highest point in the world. Nepal's biodiversity is a reflection of its unique geographic position and variations in altitude and climate. A total of 118 ecosystems with 75 vegetation types and 35 forest types have been identified in these realms.

Compared to the size of Nepal, it possesses tremendous diversity harboured in diverse climate and ecosystems of plant resources with new and higher potentialities. Medicinal plants are one of the best components of Nepalese floral diversity. Over 1900 species of medicinal and aromatic plants have been reported in Nepal out of which 250 species are endemic (Ghimire, 2008). Some of the highly valued and globally significant spp. are *S. chirayita, Taxus wallichiana*, *Neopicrorhiza scrophularii*, *Dactylorhiza hatagirea* etc.

*Swertia* is widely distributed plant genus among the 84 genus and 970 species within the Gentianaceae family (Judd *et al.* 2002). The genus *Swertia* is represented globally by about 100 species (Willis, 1996) disseminated in the temperate areas of Asia, Africa, Europe, North America and Madagascar (Struwe and Albert, 2004). 30 species of *Swertia* have been reported in Nepal from 54 districts (Barakoti *et al.* 1999) which are distributed from east to west and from tropical to alpine zone ranging from 600m to 5600m (Rijal, 2009). Of these, nine species are reported to have medicinal significance and therefore are in trade *viz. S. chirayita, S. angustifolia, S. ciliata, S. dilatata, S. multicaulis, S. racemosa, S. tetragona, S. alata, S. nervosa* (Barakoti, 2002; Joshi and Joshi, 2008; Shrestha *et al.*, 2010). Among these species, *Swertia chirayita* is the legendary species having superior medicinal properties and therefore dominant in trade. Nepal is reported to trade more than 45% of the world's total volume of *S. chirayita* (Barakoti 2004). Of the total traded volume, only 1% is used locally, the rest of which are mostly exported to India along with China, Malaysia, Singapore, Germany, Italy, France, Switzerland, SriLanka, Bangladesh, Pakistan and USA (Phoboo *et al.* 2008, Edwards, 1996) for its precious ayurvedic and allopathic values. About 66,806 kg of *S. chirayita* has been exported from Nepal according to the data from Ministry of Forest (MoFSc), Nepal (2009/010). According to the recorded data from Department of Forest (MoFSc, 2009/2010), *S. chirayita* has been traded in 61 out of 75 districts. *Swertia*

*chirayita* is mostly collected from wild habitat. Raw dried *S. chirayita* is currently exported at a price of Rs. 400 per kg (Phoboo *et al.*, 2010). World Health Organisation has estimated more than 80% of world's population in developing countries depends primarily on herbal medicine for basic healthcare needs and comparably the use in developed countries is also increasing (Canter *et al.*, 2006; WHO factsheet, 2008). For medicinal purpose, whole plant is used in crude form. Early studies documented the presence of flavonoids, xanthones, terpenoids, iridoids and secoiridoid glycosides in *S. chirayita* (Pant *et al.*, 2000). Xanthone derivative (1, 5-dihydroxy-3,8-dimethoxy) from *S. chirayita* has shown the promising anti inflammatory action in acute and experimental models in rats (Banerjee *et al.*, 2000; Mandal *et al.* 1992). Saxena *et al.* (2007) documented Swerchirin, a derivative of xanthone, as a potent hypoglycemic. Similarly, significant antihepatotoxic activity of methanol extract of *S. chirayita* was observed in mice (Karan *et al.*, 1999). Antileishmanial property of Amarogentin was reported in hamster model (Medda *et al.*, 1999) which acts as an inhibitor of topoisomerase I of *Leishmania donovani* (Ray *et al.*, 1996). Both the crude and purified extracts has shown significant inhibition of cell proliferation and induced apoptosis (Saha *et al.*, 2004). Also, the plant have been reported to have antiviral (Verma *et al.*, 2008), antihelminthic (Iqbal *et al.*, 2006) and hepatoprotective (Mukherjee *et al.*, 1997) activities.

## 1.2 Justification of study

Medicinal plants of Nepal are increasingly depleting and threats to these high value resources are from land fragmentation, degradation, overexploitation as well as prevailing climate change. Owing to its rich medicinal value, *S. chirayita* is suffering indiscriminate unscientific harvesting from its natural habitats for the commercial purposes (Joshi, 2011). As a result of which it has been already listed as critically endangered in India (CITES, cited in Phoboo *et al*., 2011) and vulnerable in Nepal (IUCN, 2004). Since, the survival index of a species is greatly determined by the percentage of polymorphisms and the gene flow between the populations, assessing genetic diversity is considered vital for formulating conservation strategies of vulnerable species such as *Swertia chirayita* (Sebastian *et al.*, 2010). An essential prerequisite for a species to survive against environmental pressures is the availability of a pool of genetic diversity and in its absence, extinction would appear inevitable (Frankel, 1993). Therefore, it is a pervert need to preserve the existing diversity of this valuable germplasm and protect its habitats from further destruction along with the sustainable utilization. Information on genetic diversity and natural distribution pattern are very important for designing conservation strategy of *S. chirayita* (Wang *et al.*, 2011).

Morphologically, the true *S. chirayita* can be distinguished from other substitutes and adulterants by its brownish-purple stem (dark color), stem that is rounded at the base and terete

at upper portion, continous, yellowish, pith, green petals with dark red distinct marking and double nectarines (Joshi and Dhawan, 2005). But, the traditional identification of folk herb *S. chirayita* by morphological characteristics has always been problematic as outlier species are very similar to each other morphologically when sold in dried bundles. Furthermore, many are sold in dried forms without flowers, rendering their authentication by morphological methods very difficult, if not impossible (Joshi, 2011). In this context the molecular marker tools hold great promise for the development of diagnostic tool for *S. chirayita* to address the problem of adulteration. Therefore, molecular identification of *S. chirayita* is essential from the viewpoint of human health safety as well as sustainable use of *S. chirayita* and its allied species. Besides, such molecular studies would complement plant breeding, Intellectual property rights (IPR) protection and pharmacological studies (Katoch *et al*., 2010).

The use of nucleic acid based molecular markers is preferred over conventional morphological and biochemical markers for genetic diversity studies, since they are not influenced by environment and development stages of plant. The advent of DNA-based genetic markers led to a whole new field of academic research to identify the indigenous species and to characterize the germplasm for conservation and sustainability purposes (Khan *et al.*, 2011). Determination of existing genetic diversity in a species and explanation of diversity in terms of its origin, organization and maintenance are thus of fundamental significance in the application of genetic principles to conservation. Various approaches are available for DNA fingerprinting such as AFLP (Amplified fragment Length polymorphism), SSRs (Simple sequence Repeats), RFLP (Restriction Fragment Length polymorphism) and RAPD (Random Amplified polymorphic DNA) (Joshi *et al.*, 1999; Weisings *et al.*, 2005). The enormous attraction of RAPD is the use of short oligonucleotides random primers involving no blotting or hybridizing steps (Williams *et al.*, 1990; Clapp, J.P., 1996), higher frequency of polymorphism, rapidity, technical simplicity, cost effectivity (Weising *et al.*, 2005), requirement of a few nanogram of DNA, no requirement of prior information of the DNA sequence and feasibility of automation (Jeya Prakash *et al.*, 2006; Subudhi *et al.*, 1999).

Therefore, present investigation on genetic diversity and population genetics of *S. chirayita* populations collected from eastern, central and western Nepal using genome based RAPD markers will be an effective way to unravel existing genetic diversity within *S. chirayita to* formulate conservation strategies and breeding for elite germplasm. Present study will also generate insights towards development of molecular diagnostic tool for S. chirayita of Nepal.

## 1.3 Objectives

### Broad Objectives

To characterize genetic diversity of *S. chirayita* populations of Nepal using RAPD marker technique.

### Specific Objectives

a) To collect available informations on *S. chirayita* populations (morphological, ecological, phytochemical and molecular).
b) To collect plant herbariums and DNA samples from the populations under study.
c) To select best DNA extraction technique for S. chirayita
d) Extraction and quantification of DNA from all collected samples
e) Optimization of RAPD-PCR reactions and cycling conditions
f) RAPD marker identification and use of NTSYS-PC software for Genetic diversity assessment
g) Population genetic analysis using POPGENE and GeNAlex software
h) Data representation using SPSS and MVSP statistical package

## 1.4 Scope of the study

RAPD-PCR technology has been applied efficiently in the genetic diversity analysis and diagnostic marker development for particular species or population for various food crops, medicinal and aromatic flora (Clapp, J.P., 1996; Joshi *et al.*, 2004; Sucher and Carles, 2008; Salem *et al.*, 2007). Such diagnostic markers are ideal tool for Pharmacobiotechnology industry, herbal drug technology (Joshi *et al.*, 2004), also can be further converted to robust marker SCARs (Sequence Characterized Amplified regions) designing specific primers for particular RAPD markers that can be effectively used in the species authentication purposes. Furthermore, DNA samples can be further utilized for other molecular studies such as PCR-based AFLP and DNA sequencing based DNA barcoding.

In context of Nepal, many *Swertia chirayita* populations are yet to be studied to know their population genetic structure and the output of this research elicit the instant need of genetic diversity study of this vulnerable species throughout its natural habitat in 54 districts (Barakoti *et al.*, 1999). Research based on molecular markers can be expanded to encompass all other species of *Swertia* of Nepal in diverse sectors of conservation genetics, genetic differentiation, molecular systematics, molecular ecology, evolutionary biology, population genetics, molecular diagnostics, Molecular breeding, DNA barcoding, Genetic mapping and recombinant DNA technology.

# 2. LITERATURE REVIEW

## 2.1 The Family Gentianaceae

Following description of family Gentianaceae is based on Struwe and Albert (2002). It consists of flowering plants with 87 genera and over 1600 species. It includes herbs, sometimes mycoparasites (with reduced leaves and lacking chlorophyll) to shrubs or small trees showing a wide range of colors and floral patterns. Stems are often winged usually with internal phloem. Leaves are usually opposite, less often alternate or in some species whorled, simple, entire, sessile, with pinnate venation. Stipules are absent. Flowers are usually bisexual and actinomorphic. Sepals are usually four or five and connate, often with colleters on adaxial surface. Petals are usually 4 or 5 connate, forming a wheel-shaped, funnel shaped or bell shaped corolla. The lobes are sometime fringed often with nectar glands or/and scales on adaxial surface of the tube. The stamens (4 or 5 in number) are attached to the inside of the petals (epipetalous) and alternate with the corolla lobes. Ovary is superior, with parietal placentation. Ovules are usually numerous, on each placenta, with one integument and a thin-walled mega-sporangium. Nectar producing disc or glands are present. The inflorescence is cymose, with simple or complex cymes. Fruits are dehiscent septicidal capsules splitting into two halves, rarely some species have a berry. Seeds are small with copiously oily endosperms and a straight embryo. Plants usually accumulate bitter iridoid substances. Distribution is cosmopolitan.

## 2.2 The Genus *Swertia*

Following description of genus *Swertia* is based on Rijal (2009). Members of the genus *Swertia* are annual, biennial or perennial herb with the roots fibrous or woody. Stems absent, scapiform, or well developed, ascending or erect, terete, striate, angled or winged, simple or branched. Leaves opposite, rarely alternate or whorled or rosulate, sessile or petiolate, margin entire. Inflorescence cymose, usually grouped into simple or paniculate thyrses, rarely strictly dichotomous, sometimes reduced to single flowers and inflorescences raceme-like or flowers solitary and terminal. Flowers pedicellate, 4 or 5 – merous. Nectaries 1 or 2 per corolla lobe, naked or covered by a scale or flaps, glabrous, fringed or fimbriate. Stamens as many as corolla lobes, attached at base of corolla lobe sinuses, sometimes surrounded by long hairs, ovary 1-celled. Style short to elongate. Stigma bilobed. Fruit, a capsule, enclosed by persistent calyx and corolla, ovoid or flattened, dehiscing into 2 valves, few to many seeded. Seeds are small.

## 2.3 Taxonomy and Phylogenetic Relationships

According to Rignanesa (2009), *Swertia chirayita* (Roxb. Ex. Fleming) H. Karst is taxonomically classified into Kingdom Plantae, Division Magnoliophyta (flowering plants), Class Magnoliopsida

(Dicotyledons), subclass Asteridae, Order Gentianales, Tribe Gentianeae and Family Gentianaceae.

*Swertia* is a paraphyletic genus and related genera include Gentianella and Halenia. Tribe Gentianeae is closely related to the tribes Helieae and Potalieae. Within the Gentianeae, there are two main clades, identified as two subtribes. Crawfurdia, Gentiana and Triterospermum belong to subtribe Gentianinae, and all other genera (eg. Bartonia, Comastoma, Gentianopsis, Halenia, Frasera, Gentianella, Jaeschkea, Latouchea, Lomatogonium, Megacodon, Obolaria, Pteridocalyx, *Swertia* and Veratrilla) belong to subtribe Swertiinae. This subtribe possess many species that are used for their medicinal properties, specially top ranking species of Gentiana and *Swertia* (Struwe and Albert, 2002).

Molecular studies attempted to clarify the circumscription of Swertiinae and its sister group relationship to Gentianinae as the only other subtribe of Gentianeae (Yuan and Kupfer 1995; Struwe and Albert, 2002). All molecular data analysed (trnL intron, matk, ITS) for the phylogenetic analysis showed the apparent subdivision of the Gentianeae into subtribes Gentianinae and Swertiinae (Struwe and Albert, 2002). In the ITS tree, Megacodon occupies a basal position in the Swertiinae, and Gentianopsis grouped with Frasera and Pterydocalyx. Because Megacodon also shows no close relationship to Gentianella in the trnL intron data and because the same is true for both Megacodon and Gentianopsis in the matK tree, generic status for both genera was justified. For the remainder of Gentianella, two major groups can be recognized in the ITS tree. The first group consists of species with two nectarines per petal and a naked corolla tube. The second group was characterized by only one nectary per petal and is part of a polytomy with several other genera in the ITS tree (Struwe and Albert, 2002). Existing molecular studies have not yet succeded in clarifying the evolution of the entire Swertiinae because of insufficient sampling and/or poor resolution (Yuan and Kupfer, 1995; Chassot *et al.*, 2001; Hagen and Kadereit, 2001; Struwe and Albert, 2002). However, the phylogenies resulting from the separate analyses of nuclear and chloroplast data (trnL intron, IGS between trnL and trnF exons and between trnS and ycf9 exons of cpDNA as well as ITS region of nrDNA) are congruent suggesting *Swertia* as strongly paraphyletic in relation to other genera.

## 2.4 World Distribution of *Swertia* Species

Genus *Swertia* is globally represented by about 100 species (Willis, 1996) distributed in the temperate areas of Asia, Africa, Europe, North America and Madagascar (Struwe and Albert, 2004). The genus *Swertia* is native to temperate Himalayas, found at an altitude of 1200-3000m (4000ft-10,000ft) from Kashmir to Bhutan and in the Khasia hills at 1200-1500m (4000ft to

5000ft) (Kirtikar and Basu, 1984; Clarke, 1985). Various species are distributed in temperate areas in Asia (100spp.), Africa (30 spp.), Europe (3 spp.), North America (1 sp.) and Madagascar (1 sp.) (Struwe and Albert, 2004) (Fig 2.1).

**Figure 2.1** world map showing distribution of *Swertia* (dark spots)

## 2.5 *Swertia* Species of Nepal

Altogether 30 species of genus *Swertia* have been reported in Nepal (Shrestha *et al.*, 2010) including endemic species *S. acaulis, S. nepalensis* and *S. dilatata* var*. pilosa* (Press *et al.*, 2000; Joshi, 2011). Of these, nine spp. are being traded due to having medicinal properties *viz.* 1) *S. chirayita* (Roxb. Ex Fleming) H Karst, 2) *S. angustifolia* Buch. – Ham. Ex D. Don, 3) *S. tetragona* Edgew, 4) *S. racemosa* (Griseb) C.B Clark, 5) *S. ciliata* (D. Don Ex G. Don) B.L. Burtt, 6) *S. dilatata* C.B. Clarke, 7) *S. multicaulis* D. Don, 8) *S. alata* (Royle ex D. Don) C.B. Clarke, 9) *S. nervosa* (G.Don) C.B. Clarke (Joshi and joshi, 2008; Internet visit [7], 2007*;* Barakoti , 2002).

Nepalese species of *Swertia* are distributed from east to west and from tropical to alpine landscapes. Distribution ranges from 600 m a.s.l. (*S. angustifolia*) to 5600 m a.s.l. (*S. petiolata*) (Rijal, 2009).

**Table 2.1** *Swertia* species of Nepal

| SN | Distribution in Nepal | Altitude | Distribution in World | Species |
|---|---|---|---|---|
| 1 | Nepal | 3700-5500 m | - | *Swertia acaulis* |
| 2 | Central and Western Nepal | 2000-3600m | Himalaya (Kashmir to Nepal) | *Swertia alata* |
| 3 | Central Nepal | 3000-4000m | Himalaya (Kashmir to Nepal) | *Swertia alternifolia* |
| 4 | Western, Central and Eastern Nepal | 600-2600m | Himalaya (Kashmir to Bhutan), North India, Myanmar, South China | *Swertia angustifolia var angustifolia* |
| 5 | Western, Central and Eastern Nepal | 2000m | Himalaya (Uttar Pradesh to Bhutan), India , Myanmar, China | *Swertia angustifolia var pulchella* |
| 6 | Central and Eastern Nepal | 600m | Himalaya (Nepal and Sikkim) | *Swertia angustifolia var wallichiana* |
| 7 | Eastern Nepal | 900-2700m | Himalaya (Nepal to Bhutan), NE India( | *Swertia bimaculata* |

| | | | | |
|---|---|---|---|---|
| | | | Assam, Nagaland), China , Japan) | |
| 8 | Central Nepal | 4800m | Himalaya (Nepal to Bhutan) | *Swertia candelabrum* |
| 9 | Central and Eastern Nepal | 1500-2500m | Himalaya (Kashmir to Bhutan), North east India | *Swertia chirayita* |
| 10 | Western, central and Eastern Nepal | 2800-4000m | Afganistan, Himalaya (Kashmir to Sikkim) | *Swertia ciliate* |
| 11 | Western, Central and Eastern Nepal | 2000-3000m | Pakistan (Chitral), Himalaya (Kashmir to Bhutan), North East India (Meghalaya), Myanmar, China (Xizang) | *Swertia cordata* |
| 12 | Western, Central and Eastern Nepal | 3900-5000m | Himalaya (Uttar Pradesh to Sikkim), North East India, China (Xizang) | *Swertia cunecta* |
| 13 | Central Nepal | 2000-3000m | Nepal | *Swertia dilatata var pilosa* |
| 14 | Central Nepal | 4000-4200m | Nepal, china (Xizang) | *Swertia hispidicalyx var hispidicalyx* |
| 15 | Eastern Nepal, | 3800-4300m | Himalaya (Nepal to Bhutan), China (Xizang) | *Swertia hookeri* |
| 16 | Central and Eastern Nepal | 3100-4500m | Himalaya (Nepal to Bhutan), China (Xizang) | *Swertia kingii* |
| 17 | Western and Central Nepal | 2500m | Himalaya (Kashmir to Nepal) | *Swertia lurida* |
| 18 | Central and Eastern Nepal | 2000-3200m | Himalaya (Nepal to Bhutan), North east India (Meghalaya), Myanmar | *Swertia macrosperma* |
| 19 | Central and Eastern Nepal | 4000-4900m | Himalaya (Nepal to Bhutan), China (Xizang) | *Swertia multicaulis* |
| 20 | Central Nepal | 3850m | Nepal | *Swertia nepalensis* |
| 21 | Western, Central and Eastern Nepal | 700-3000m | Himalaya (Himanchal Pradesh to Bhutan), North east India (Asam, Nagaland), West China | *Swertia nervosa* |
| 22 | Western, Central and Eastern Nepal | 1500-4000m | Himalaya (Kashmir to Bhutan), North east India, Myanmar, China (Xizang) | *Swertia paniculata* |
| 23 | Eastern Nepal | - | Himalaya (Nepal, Sikkim, West Bengal) | *Swertia pedicellata* |
| 24 | Western Nepal | 5600m | Himalaya (Kashmir to Nepal), China (Xizang) | *Swertia petiolata* |
| 25 | Western, Central and Eastern Nepal | 3000-5000m | Himalaya (Nepal to Bhutan), North east India, China (Xizang) | *Swertia racemosa* |
| 26 | Eastern Nepal | 4000-4200m | Himalaya (Nepal to Bhutan), China (Xizang) | *Swertia ramose* |
| 27 | Western, Central and Eastern Nepal | 1400-3000m | Pakistan (Chitral), Himalaya (Kashmir to Bhutan) | *Swertia speciosa* |
| 28 | Eastern Nepal | 3800-4400m | Himalaya (Nepal to Bhutan) | *Swertia staintonii* |
| 29 | Western, Central and | 3000-5000m | Himalaya (Nepal to Bhutan), North east | *Swertia teres* |

| | | | | |
|---|---|---|---|---|
| | Eastern Nepal | | India, China (Xizang) | |
| 30 | Western Nepal | 2400-3300m | Pakistan (Chitral), Himalaya (Kashmir to Nepal) | *Swertia tetragona* |

***Source***: Annotated Checklist of the Flowering Plants of Nepal (Press *et al.*, 2000; Joshi and Joshi, 2008)

## 2.6 Ethnobotany of *Swertia* species

The people inhabiting in areas with *Swertia* spp. rely on traditional medicine for their primary health care needs. They have developed unique indigenous practices on the use of existing plant resources due to constant association with the forest and agro-ecosystems (Joshi and Joshi, 2008; Rajbhandari, 2001). Various ethnic groups of Nepal are using different *Swertia spp* as traditional medicines for various ailments since time immemorial. Due to its pharmacological importance, *Swertia chirayita* is popular in ayurvedic, allopathic as well as herbal drug system. One of the ethnic groups of Nepal, the Magars, use *S. chirayita* as herbal tea. The bitter juice is also used to cure malarial fever. The decoction of the plant is used as tonic that influence on the digestive organs and also used as antihelminthic, especially for children. Decoction of root of *S. angustifolia* is used as antipyretic by the Tamang community of Kathmandu valley. Also, boiled juice of *S. chirayita* is used to relieve fever in Jumla. It is used to cure stomach disorder at Chaubas village (Rajbhandari 2001). Decoction of aerial part of the plant is taken by the Sherpas of Helambu as an antipyretic and to treat body ache. Moreover, other species of *Swertia* like *Swertia nervosa* is also used as antipyretic in Karnali zone and to treat skin diseases along with stomach problem in different parts of Nepal. Juice of *S. multicaulis* is used as antipyretic and antihelminthic and to prevent infection and blood clotting of wounds at Rolwaling. Similarly, aerial part of decoction of *S. speciosa* is used as an effective febrifuge and good appetizer in Makwanpur district. Plant paste of *S. pedicellata* is applied externally on forehead to get relief from headache. Decoction of *S. racemosa* is used to treat fever, eczema and pimples and also to treat jaundice (Joshi and Joshi, 2008; Rajbhandari, 2001).

## 2.7 *Swertia chirayita* (ROXB. EX FLEM) H. KARST

### 2.7.1 Distribution

*Swertia chirayita* is distributed throughout temperate Himalaya (Kashmir to Bhutan, and Khasia hills) between 1200 and 3000 m a.s.l. (Garg 1987; Press *et al.*, 2000; Bhatt *et al.*, 2006). In Nepal, its distribution has been reported in 40 districts (Bhattarai, 1996) whilst Barakoti *et al.* (1999) has reported its distribution in 54 districts (Fig 2.2).


**Figure 2.2** Map of Nepal showing *S. chirayita* distribution

## 2.7.2 Taxonomy/Morphology

*Swertia chirayita* is biennial herb of about 90 cm (2-3 feet) high. The root is simple, tapering and stout, generally small about 5 to 10 cm. Stem is erect, hollow, terete, glabrous, branching, robust and the middle portion is round while upper is four angled with a prominent line at each angle. The stems are orange brown or purplish in colour with large continous yellowish pith. Leaves are cauline, opposite, united at base, subsessile, broadly lanceolate, 5 nerved. Flowering in *S. chirayita* is in the form of numerous small axillary, opposite, lax cymes arranged as short branches and the whole inflorescence is two feet long. Flowers are small, green-yellow, stacked tinged with purple colour, rotate and tetramerous. The corolla is twice as long as the calyx and divided near the base into four ovate-lanceolate segments. The upper surface of the petal has a pair of nectaries covered with oblong scales ending as fringes. Fruit is a small, one- celled capsule with a transparent yellowish pericarp. It dehisces from above, septicidally into two valves. Seeds are numerous, minute, many sided and angular (Joshi and Dhawan, 2005).

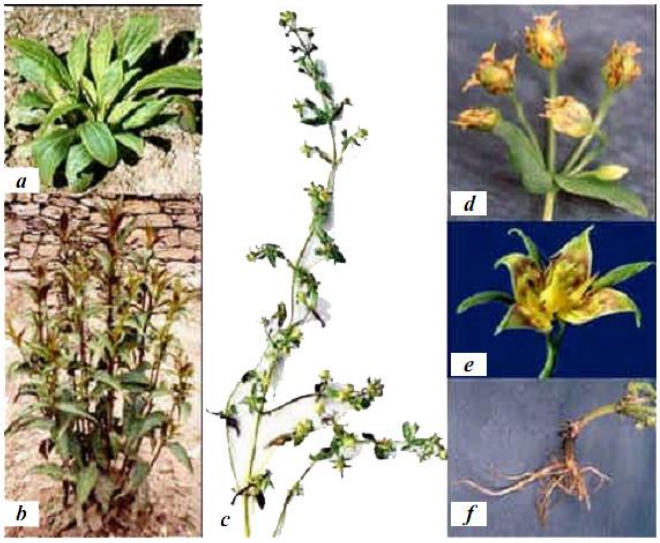



**Figure 2.3** *Swertia chirayita*: a) Plant in vegetative phase; b) A 2 feet tall plant before flowering; c) Flowering twig; d) Flowering panicle during seed set; e) Single tetramerous flower; f) Root of a mature two year-old plant [*Source*: Joshi and Dhawan (2005)]

## 2.7.3 Ecology

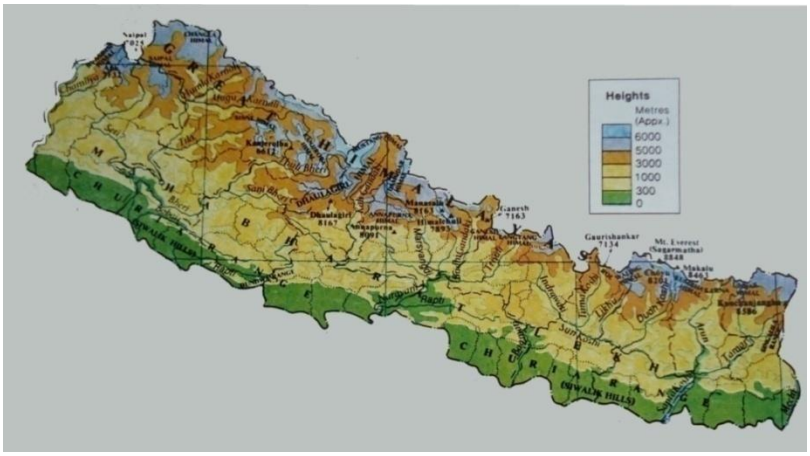



**Figure 2.4** Different geographical regions of Nepal according to the altitude. *S. chirayita* is found at 1200 to 3000m; distinguished by different colors (*Source*: Barakoti, 2002)

Nepal occupies one third of Himalayas lying at $80^0$ 04' to $88^0$ 12' E and $26^0$ 22' to $30^0$ 27' N in meeting point of Central Himalayas and Eastern Himalayas. Nepal has rich floral diversity due to high altitudinal, topographic, climatic and edaphic variations. Geographically, *S. chirayita* is distributed in the hills of eastern, central and western Nepal at an altitude of 1200 to 3000m in the swamp meadows, marshes, grassland, open forests and shady habitats. The plant can be grown in a variety of soils with sandy loam rich in carbon and humus. Population density is high

in steep slopy land. It's distribution is not uniform and it depends upon the altitude and slope. It prefers to grow on north facing slopes. It grows in south facing slope between 1500m and 3000m. While on the north facing slope, it descends below 1500m. In general, 2000m altitude is most preferable range for *S. chirayita* (Bhattarai, 1996).

*S. chirayita* is usually found growing with other species of plant like *Anaphalis* spp*, Bidens* spp*., Eupatorium adenophorum, Centella asiatica, Viola* spp*., Polygonum amplexicaule, Rhododendron arboreumn, Quercus* spp. and *Acer* spp. (Phoboo *et al.*, 2008).

### 2.7.4 Cytology

Very few cytological studies have been carried out in *Swertia* spp together with *S. chirayita*. Few studies reveal that *Swertia* shows variation in chromosome number. Khoshoo and Tandon (1963) used pollen-mother cells for cytological studies in some Himalayan species of *Swertia*. The authors counted thirteen bivalents at metaphase I, and observed that one of them was bigger than the rest.

### 2.7.5 Reproductive Biology

*Swertia chirayita* being a biennial herb, shows a rosette form in the first year whereas two year old plant has elongated stem with yellow flower. An understanding of the breeding system, (self or cross) which was not known regarding this plant is imperative and fundamental to cultivation of any genetic improvement programme for better conservation of any germplasm. A perusal of the literature revealed that very few cytological works were carried out on this particular species (Khoshoo and Tandon, 1963) and represented the plant as cross pollinated without any field experiment. In one investigation, bagging experiments were carried out in the field condition in conjunction with the cytological study to confirm the mode of pollination (Chakraborty *et al.*, 2009). Seed setting in the capsules of different parameters were taken into account in order to determine the mode of pollination of this plant. The result showed highly significant and more than four fold increase in seed setting than controlled cross and open cross treatments. The natural regeneration of plant takes place by seeds and when seeds become biologically mature, have high potentiality of viability during the month of November (Bhattarai, 1996).

Flowering takes place during July – September. Seed setting commences around October – November and seeds germinate immediately after shedding (Joshi and Dhawan, 2005). Vegetative growth occurs in first year while flowering and fruiting takes place in the following

year of vegetative growth. The plants are harvested from wild for the drug industry when it sets into flowering in July – September (Joshi and Dhawan, 2005).

### 2.7.6 Domestication and Cultivation Practices

*Swertia chirayita* fall under the IUCN threat category "vulnerable plant" (IUCN, 2004). Haphazard collection of this species from the wild habitat is still a common practice. The continued commercial exploitation of this species has resulted into depletion of population size in their natural habitats. Vacuum is likely to occur in the supply of raw plant materials that are used extensively by the pharmaceutical industry as well as the traditional practitioners. Consequently, commercial cultivation of these plants is urgently needed to fulfill their increasing demand. If timely steps are not taken for its conservation, cultivation and mass propagation, they may be lost from their natural habitat forever. *In situ* conservation of the resources alone cannot meet the ever increasing demand of pharmaceutical industry. It is, therefore inevitable to develop cultural practices and propagate these plants in suitable agroclimatic regions. Commercial cultivation will definitely check the continued exploitation from wild sources and serve as an effective means to conserve the rare floristic wealth and genetic diversity (Joy *et al*, 1998). For the proper and sustainable harvesting of *S. chirayita*, which is suffering from immature collection, Government of Nepal (GON) has proclaimed a regulation act, Forest Rule (1995) which forbids both collection and trade from May to September. If the rule is found to be breached, both the buyer and seller can be jailed or fined or both.

Practices for commercial cultivation of *S. chirayita* have been initiated within community forest and marginal land of eastern hilly districts of Nepal at an altitude of 1500 – 2500m (Barakoti, 1999; Pant, 2005). The nursery practices start from March to April. Since seeds are very small, they are mixed with sand before sowing in the bed and it takes nearly two years for the plant to mature. Germination of seed depends upon the seed quality and environment that may take about 2 weeks to 2 months (Raina *et al.*, 1994). The optimum period for germination in field is April to June.

Seed biological research are vital in generating valuable information on viability, germination and dormancy of seed which in turn can be used for enhancing productivity for income generation. Study concerning the seed germination potentiality and the nursery practices of *S. chirayita* are still on demand (Raina *et al*, 1994; Basnet 2001). 91% seed germination has been reported after chilling treatment for 15 days at $3^0$C (Raina *et al*, 1994). Similarly, another study reported 81% germination (Basnet, 2001). One study looked at assessment of seed germination potential of *S. chirayita* seeds procured from *ex-situ* and found enhancement in their germination using various

pre sowing chemical treatments. Those seed showing poor germination at initial testing after subjecting to the pre-sowing chemical treatments with gibberelic acid (50 to 350 µM) was found to be most effective for stimulation of seed germination with reduced mean germination time, (Pradhan and Badola, 2010). Study confirmed that *ex-situ* produced seeds attained physiological dormancy which was broken by pre-sowing treatments. Low germination percentage and viability of seeds, long gestation periods and delicate field handling requirements are some of the factors that discourage commercial cultivation.

The need for cultivation of medicinal plants has been voiced for decades since cultivation lessens pressure on wild populations. Cultivation is a sustainable alternative and offers the opportunity to overcome problems that are inherent in herbal extracts, misidentification, genetic and phenotypic variability, extract variability and instability, toxic components and contaminants (Canter *et al.*, 2006). Although the cultivation of *S. chirayita* has been attempted in various parts of the country, these are often small scale-ventures of local farmers (Phoboo *et al.*, 2008). There is huge gap in information and lack of knowledge sharing especially on important issues like the possible market opportunities and future trade predictions (Phoboo *et al.*, 2010). The Agriculture Research station at Pakhribas, Dhankuta, and east Nepal has been promoting cultivation of *S. chirayita* for about a decade with only a limited success (Barakoti, 2004). Therefore, to meet the increasing national and international demand, due priority should be given towards domestication and conservation of this valuable germplasm. Moreover, the novel techniques of *in vitro* and micropropagation conservation can help in production of large number of disease free true to type plants (Joshi and Dhawan, 2005). Due attention has to be paid on prior incidentssuch as the one released after the survey in 2050 BS which stated that *S. chirayita* had been extinct from few places of country due to ill practice of uprooting the plants, deforestation, lack of seed collection and ignorance (DOF, 1996/97).

### 2.7.7 Sustainable Harvesting and Conservation Initiatives

*Swertia chirayita* is mostly collected from the wild by the local collectors. As the whole plant is used for medicinal purpose, entire plants are uprooted during collection. The best time to harvest plants is after seed set. Harvesting after seed dispersal ensures more plant multiplications in its natural habitats and therefore a sustainable harvesting practice is necessary in order to conserve the remaining populations of *S. chirayita* in the wild habitats (Phoboo and Jha, 2010). Due to its high price, collectors have high competition for collection and it is collected before maturation. Thus, unmanaged exploitation of *S. chirayita* has resulted in the decrease in natural production. As the collection of *S. chirayita* is taking place at a faster rate than its replenishment, it is important to devise sustainable conservation practices, although there are

many *in-situ* management problems such as illegal collection, stealing, grazing and collection along with fodder (Bhattarai, 1996).

The increasing awareness in the national level conservation and economic utilization of biological and genetic resources is closely associated with the enforcement of international agreements such as Trade Related Aspects of Intellectual property right (TRIPs) of the World Trade Organization (WTO), the Convention of Biological Diversity (CBD) and International convention for the Protection of New varieties of Plants (UPOV). Nepal, being a repository of plant genetic resources has responded to international movement and became a member of WTO on 23rd April 2004. Draft of plant variety protection and Farmer's rights Act 2002 and 2004 have been prepared and under review possessing legislation to plant genetic resources, traditional knowledge with equitable benefit sharing (Access to benefit sharing, ABS). Nepal has also made commitments to fully implement the TRIPS Agreement by 1 Jan, 2007 that clearly indicates that Nepal requires to give effect to the article 27.3 (b) of the TRIPS agreement that is related to the protection of plant varieties either through patents or through effective Sui-generis system of combination there of (Bhandari, 2004; Internet visit 2011[1], [2]). As a signatory to the CBD at the Earth summit (1992), Nepal has fulfilled its commitment and has developed a Nepal Biodiversity Strategy (NBS) in 2002 to meet the obligations of the convention *viz.* a) serve as an overall framework for the conservation and sustainable use of its rich biodiversity and biological resources and equitable sharing the benefits b) preparation and implementation of national strategies, plans or programmes for the conservation and sustainable use of biodiversity and c) conservation in *in-situ* and *ex-situ* conditions and promotion of biotechnology and genetic research. Similarly, pertinent to the biodiversity conservation and sustainable development, Nepal has been affiliated with The Wolrd Conservation Union – IUCN and Nepal has also followed the spirit of the world conservation strategy (1980) and world charter for nature (1982) (Bhuju *et al.*, 2007)

### 2.7.8 Economic Importance of *S. chirayita*

In Nepal, about 90% of the medicinal plants traded are collected from the wild (Phoboo *et al.*, 2008). Some 104 non-timber forest products (NTFPs) items are commonly traded in Nepal. Among the high valued NTFPs, *Swertia* species occupy one of the major positions in the trade. Among the nine medicinally valued *Swertia* spp., *S. chirayita* plays a dominant role in trade covering about 80% of total traded volume of *Swertia spp* and also highly superior in quality to other species (Pant, 2005). *Swertia chirayita* enjoys a good domestic as well as international market (Joshi and Dhawan, 2005). The plant has a huge demand in the medicinal market and has played an important role in the Nepalese economy. According to Department of Forests (DOF

2009/2010), *S. chirayita* is traded in 61 out of 75 districts of Nepal encompassing all the five development regions. 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, much of the local *S. chirayita* is exported to India for their Ayurvedic, Unani and Sidha medicinal market while some are exported to China, Malaysia, Singapore, Germany, Italy, France, Switzerland, Srilanka, Bangladesh, Pakistan and USA (Edwards, 1996). The highest volume of *S. chirayita* comes from eastern Nepal. In the last thirteen years, East Nepal contributed more than 50% of the total *S. chirayita* traded in Nepal (Department of forest 1996/97 – 2008/09). The total revenue generated by the Department of Forest from the taxes and duties levied on medicinal and aromatic plants (MAPs) in 2008/2009 was Rs 38.9 millions out of which the trade of *S. chirayita* generated Rs 9.9 hundred thousands. The highest amount of *S. chirayita* in the last thirteen years came from Taplejung (263,572 Kg) and Terathum (213,837 Kg) districts of East Nepal. (Phoboo and Jha, 2010). There has been a general decline in volume of *S. chirayita* traded from Nepal in the last twelve years (1997-2009) although there seems to be slight increase in total volume in the last two years (DOF 1996/97- 2008/09).

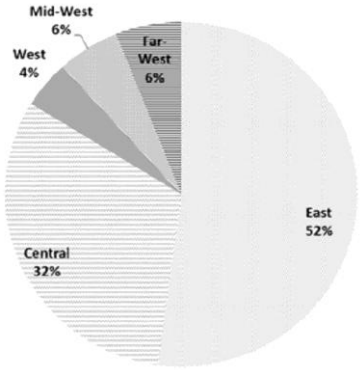



**Figure 2.5** Share of total *Swertia chirayita* traded in the last thirteen years (1996/1997-2008/2009) from five development regions of Nepal (*Source*: Department of Forest 1996/1997-2008/2009).

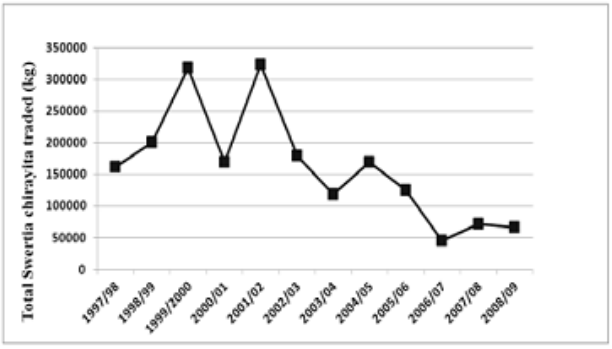



**Figure 2.6** Total volume of *S. chirayita* traded from Nepal (*Source*: DOF 1997/98-2008/09).

Frequent fluctuation in market price of *S. chirayita* has been observed in the past decade. It ranged from NRs 80-150/Kg to NRs 400/Kg in 2000 (Pant, 2005). According to Forest Rules, 1995, the Royalty rate for entire Plant is NRs. 3.00/Kg.

**Table 2.2** Market price of *S. chirayita* in different fiscal years

| SN. | Year (AD) | Price in Rs. (per Kg in dry weight) |
|---|---|---|
| 1 | 1998 | 80-100 |
| 2 | 1999 | 80-100 |
| 3 | 2000 | 80-100 |
| 4 | 2001 | 200-300 |
| 5 | 2002 | 400-450 |
| 6 | 2003 | 200-300 |

***Source***: Chiraito Trade centre Hile Bazar, Dhankuta (Raskoti, 2004)

The current market price (2010) of raw dried *S. chirayita* is Rs. 300 per kg while the local collectors are paid only Rs. 110 per Kg and these are exported at a price of Rs. 400 per Kg (Phoboo *et al.*, 2008).

### 2.7.9 Adulteration Problem

In comparision to other species of *Swertia*, *S. chirayita* plays a dominant role in trade due to its superiority and high medicinal properties. But its trade is affected by adulterants (Rijal, 2009). Due to high commercial demand, locally many species of *Swertia* including *S. angustifolia* and *S. nervosa* are used as substitutes for *S. chirayita* in case of its unavailability. This is a serious issue in terms of human health safety. Furthermore, adulteration with low-valued inferior species causes problematic trade issues and product devaluation. Such activities are depleting not only *S. chirayita* but also other allied species. Some of the main adulterants of *S. chirayita* are: *Andrographis paniculata, Exacum tetragonum, E. pendunculatum, Slevolgia orientalis, Swertia alata, S. angustifolia, S. bimaculata, S. ciliata, S. densifolia, S. elegans, S. lawii, S, minor, S. minor, S. paniculata, S. multiflora* and others (Pant *et al.*, 2000).

### 2.7.10 Medicinal properties and Chemical constituents of *S. chirayita*

*Swertia chirayita* is regarded as the multifaceted and high value cash crop of Nepal having broad spectrum, Ayurvedic and Allopathic values. Of the 30 species of *Swertia* identified in Nepal, nine spp. have been reported to have medicinal value (Joshi and Joshi, 2001). *Swertia chirayita* is powerful /effective non toxic cure for various diseases. Its whole plant is used in crude form and

to manufacture different Ayurvedic /Allopathic medicines (Pant, 2005). In Ayurveda, *S. chirayita* is described as bitter (Tikta) in taste and its thermal action defined as cooling (Shita), easily digestible (laghu) and dry (ruksha) (Joshi and Joshi, 2001). The whole plant is extremely bitter and is used for chronic fever, malaria, anemia, bronchial asthma, liver disorders, jaundice, hepatitis, gastritis, constipation, dyspepsia, skin diseases, worms, epilepsy, ulcer, scanty urine, hypertension, melanocholia, cold cough, diabetes, urinary disorders, febrifuge, inflammation, burning sensation, leucorrhea, obesity, wounds, typhoid fever, vomiting in pregnancy and certain type of mental disorder, secretion of bile, blood purification (Karan *et al.*, 1999; Banerjee *et al.*, 2000; Pant, 2005). For medicinal purpose, whole plant is used in crude form.

*Swertia chirayita* is rich in various chemical compounds that are responsible for its therapeutic properties. They are xanthones, flavonoids, terpenoids, iridoids and secoiridoid glycosides (Pant *et al*, 2000). Derivatives of these compounds *viz.* Chiretta, Swertin, Swertiamarin, Swerchirin and Amarogentin are widely accepted therapeutic compounds. Bajpai *et al.*(1991) isolated xanthone derivative from xanthone fraction and identified as 1,8 dihydroxy 3,5 dimethoxy xanthone (Swerchirin) which has shown the hypoglycemic properties (Bajpai *et al.*, 1991, Saxena *et al.*, 2007) and a protective effect on haematopoiesis (Ya *et al.*, 1999) in animal model. Bauerenyl acetate, isobauerenyl acetate and multiflorenyl acetate was recorded by Chakravarty *et al.* (1991) as significant triterpenoids. Comparatively, Phoboo *et al.* (2010) quantified major phytochemicals of *S. chirayita* from nine districts of Nepal in crude aqueous and ethanolic extracts of different plant parts using HPLC-DAD [High Performance Liquid Chromatography – Diode Array Detection]. Amarogentin, magniferin, *Swertia*marin were the main phytochemicals in all extracts observing highest quantity of all in inflorescence and leaf mixture in all collected plant samples. Quantities of those phytochemicals were also compared between wild and cultivated plant parts of *S. chirayita*, the result of which substantiates the validity of cultivated *S. chirayita* for medicinal purpose and trade as no significant difference in amount of those 3 phytochemicals was observed between wild and cultivated plant. In addition to the different studies, a number of secondary metabolites have been reported from *S. chirayita* (Joshi and Dhawan, 2005) (Appendix 2).

Xanthone derivative (1,5 – dihydroxy -3,8- dimethoxy) from *S. chirayita* has shown the promising anti inflammatory action in acute experimental models in rats (Mandal *et al.*, 1992; Banerjee *et al.,* 1999). Saxena *et al.* (2007) documented Swerchirin, derivative of xanthone is a potent hypoglycemic. Similarly, significant antihepatotoxic activity of methanol extract of *S. chirayita* was observed in mice (Karan *et al.*, 1999). Antileishmanial property of Amarogentin was reported in hamster model (Medda *et al*, 1999) which acts as an inhibitor of DNA topoisomerase I of

*Leishmania donvani*, which is a vital enzyme for the survival of the parasite, the causative agent of visceral leishmaniasis. Both the crude and purified extracts has shown significant inhibition of cell proliferation and induced apoptosis (Saha *et al.*, 2004) showing *S. chirayita* as anticarcinogenic agents which may be useful for prevention of cancer. Swerchirin is reported in lowering the blood sugar level by stimulating islets of langerhans to release insulin (Raskoti, 2004). Also, the plant have been reported to have antiviral (Verma *et al*, 2008), antihelminthic (Iqbal *et al.*, 2006), hepatoprotective (Mukherjee *et al*, 2006), antipyretic (Bhargava *et al.*, 2009), anticholinergic (Rafatullah *et al.*, 1993), antimicrobial, Central Nervous System (CNS) depressant (Leslie and Chungath, 1987), analgesic (Alam *et al.*, 2010), chemopreventive (Saha *et al.*, 2004), antioxidant (Balasundari *et al.*, 2005), antidiarrhoeal, antimalarial (Phoboo *et al.*, 2010) and anti diabetic (Suryawanshi *et al.*, 2006) properties. Paste of plant is used to treat skin diseases like pimples and eczema (IUCN, 2004). *Swertia chirayita* in presence of Magniferin (which have potent anti-inflammatory property) was found to be responsible for reducing Tumor Necrosis Factor, Interleukin IL-1, IL-6 and IFN and/or elevating IL-10 in the joint homogenates of arthritic mice (Kumar *et al.*, 2003).

Mineral elements, based on their proportion can play different roles in human health and plant physiology. The quantity and quality of secondary metabolites are affected by the availability of active minerals. Negi (2009) has studied trace elements and electrolytes in *S. chirayita* and found the order of concentration as K>Ca>Fe>Na>Mn>Zn>Co>Cu>Li in different samples of *S. chirayita* from India.

### 2.7.11 Other Uses

In industrial use, it is reported being used in brewing industry due to its bitter taste and in dying to colour cotton clothes yellow. *S. chirayita* is also known as appetizer, bitter tonic, used in alcoholic preparation as bitter flavouring agent (IUCN, 2004), used in the skin tonic 'Safi' as well as skin soaps and broadly being used in cosmetic industry as an ingredients in facial creams, cleansers, scrubbers and hair oil (Joshi, 2008; phoboo *et al.*, 2008). Recently, it has also been reported to be effective as an insecticidal in killing larvae of *Aedys aegyptii* mosquito, carrier of dengue virus (Mallikarjun *et al.*, 2010).

## 2.8 Genetic Markers and Types

Markers are "characters" whose pattern of inheritance can be followed at the Phenotypic/morphological (eg. Flower color), biochemical (eg. Protein and/or enzymes) or molecular (DNA) levels (Chawla, 2009). They are so called because they can be used to elicit,

albeit indirectly, information concerning the inheritance of "real" traits. Even though genetic diversity is at the lowest hierarchy, without genetic diversity, a population cannot evolve and adapt to environmental changes (Templeton, 1991).

A difference, whether phenotypic or genotypic, may act a genetic marker if it identifies characteristics of an individual's genotype and/or phenotype and if its inheritance can be followed through different generations. A genetic trait may not have necessarily observable consequences on an individual's performance. Sometimes, however, this trait may be linked to other traits that are more difficult to measure and do affect the individual's performance. In such cases, these unobservable genetic traits may be used as genetic markers for the linked traits because they indirectly indicate the presence of the characteristics of interest (De Vicente and Fulton, 2004). Studies of genetic variation in wild populations usually are designed to identify the processes causing microevolutionary change. The availability of easily accessible, locus-specific genetic markers is crucial to this endeavour (Arnold and Emms, 1992).

Molecular markers mainly deal with biological macromolecules either nucleic acids (i.e RNA or DNA from coding and non-coding parts of genome) themselves or gene products (i.e proteins resulting from the coding parts of the genome) that are able to reveal genetic variation (or polymorphism) among organisms and can be employed to address various biological problems. These naturally occurring protein-based and DNA-based markers are interpreted as genealogical markers which offer extraordinary power to illuminate the plethora of information regarding human forensics, disease diagnostics, wild life forensics, genetic parentage, reproductive modes, mating systems, kinship, population structure, dispersal and gene flow, intra-specific phylogeography, speciation, hybridization, introgression, phylogeny, taxonomy, systematics and conservation biology (Avise, 2004).

The polymorphism in the marker can be detected at three levels *viz.* 1) phenotypic (morphological) 2) differences in proteins (biochemical) or 3) differences in the nucleotide sequence of DNA/ RNA (nucleic acids).

### 2.8.1 Morphological/Taxonomical Markers

Traditionally diversity within and between population was determined by assessing differences in the morphology. Morphological markers also called as phenotypic markers generally correspond to the qualitative traits that can be scored visually. Morphological markers are usually dominant or recessive (Chawla, 2009). Much morphological variation depends on environmental/seasonal or developmental changes that affect many individuals in a population regardless of genotype

(Kapila *et al.*, 1997). Morphological characters have long been used to identify species, families and genera. These measures have the advantage of being readily available, do not require sophisticated equipment and are the most direct measure of phenotype, thus they are available for immediate use, an important attribute. However, morphological determinants and plant identification includes (1) Expert determination (2) Recognition (3) Comparison and (4) Use of taxonomic keys and similar devices. Identification is a basic activity and one of the primary objectives of systematic (De Vicente and Fulton, 2004).

On the other hand, various constraints of morphological markers have limited their use in genetic marker systems. The limitations include 1) they cause such large effects on phenotype that they are undesirable to be used in breeding programs 2) they mask the effects of linked minor gene(s), making it almost impossible to identify desirable linkages for selection 3) they are highly influenced by the environment. They are subject to changes due to environmental factors and may vary at different developmental stages and their number is limited. The morphology or phenotype is the outcome of genetic constitution (G) and its interaction with environment (E). Due to varying levels of G x E interaction, it is not appropriate to compare the morphological data of varieties that have been collected across different years and/or locations (Chawla, 2009). However, in the meantime it has been stated that characterization for morphological traits can not be replaced by any of the molecular techniques.

Also, Molecular Cytogenetics (the study of chromosomes) has occupied a prominent place in genetics in comparative biology and phylogenetic study (Sessions, 1996). A karyotype describes the phenotypic aspects of the chromosome complement of a species in terms of number, size, arm ratio (or centromere position), and other landmark features of its chromosomes (Levin, 2002). In the modern era, cytogenetics includes the incorporation of molecular methods that was initiated by the development of four main technological breakthroughs *viz.* (1) the discovery that hypotonic treatment spreads metaphase chromosomes, (2) the development of chromosome banding techniques, (3) the development of techniques for *in situ* hybridization (ISH) of nucleic acid probes to cytological preparations of chromosomes (Hsu, 1979; Macgregor, 1993; Sessions, 1996), and (4) the use of immunochemistry, in conjunction with ISH were applied not only for mapping sequences on chromosomes (Sessions, 1996).

### 2.8.2 Protein Based Markers /Biochemical Markers

To overcome the limitations of morphological traits, other markers have been developed at both proteins level (phenotype) and the DNA level (genotype) (De Vicente and Fulton, 2004). Biochemical markers are protein produced by gene expression (Chawla, 2009). Protein based

techniques can be either serological or electrophoretic (Rieseberg, 1997). For the generation of molecular markers based on protein polymorphisms, the most frequently used techniques is the electrophoretic separation of proteins, followed by specific staining of a distinct protein subclass (Weising *et al.*, 2005) and are compared (Rieseberg, 1997). Less commonly specific proteins are detected by monoclonal antibodies attached with an attached fluorescent label. The protein based techniques include isozymes, Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis, Western Blot etc.

#### 2.8.2.1 Isozymes / Allozymes

The advent of isozymes /allozymes as genetic markers in the early 1970s heralded a great advance for genetic studies of plant populations, since only morphological and in some cases cytological markers were available up to that time. Even today, isozyme markers are used to answer many research questions in analysis of genetic variation. Market and Moller coined the term 'isozyme' in 1959 to describe the multiple molecular forms of enzymes that exhibit the same enzymatic specificity. They are all functionally similar forms of enzymes, including all polymers of subunits produced by different gene loci or by different alleles at the same locus (Markert and Moller, 1959). Allozymes consists of another data set, which is a sub-set of isozymes which are variants of polypeptides representing different allelic alternatives of the same gene locus. It should be conceptually clear that Isozymes are enzymes that convert the same chemical substrate, but are not necessarily products of the same gene whereas Allozymes are isozymes produced by orthologous genes, but which differ in composition by one or more amino acids due to allelic differences (Weising *et al.*, 2005).

Isozymes, due to their net molecular charge have different electrophoretic mobilities. This difference in electrophoretic mobility provides a means of monitoring differences in isozymes and hence differences in species. Even so, isozymes are a robust complement to the simple morphometric analysis of variation (De Vicente and Fulton, 2004). Isozyme may be active at different life stages or in different cell compartments (Weising *et al.*, 2005). The use of isozymes as markers for identifying cultivars or genotypes is recommended as a supplementary molecular technique due to their low input cost (Sharma and Maloo, 2006). The isozyme electrophoretic technique has been extensively employed in the study of genetic variation in variety of agricultural crops including medicinal plants and also proven successful in varietal identification (Lebot *et al.*, 1991; Isshiki and Umezaki, 1997; Sonnante *et al.*, 1997; Yu *et al.*, 2001; Dias *et al.*, 2008; Mendioro *et al.*, 2008; Sanal kumar *et al.*, 2010).

Allozymes can be used in identification of clones, paternity analysis, characterization of various materials and population, predominant characteristics of reproduction (inbreeding) and genetic consequences of domestication and environmental impacts on populations (Muller-Starck, 1998). Allozyme markers have been successfully applied to many organisms from bacteria to numerous fungal, plant and animal species due to its codominant inheritance, the technical simplicity and low cost (Weising *et al.*, 2005). The utility of the isozyme/allozyme technique is limited due to the relatively low levels of polymorphisms detected among closely related genotypes (Golembewski *et al.*, 1997), restricted number of suitable allozyme loci in genome, the requirement of fresh tissue and sometimes limited variation (Weising *et al.*, 2005).

#### 2.8.2.2 Chemotaxonomic Markers

The rapidly expanding field of plant chemotaxonomy (chemosystematics) employs chemical constituents as a means of determining inter- and intraspecific relationships to infer phylogeny (Crawford and Giannase, 1982). However, multifarious chemical studies have come under the plant chemosystematics. Variation in medicinal plants is often noticed at chemical level which is due to the synthesis and accumulation of a wide variety of micromolecules that are often plant-specific. These compounds collectively grouped as secondary metabolites are 'high-value low–volume' compounds biosynthetically derived from primary metabolism which help to defend, tolerate, adapt and adjust themselves against abiotic and biotic stresses including insect pests and fungal and other pathogenic diseases. These phytochemical constituents may also reflect the genetic diversity or epigenetic responses or both (Van-Seters, 1997). Relatively small changes caused by mutations can give rise to large differences in the production of secondary plant products (Erdtman *et al.*, 1963).

Phytochemical variation is also possible among population, local races etc. and among different organs of the same plant. Ahmad *et al.*, 2010 developed the chemotaxonomic markers for misidentified medicinal plant *Onosma hispida* Wall. and G. Don. Covering detailed morpho-anatomical, palynological, features of crude drugs (roots) with UV, IR analysis and TLC fingerprinting (Flavonoids) serving the standard reference for correct identification in commercialization. Characteristic flavonoids as chemotaxonomic markers for *Erythroxylum austral* have been reported by Johnson and Schimidt (2004).

### 2.8.3 Nucleic Acid Based Molecular Marker Techniques

At present, many powerful DNA based techniques are available which are not confounded by environmental, pleiotropic and epistatic effects as morphological and biochemical markers

(Mondini *et al.*, 2009). Studies of nucleic acids (DNA, RNA) represent the latest and most powerful approach to plant identification and systematics and in recent years, increasing interest has been focused on DNA polymorphisms. Direct study of DNA enables the most fundamental and objective assessment to be made of the genetic similarities and differences of plant taxa. Like other taxonomic characters, nucleic acid data are comparable at all taxonomic levels (Rieseberg, 1997). They offer numerous advantages over conventional, phenotype based alternatives as they are stable and detectable in all tissues regardless of growth, differentiation, development or defence status of the cell (Mondini *et al.*, 2009).

The following properties would generally be desirable for a molecular marker (De Vicente and Fulton, 2004; Weising *et al.*, 2005; Mondini *et al.*, 2009): 1) Moderately to high polymorphic 2) Codominant inheritance (Which allows the discrimination of homo and heterozygous state in diploid organisms) 3) Unambigous assignment of alleles 4) Frequent occurrence in the genome 5) Even distribution throughout the genome 6) Selectively neutral behavior (i.e. no pleiotropic effects) 7) Easy access/ Inexpensive (i.e. by purchasing or fast procedures) 8) Easy and fast assay (e.g., by automated procedures) 9) High reproducibility 10) Easy exchange of data between laboratories 11) Low cost for both marker development and assay. But no single type of molecular marker fulfils all of these criteria. However, one can choose the one with most of above mentioned characteristics (Weising *et al.*, 2005).

Due to the rapid developments in the field of molecular genetics, a variety of different techniques have emerged to analyze genetic variation during the last few decades (Whitkus *et al.,* 1994; Karp *et al.,* 1996, 1997a, b; Parker *et al.,* 1998; Schlötterer, 2004; Weising *et al.*, 2005; Spooner *et al.*, 2005). These genetic markers may differ with respect to important features, such as genomic abundance, level of polymorphism detected, locus specificity, reproducibility, technical requirements and financial investment. No marker is superior to all others for a wide range of applications. The most appropriate genetic marker will depend on the specific application, the presumed level of polymorphism, the presence of sufficient technical facilities, time constraints and financial limitations (Spooner *et al.*, 2005; Mondini *et al.*, 2009). Based on the need for ecological, evolutionary, taxonomical, phylogenetic and genetic studies, various marker techniques can be selected (Mondini *et al.*, 2009).

The advanced marker techniques also utilize newer classes of DNA elements such as retrotransposons, mitochondrial and chloroplast based microsatellites, allowing increased genome coverage with better resolution and sensitivity (De Vicente and Fulton, 2003; Mondini *et al.*, 2009). As each individual has its own unique DNA sequence, this can be easily exploited in genetic variation studies (Weising *et al.*, 2005).

The different techniques employed are either Non–PCR based techniques (based on restriction-hybridisation of nucleic acids) or techniques based on Polymerase Chain Reaction (PCR) or both or sequencing based techniques (Joshi *et al*, 1999; Weising *et al.*, 2005; Spooner *et al.*, 2005; Semagn *et al.*, 2006)

### i) Non-PCR based techniques

- Restriction Fragment Length Polymorphisms (RFLP, Botstein *et al.* 1980; Neale and Williams 1991)
- Minisatellites or Variable Number of Tandem Repeats (VNTR, Jeffreys *et al.*, 1985a, b)

### ii) PCR-based techniques

#### *PCR with Arbitrary primers:*

-Random Amplified Polymorphic DNA (RAPD, Williams *et al.* 1990)

-Arbitrarily Primed Polymerase Chain Reaction (AP-PCR, Welsh and McClelland 1990; Williams *et al.* 1990)

-DNA Amplification Fingerprinting (DAF, Caetano-Anolles *et al.* 1991)

- Amplified Fragment Length Polymorphism (AFLP, Vos *et al.* 1995)

-Inter-Simple Sequence Repeat (ISSR, Zietkiewicz *et al.*, 1994; Godwin *et al.*, 1997)

#### *Specific Primed PCR (Site-Targeted PCR)*

- Sequence Characterized Amplified Region (SCAR, Paran and Michelmore, 1993)

-Cleaved Amplified Polymorphic Sequence (CAPS, Konieczny and Ausubel 1993)

-Microsatellites/ Simple Sequence Repeats (SSR)/ Short Tandem Repeats (STRs), Sequence Tagged Microsatellite (STMs) or Simple Sequence Length Polymorphism (SSLP) (Hearne *et al.* 1992; Morgante and Olivieri 1993; Queller *et al.* 1993; Jarne and Lagoda 1996)

### iii) DNA sequencing based markers

-Nuclear rDNA sequences such as Internal Transcribed Spacer (ITS), 18S, 26S etc.

-cpDNA and mtDNA sequences such as rbcL, matK, COI etc coding and non coding parts of genome

-Single Nucleotide polymorphisms (SNP)

They assess either multi-locus or single-locus markers. Multilocus markers allow simultaneous analyses of several genomic loci, which are based on the amplification of casual chromosomic traits through oligonucleotide primers with arbitrary sequences. These types of markers are defined as dominant since it is possible to observe the presence or the absence of a band for any locus, but it is not possible to distinguish between heterozygote (Aa) and homozygote (AA) conditions thus estimation of allele frequencies cannot be estimated using the dominant markers like RAPD, AP-PCR, ISSR etc. (Weising *et al.*, 2005). In contrast, single-locus markers employ probes or primers specific to genomic loci, and are able to hybridize or amplify chromosome

traits with well-known sequences. They are defined as co-dominant as they allow discrimination between homozygote and heterozygote loci eg. SSR markers (Mondini *et al.*, 2009).

### 2.8.3.1 Non-PCR Based Marker Techniques

These marker techniques does not depends on the amplification of the nucleic acid, instead variation analysis is performed to visualize DNA profiles by hybridizing the restriction enzyme – digested DNA, to a labeled probe, which is a DNA fragment of known origin or sequence (Joshi *et al.*, 2004). Variation in this DNA sequence is the basis for the genetic diversity within a species. Restriction endonucleases are bacterial enzymes able to cut DNA, identifying specific palindrome sequences and producing polynucleotide fragments with variable dimensions. Any changes within sequences (i.e. point mutations), mutations between two sites (i.e. deletions and translocations) or mutations within the enzyme site, can generate variations in the length of restriction fragment obtained after enzymatic digestion. RFLP and VNTRs markers (Mondini, 2009), probes for microsatellites and minisatellites (Kurane, 2009) are examples of molecular markers based on restriction-hybridisation techniques.

#### *Restriction Fragment Length Polymorphism (RFLP)*

All organisms have numerous differences in their genomic DNA sequence and therefore are genotypically distinct (Semagn *et al.*, 2006). This difference results in a restriction fragment length polymorphisms upon digestion with Restriction Enzymes. The homology of the restriction digested fragments can be detected by Southern hybridization (Somasundaram and Kalaiselvan, 2009). RFLP is the most widely used hybridization-based molecular marker. In RFLP analysis, restriction enzyme-digested genomic DNA is resolved by gel electrophoresis and then blotted (Southern, 1975) on to a nitrocellulose membrane. Specific banding patterns are then visualized by hybridization with labeled probe. These probes are mostly species – specific single locus probes of about 0.5 to 3 kb in size, obtained from a cDNA library or a genomic library (Joshi *et al.*, 1999) and ultimately the scoring of RFLP bands is done by observing autoradiographs (Kochert, 1994). Labelling of the probe may be performed with a radioactive isotope or with digoxigenin or fluorescein (non-radioactive).

RFLP is a codominant marker system. They are reliable markers in linkage analysis and breeding and can easily determine if a linked trait is present in a homozygous or heterozygous state in individual (Winter and Kahl, 1995). It has been widely practiced for economically important crops (De Vicente and Fulton, 2004). RFLP have thus been widely employed in determining linkage maps, in identifying genes linked to characters of agronomic importance and in mapping the

quantitative trait loci (QTL) (Young *et al.*, 1988; Paterson *et al.*, 1990). These maps are significant tools for marker assisted selection in breeding programs and map based cloning of genes (Nienhuis *et al*, 1987; Tanksley *et al.*, 1992). Applications of RFLP are also observed in areas of phylogenetic studies (Schmit *et al.*, 1993; Vekemans *et al.*, 1998; Ando *et al.*, 2005) and cultivar identification (Corniquel and Mercier, 1994; Powell *et al.*, 1996; Busti *et al.*, 2002). Restriction site analysis of cpDNA genome remained the molecular tool of choice for inferring phylogenetic relationships for nearly a decade (Soltis and Soltis, 1998).

Although being a highly robust technology, some of its inherent complexities lead substitute RFLP with alternative PCR based techniques (De Vicente and Fulton, 2004). The assay is time consuming and labour intensive too (Moodie *et al.*, 1997; Joshi, 1999; Mondini, 2009). Moreover, the prerequisite of prior sequence information for probe construction contributes to the complexity of the methodology. Also, use of RFLP technique has been hampered due to the requirement of large amount of DNA for restriction digestion and Southern blotting. The requirement of radioactive isotope makes the analysis relatively expensive and hazardous. Therefore, RFLP based studies are rarely applied for population genetics and biodiversity studies of wild species (Mondini, 2009).

### *Variable number of tandem repeats (VNTRs)/Microsatellites/Minisatellites*

Interspersed within the genomes of plants and other higher organisms are hypervariable regions which are comprised of tandemly repeated DNA sequences. There are two classes: 'microsatellites' or 'Simple Sequence Repeats'(SSRs/STRs) where the basic repeat unit is around 2-8 base pairs in length and 'minisatellites' for longer units of around 16-100 base pairs. Microsatellites are also known as Simple Sequence Repeats (SSRs; Jacob *et al.*, 1991) or Short Tandem Repeats (STRs; Edwards *et al.*, 1991). Minisatellite analysis, like RFLPs, also involves digestion of genomic DNA with restriction endonucleases, but minisatellites are a conceptually very different class of marker (Spooner *et al.*, 2005). Hybridisation to restricted DNA with micro or minisatellite probes gives multilocus patterns which can resolve variation at the level of populations and individuals (Beyerman *et al.*, 1992; Karp *et al.*, 1997b; Spooner *et al.*, 2005). The variation results from changes in the number of copies of the basic repeat and is often referred to as Variable Number of Tandem Repeats (VNTRs). In principle, VNTR loci are co-dominant markers (Arens *et al.*, 1995; karp *et al.*, 1997b).

It is a variation of traditional RFLPs that used synthetic oligonucleotides of simple sequence repeat complexities to detect variations in hypervariable regions of the genome and is best suited for identification of genotypes. The most commonly used sequences are $(AT)_8$, $(AG)_8$, $(CT)_8$,

$(GACA)_4$ and $(GATA)_4$ repeats. They can also be used for cultivar identification in the same way as RFLPs (Bhat, 2001).

Microsatellite markers have been characterized in number of crops, particularly in the development of linkage maps (Wang *et al.*, 1994). The marker has been used to study heterozygosity in wild yam species, *Dioscorea tokoro* (Terauchi and Konuma, 1994). The analysis of VNTR loci are proven to be economic, fast and simple technique, beside being reproducible and reliable for fingerprint generation and establishment of genetic relations as undertaken in buck eyes (*Aesculus* Sp) (Lim *et al.*, 2002); *Rubus moluccanus* L. (Busemeyer *et al.*,1997).

### 2.8.3.2 PCR-Derived Marker Techniques

PCR is a molecular biology technique for enzymatically replicating (amplifying) small quantities of DNA without using a living organism. It is used to amplify a short (usually up to 10 kb), well-defined part of a DNA strand from the genome of organism (Semagn *et al.*, 2006). The invention of Polymerase Chain Reaction (PCR) by Kary B. Mullis and FA faloona (Saiki *et al.*, 1985) has revolutionized the methodological repertoire of molecular biology and commenced in the development of variety of PCR-based techniques in the field of research in plant identification as well as analysis of genetic variation (Aert *et al.*, 1998; Weising *et al.*, 2005). Even though traditional locus specific hybridization based techniques and RFLPs are still in use in some laboratories, they have already been largely replaced by more sensitive and convenient PCR-based marker technologies. To amplify a particular DNA sequence, two single stranded oligonucleotide primers are designed complementary to motifs on the template DNA. Addition of a thermostable DNA polymerase in a suitable buffer system and cyclic programming of primer annealing, primer extension and denaturation steps results in the exponential amplification of the sequence between the primer binding sites.

The major advantages of PCR techniques compared to hybridization-based methods include: 1) requirement of only small amount of DNA 2) elimination of radioisotopes in most techniques 3) can amplify DNA sequences from preserved tissues 4) accessibility of methodology for small labs in terms of equipment, facilities, and cost 5) no prior sequence knowledge is required for many applications, such as AP-PCR, RAPD, DAF, AFLP and ISSR 6) detect high polymorphism that enables to generate many genetic markers within a short time, and 7) simultaneous screening of many genomes for direct collection of data or as a feasibility study prior to nucleotide sequencing efforts (Wolfe and Liston, 1998). These advantages, however, can vary depending on the specific technique chosen by the researcher.

The various PCR-based techniques are of two types depending on the primers used for amplification *viz.* 1) Arbitrary primed PCR techniques developed without prior sequence information (e.g., RAPD, AP-PCR, DAF, AFLP, ISSR). 2) Specifically primed site-targeted PCR techniques that developed from known DNA sequences (e.g., EST, CAPS, SSR, SCAR, STS etc.).

### *PCR with Arbitrary primers*

Unlike PCR reactions that amplify specific targeted regions of the genome i.e specifically primed PCR, PCR with arbitrary primers does not require sequence information for construction of flanking primers. Furthermore, the single primer is used which amplifies multiple genomic regions that vary in their presence among individuals and species (Weising, *et al.*, 2005). It offers an inexpensive means to detect genetic variation at a large number of genomic regions in a single PCR reaction. These polymorphisms provide a powerful tool to discriminate closely related species even down to the level of subspecies. This technology has been extensively used in different areas of genetics including DNA fingerprinting, population genetics, genome mapping and molecular taxonomy (Clapp, 1996).

### *1) Random Amplified Polymorphic DNA (RAPD)*

RAPDs were the first PCR-based molecular marker to be employed in genetic variation analysis which can be used as a superior alternative to isozyme and RFLP analysis (Williams *et al.*, 1990). It is technically simple and employs the random amplification of genomic DNA using short primers (decamer with GC content of at least 50%), separation of the obtained fragments on agarose gel and detected by staining with ethidium bromide and finally visualization under ultraviolet light. Although, the primer sequences are random, they are able to find homologous sequences suitable for annealing. To obtain effective amplification products via RAPD, two identical target sequences for primer must exist in close proximity on opposite DNA strands to allow DNA amplification to take place. The distance between those two sites should not exceed 3 kb (Black, 1996). DNA polymorphisms are then produced by rearrangements, insertions, substitutions or deletions at or between oligonucleotide primer binding sites in the genome (Williams *et al.*, 1990). As this approach requires no prior knowledge of the genome analyzed it can be employed across species using random primers. The simplicity, feasibility of automation and wide applicability of the RAPD technique have captivated many scientists' interests. Perhaps the main reason for the success of RAPD analysis is the gain of a large number of genetic markers that require less amounts of DNA (10-100ng per reaction) (Karp *et al*, 1997a) without the

requirement of cloning, sequencing or any other form of the molecular characterization of the genome (Clapp, 1996; Bardakci, 2001).

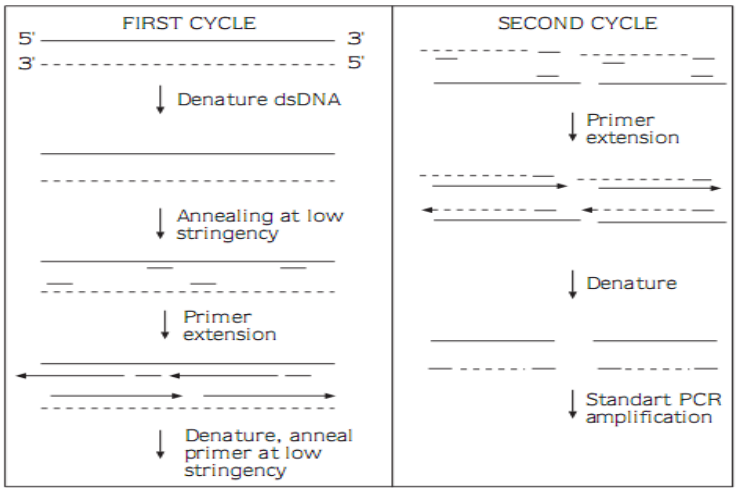



**Figure 2.7** Schematic diagram of RAPD reaction for 2 loci (derived from Welsh and McClelland, 1994)

RAPD markers have been used in the detection of DNA polymorphisms, cultivar fingerprinting (Xu *et al*; 1995) and in identification of markers for specific genes (Martin *et al.*, 1991; Paran *et al.*, 1991). Among the different types of molecular markers available, RAPD has been widely used for the assessment of genetic diversity among rare species (Williams *et al.*, 1990), despite its existing limitations (dominant mode of inheritance of RAPD loci), because of their simplicity, speed and relatively low costs as compared to other molecular markers. One of the most widely used applications of the RAPD technique is the identification of markers linked to traits of interest without the necessity for mapping the entire genome. Martin *et al.* (1991) have described an efficient method based on the RAPD technique to isolate DNA segments linked to certain traits. This approach based on near-isogenic lines (NILs) is accomplished by repeatedly backcrossing a line carrying a gene of interest (donor parent) to a cultivated line having otherwise desirable characteristics (recurrent parent). The introgression of the target gene produces a line with a small segment of donor parent genome in a genetic background, which is almost exclusively from the recurrent parent. Thus, markers that show polymorphisms between these 2 lines are likely to be linked to the gene of interest. RAPD analysis of NILs has been successful in identifying markers linked to disease resistance genes in *Lycopersicon* sp. (Martin *et al.*, 1991), in *Lactuca* sp. (Paran *et al.*, 1991) and in *Phaseolus vulgaris* (Adam-Blondon *et al.*, 1994). Klein-Lankhorst *et al.* (1991) identified chromosome specific RAPD markers in tomato.

Some problems may be associated with RAPD like Reproducibility (Jones *et al.*, 1997) and co-migration of some non-homologous fragments (Rieseberg, 1996). However, reproducibility can

be considerably improved by making the reaction condition optimum and uniform across samples and by thorough screening of primers for reproducibility (Skroch and Nienhuis, 1995). The problem associated with co-migration of non-homologous fragments is minimal when RAPD is applied to study closely related populations or species (Rieseberg, 1996).

### *2) Arbitrary primed PCR (AP-PCR)*

This is a special variant of arbitrarily primed PCR introduced by Welsh and McClelland (1990), where in discrete amplification patterns are generated by employing single primers of 20 or more bases in length in PCR of genomic DNA. Radio-labelled nucleotides are included in the last 20 to 30 cycles only. PCR products are separated by polyacrylamide gel electrophoresis and made visible by autoradiography, so not popular as compared to DAF. However, recently it has been simplified by separating the fragments on agarose gels and using ethidium bromide staining for visualization. It was found used for fingerprinting of genomes (Welsh and McClelland, 1990; Joshi *et al.*, 1999).

### *3) DNA Amplification Fingerprinting (DAF)*

DNA amplification fingerprinting (DAF) is another variant of arbitrarily primed PCR which employ single very short (often only five to eight nucleotide long) arbitrary primer (Caetano-Anolles *et al.*, 1991) at relatively high concentrations (~3µM). The resulting fragments are resolved in polyacrylamide gels and visualized by silver staining instead of Ethidium Bromide (Weising *et al.*, 2005). DAF requires careful optimization of parameters. However it is extremely amenable to automation and fluorescent tagging of primers for early and easy determination of amplified products. This technique has been useful in genetic typing and mapping based on its complex and simple patterns of bands it produce (Joshi *et al.*, 1999).

### *4) Amplified Fragment Length Polymorphism (AFLP)*

AFLP technology introduced by Vos *et al.* (1995) represents an ingenious combination of RFLP analysis and PCR. It is applicable to all organisms without previous sequence information and generally results in highly informative fingerprints. It rapidly become one of the most popular and powerful approaches to detect DNA polymorphisms (Weising *et al.*, 2005). The technique involves 3 steps: a) Restriction of the DNA and ligation of oligonucleotide adapters, b) Selective amplification of sets of restriction fragments and c) gel analysis of the amplified fragments (Vos *et al.*, 1995). The PCR primers consist of a core sequence (part of the adapter), and a restriction enzyme specific sequence and 1–5 selective nucleotides (the higher the number of selective

nucleotides, the lower the number of bands obtained per profile). The AFLP banding profiles are the result of variations in the restriction sites or in the intervening region (Spooner *et al.*, 2005)

AFLP is applied as versatile tool in molecular taxonomy, population genetics, germplasm cahracterization, identification of clones, cultivars, genetic linkage maps construction (Joshi *et al.*, 1999; Weising *et al.*, 2005). AFLP has already been used successfully for variation analysis of number of plants; *Swertia* species (Misra *et al.*, 2010); almond (Sorkheh *et al.*, 2007); barley (Russel *et al.*, 1997); *Jatropha curcas* (Tatikonda *et al.*, 2009); *Cynodon* species (Wu *et al.*, 2005); tea (Paul *et al.*, 1997); *Olea europaea* L. (Sensi *et al.*, 2003).

### *5) Inter Simple Sequence Repeats (ISSR)*

Inter Simple Sequence Repeats (ISSRs) are DNA fragments of about 100–3000 bp located between adjacent, oppositely oriented microsatellite regions. ISSR marker technique is a PCR based method, which involves amplification of DNA segment present at an amplifiable distance in between two identical microsatellite repeat region oriented in opposite direction. The technique uses microsatellites, usually 16-25 bp long, as primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly the inter – SSR sequences of different sizes. The microsatellite repeats used as primers can be dinucleotide to pentanucleotide repeats. The primers used can be either unanchored (Gupta *et al.*, 1994; Wu *et al.*, 1994; Spooner *et al.*, 2005; Semagn *et al.*, 2006) or more usually anchored at 3’ or 5’ end with 1 to 4 degenerate bases extended into the flanking sequences (Zietkiewicz *et al.*, 1994). However, they have also been shown to segregate as co-dominant markers in some cases thus enabling distinction between homozygotes and heterozygotes (Wu *et al.*, 1994; Wang *et al.*, 1998). The technique is useful in areas of genetic diversity, phylogenetic studies, gene tagging, genome mapping and evolutionary biology in wide range of plant species. Recently ISSR-based markers have found wide applicability in pharmacognostic characteristics of medicinal plants (Kurane *et al.*, 2009) such as *Swertia chirayita* (Joshi and Dhawan, 2007), *Glycyrrhiza uralensis* (Yao *et al.*, 2008), *Humulus lupulus* (Patzak *et al.*, 2001) and *Psychotria ipecacuanha* (Rossi *et al.*, 2009).

### *Specific primed PCR (Sequence targeted PCR)*

The approach of the specific primed PCR is opposite to the arbitrarily amplicon profiling procedure in which primers are designed to target specific regions of the genome (Chawla, 2009). A particular DNA sequence can be amplified by a pair of specific primers, the sequence of which is designed on the basis of sequence information of that particular organism under study (Karp *et al.*, 1997b; Chawla, 2009). A Sequence tagged sites (STS) is the general term given to a

marker defined by its primer sequences. STS is a short unique sequence (60 to 1000 bp) that can be amplified by PCR, which identifies a known location on a chromosome.

### *1) Sequence Characterized Amplified Regions (SCARs)*

Paran and Michelmore (1993) introduced a new type of RAPD derived molecular marker, which circumvented several of the drawbacks inherent to RAPDs. Although RAPD is a useful tool for genetic analysis, it is very sensitive to reaction conditions, which renders it less useful for routine analysis of large number of plant samples. The new markers were generated by cloning and sequencing RAPD derived fragments of interest and designing long (24 - mer) oligonucleotide primers complementary to the ends of the original RAPD fragment. When these primers were used in a PCR with the original template DNA, single loci called Sequence Characterized Amplified Regions (SCARs) were specifically amplified. Thus, SCAR is an example of a specific-primed PCR (Kesseli *et al.*, 1992; Paran and Michelmore, 1993). Conversion of RAPD markers to SCAR markers is an efficient advent for the development of robust diagnostic PCR markers in which RAPD fragments of interest have to be isolated from the gel, purified and cloned into an appropriate vector. Further sequencing is carried out to design longer primers for SCAR-PCR that can be applied effectively in robust diagnostic and typing (Paran and Michelmore, 1993; Shrestha, 2001; Gostimsky *et al.*, 2005; Weising *et al.*, 2005).

Study of species - specific SCAR markers have been widely accepted for fingerprinting and authentication of the major valued plants such as *Sinocalycanthus chinensis* (Qian *et al.*, 2006); *Amaranthus* species (Ray and Roy, 2008); *Phyllanthus emblica* (Dhyaneshwar *et al.*, 2006); *Ipomoea mauritiana* (Devaiah *et al.*, 2010); *Panax* species (Wang *et al.*, 2001); *Momordica charantia* L. (Paul *et al.*, 2010); *Artemisia princeps* (Lee *et al.*, 2006); *Aquilaria* species (Lee et a., 2011) etc.

### *2) Cleaved Amplified Polymorphic Sequence (CAPs)/PCR - RFLPs*

Williams *et al.* (1991) propounded molecular markers by digesting PCR products with restriction enzymes. CAPs markers are generated in two steps. At first, a defined DNA sequence is amplified using a sequence – specific primer pair. This may already result in differently sized and hence informative PCR fragments (Williams *et al.*, 1991). In the second step, the PCR product is digested with a restriction enzyme and subjected to agarose gel electrophoresis and visualized by ethidium bromide staining (Chawla, 2009). CAPs markers can be generated from either nuclear or organellar DNA. The CAPs approach doesnot require radioactivity or blotting steps, small

quantity of DNA is sufficient, the technique is quick and robust and marker is co dominant (Chawla, 2009; Weising *et al.*, 2005).

One way of discriminating individual using its ITS region is amplifying the ITS region and then amplified DNA is cut with a series of restriction fragment patterns as done for rapid identification of *Phytophthora* species (Ristaino *et al.*, 1998); *Amaranthus* spp.(Lanoue *et al.*, 1996).

### *3) Simple Sequence Repeats (SSRs) or Microsatellites or Short Tandem Repeats*

Microsatellites also known as Simple Sequence Repeats (SSRs) or Short Tandem Repeats (STRs), are polymorphic loci present in DNA that consist of repeating units of one to six base pairs in length (Bidichandani *et al.*, 1998). Microsatellites are ubiquitous components of all eukaryotic genomes and were also found in prokaryotes (Weising *et al.*, 2005) eg. $(CA)_n$ repeat, where n is variable among different alleles. The repeat present consist of frequent di-, tri- and tetra – nucleotides (eg. A, T, AT, GA, AGG, AAAC) which can be repeated many times. Polymorphisms associated with a specific locus are due to the variation in length of the microsatellite that in turn depends on the number of repetitions of the basic motif. As compared to other neutral regions of DNA, Microsatellite owe their variability due to an increased rate of mutation. These high rates of mutation can be explained most frequently by slipped strand mispairing (slippage) during DNA replication (Somasundaram and Kalaiselvam; 2009).

Microsatellites can be amplified for identification by PCR using the unique sequences of flanking regions as primers. The most common way to detect microsatellites is to design PCR primers that are unique sequences of flanking regions (Chawla, 2009). Therefore, a single pair of PCR primers works for every individual in the species and produce different sized products for each of the different length of microsatellites (Arif *et al.*, 2010). They are popular as they possess: co-dominant inheritance, high abundance, enormous extent of allelic diversity, ease of assessing SSR size variation through PCR and high reproducibility.

The extensive length polymorphism observed at SSR loci has made them a very popular marker type for linkage mapping and genetic diversity studies (Henry, 2006). Microsatellite based marker technique has been used to establish conservation strategy of endangered plants such as *Tricyrtis ishiiana* (Setoguchi *et al.*, 2010); *Calystegia soldanella* (Noda *et al.*, 2009) and *Galium catalinense* subspecies acrispum (Mcglaughlin *et al.*, 2009). Resulting markers were variously called Simple sequence length polymorphisms (SSLPs), Sequence – tagged microsatellite sites (STMs), SSRs or microsatellite markers (Weising *et al.*, 2005).

### 2.8.3.3 DNA Sequencing Based Markers

In recent years, DNA sequencing also has become popular for genetic studies. Among the numerous approaches to the study of polymorphism at DNA level, certainly the most direct strategy is determination of the nucleotide sequence of a defined region. Two basic strategies of DNA sequencing were devised in the mid 1970s: a) Chemical based method (Maxam and Gilbert, 1977) and b) Enzyme based method (Sanger *et al.*, 1977). DNA sequencing provides highly robust, reproducible, and informative datasets, and can be adapted to different levels of discriminatory potential by choosing appropriate genomic target regions. On the negative side, DNA sequencing can be prohibitively tedious and expensive when very large numbers of individuals have to be assayed (eg. In population genetics, phylogeography and marker assisted plant breeding programs) (Weising *et al.*, 2005). DNA sequencing of different genomes (nuclear, chloroplast and mitochondrial) and different genes (matK, rbcL, ndhF of chloroplast genome and ribosomal DNA sequences such as 18S, 26S, ITS and 5S of nuclear genomes) are widely applied in the phylogenetic reconstruction and diagnostic development for DNA based studies (Shrestha *et al.*, 2003).

#### *1) Internal Transcribed Spacer (ITS)*

The nuclear rDNA region has frequent insertion/deletions which can be phylogenetically informative (Baldwin *et al.*, 1995). The nuclear ribosomal transcriptional unit (NRTU) is comprised of 18S, 5.8S, and 28 S genes with two Internal Transcribed Spacer (ITS) (ITS-1 and ITS-2). Genes encoding ribosomal RNA and spacers occur in tandem repeats that are hundred to thousands of copies long; each separated by regions of non-transcribed DNA termed intergenic spacer (IGS). ITS and IGS are used for genetic variation studies at the genus, species and population levels (Soltis and Soltis, 1998; Alvarez and Wendel, 2003).

Besides, sequence comparison of the ITS region is widely used in taxonomy and molecular phylogeny because it a) is easy to amplify even from small quantities of DNA and b) has a high degree of variation even between closely related species. ITS of nrDNA have been widely used for resolving phylogenetic relationships among closely related species (Lanoue *et al.*, 1996; Shrestha *et al.*, 2003; Li *et al.*, 2009; Joshi, 2011); molecular authentication of herbal materials (Zhang *et al.*, 2007); genetic diversity assessment (Mondini *et al.*, 2009); intra-specific variation study (Haque *et al.*, 2009) and DNA barcoding (Zuo *et al.*, 2010).

#### *2) Single Nucleotide Polymorphisms (SNPs)*

SNP represent the most abundant source of DNA polymorphisms in organisms which is characterized by a single base substitution at a particular position (Weising *et al.*, 2005). They represent a defined position at a chromosomal site at which the DNA sequence of two individuals differs by a single base (A, T, C or G). Direct sequencing of DNA fragments (amplified by PCR) from several individuals and aligning is the most direct way to identify SNP polymorphisms (Chawla, 2009). In addition, the dramatic increase in the number of DNA sequences submitted to the database has made it possible to identify SNPs for several crops by electronic mining (e-minning) without the need for sequencing (Taillon-Miller *et al.*, 1998; Somers *et al.*, 2003). This approach consists in the identification and alignment of sequences from the same locus from different sources (genotypes) allowing the detection of SNPs along these DNA sequences. The availability of Expressed Sequence Tag (EST) databases makes it possible to target polymorphisms in functional regions of the genomes and even to specific gene (Chawla, 2009).

They are used for a wide range of purposes, including rapid identification of crop cultivar and construction of ultra high density genetic maps (Spooner *et al.*, 2005; Mondini *et al.*, 2009).

### 2.8.4 Recent Advancements

DNA microarray is a multiplex technology used in molecular biology and in medicine. It consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides, called features, each containing picomoles of a specific DNA sequence known as probes (reporters) (Hao *et al.*, 2010) printed on an impermeable solid support, usually glass, silicon chips or nylon membrane. Several novel technologies differential display PCR, northern blots, quantitative PCR, serial analysis of gene expression (SAGE) and TIGR (The Institute for Genome Research) orthologous gene alignments (TOGA) are used alongside microarrays as research tool allowing rapid and detailed analysis of thousands of transcripts, providing a revolutionary approach to the investigation of gene expression. DNA microarray are efficiently applied in pharmacodynamics (for discovery of new diagnostic, prognostic indicators and biomarker of therapeutic response with elucidation of molecular mechanism of action of herb); in pharmacogenomics; in pharmacognosy (for correct botanical identification and authentication of crude plant materials as a part of standardization and quality control, safety and efficacy (Khan *et al.*, 2009). Microarray based DNA polymorphism assays have been developed for the identification of herbal medicines (Joshi *et al.*, 2004).

## 2.9 Applications of Nucleic Acid Based Molecular Marker Techniques

Applications of molecular marker techniques can be diverse (Shrestha, 2011, pers.comm) which are reviewed in the following section.

### 2.9.1 Genetic Diversity / Genetic resources conservation / Conservation genetics / Population genetics

Determination of existing genetic diversity in a species and explanation of diversity in terms of its origin, organization and maintenance are thus of fundamental significance in the application of genetic principles to conservation. An essential prerequisite for a species to survive against environmental pressures is the availability of a pool of genetic diversity and in its absence, extinction would appear inevitable (Frankel, 1993).

Evolution results from natural selection acting on diversity in populations, which ultimately stems from mutations. Extensive comparative analyses across genomes of model organisms elucidate diversity in nature. Genetic diversity is genealogically arranged across diverse temporal scales from family units, extended kin-groups and phylogeographic population structures within species to graded magnitudes of genetic divergence among species that became phylogenetically separated at various times in the past. It has now been well established that phenotypic diversity is not reliable guide to the understanding the way of arrangement in this genealogical diversity (Avise, 2004).

The importance of plants as valuable sources for chemical compounds of medicinal value is well known from time immemorial. The general principle that governs over the conservation of any species is the inclusion and maintenance of maximum genetic diversity (Wickneswari and Norwati, 1993; Stewart and Porter, 1995). Apart from habitat degradation and loss, in medicinal plants in particular, injudicious collection is yet another important reason for genetic depletion and endangerment of species. Understanding the molecular basis of the essential biological phenomena in plants is crucial for the effective conservation, management, and efficient utilization of plant genetic resources (PGR). In particular, an adequate knowledge of existing genetic diversity in plant population is of fundamental significance for basic science and applied aspects like the efficient management of the medicinal valued plant *in situ* environment (Mondini, 2009).

Molecular markers are routinely used indispensable tools for assessing genetic diversity within and between populations of plant species. Low assay cost, affordable hardware, throughput,

reproducibility, the level of information expecting, convenience and ease of assay development and automation are important factors when choosing a molecular assessment technology (Rafalski and Tingey, 1993; Rafalski, 2002, Arif *et al.*, 2010). Molecular genetic variability assessment can produce valuable insights of within population genetic variation in terms of heterozygosity estimates and allele frequency estimates, which can be correlated with populations' survival probability over ecological or evolutionary history (Avise, 2004).

The general goals of population genetic studies are to account for and characterize the extent of genetic variation within species. Variation provides the raw material for future evolutionary change and may provide evidence for different evolutionary events in the past (Weir, 1996). The genetic structure of natural populations is one of the central issues in population genetic studies that play significant role in understanding of speciation, adaptation or genetic change in the species or population level (Syamsuardi and Okada, 2002). Long term survival and evolution of species depend on the maintenance of sufficient genetic variation within and among populations to adapt to new selection pressures as those exerted by environmental changes (Barrett and Kohn, 1991). Therefore, understanding of the genetic variation within and between populations together with the information of genetic drift and gene flow between populations are required for the establishment of effective conservation strategies for endemic, vulnerable and endangered plant species (Hamrick and Godt, 1996; Lande, 1999).

Molecular markers have been widely used for population genetic studies in plants such as RAPD (Dharmar and Britto, 2011; Sebastian *et al.*, 2010; Roy and Chakraborty, 2009; Hoque and Rabbani, 2009; Sozen and Ozaydin, 2010; Shi *et al.*, 2008), ISSR (Hollingsworth *et al.*, 1998; Hodkinson *et al.*, 2002; Wang *et al.*, 2004), AFLP (Issagi *et al.*, 2004 and SSR (Reusch *et al.*, 2000; Rossetto *et al.*, 2004).

The variations detected in chloroplast DNA (cpDNA) using PCR-RFLP technique are useful for population genetic studies at both the interspecific and the intraspecific level (McCauley, 1995; King and Ferris, 1998; Fineschi *et al.*, 2000). In some studies, mitochondrial DNA (mtDNA) variations have been very informative. Polymorphisms in mtDNA are geographically structured at the local scale in *Thymus vulgaris* (Manicacci *et al.*, 1996) and at the regional scale in *Beta vulgaris* ssp *maritima* (Desplanque *et al.*, 2000). Mohanty *et al.* (2003) investigated cpDNA and mtDNA diversity of *Prunus spinosa* characterizing cpDNA and mtDNA haplotypes and phylogenetic and geographic relationship between them which can be useful for identifying populations for conservation and to formulate their management strategies.

### 2.9.2 Molecular diagnostics / Authentication/DNA fingerprinting / DNA barcoding

Based on the positive therapeutic results, herbal medicines are gaining popularity worldwide for human wellbeing and healthcare. Unfortunately, one major hurdle that might impair their potential future as 'medicine of choice' is the lack of standardisation. Many scientific studies on adulteration of herbal medicines have demonstrated that health consequences of adulterants may vary from life threatening to death. The breakthrough in genetic analysis and identification promise herbal medicines challenging era. Genetics permit the capacity to police adulterants to protect patients and public from dangerous fraud (Tewfik, 2008). For this, Genuine sample specific markers are required to maintain the quality of medicinal herb for medicine formulation. Identification of herbal medicinal materials by DNA technology has been widely applied, initiated from the mid 1990s.

Even today, 80% of the world's population uses traditional medicine for healthcare and therapeutic purposes (WHO, 2008). However, adulteration of herbal medicines remains a major concern of users and industry for reasons of safety and efficacy (Li *et al.*, 2011). According to the need of sensitivity, reliability, reproducibility and running cost, most commonly applied DNA-based technologies are RAPD, RFLP, CAPs, AFLP, DAF, ISSR, Sequencing, hybridization and microarrays (Heubl, 2010).

Recent achievements in the field of DNA barcoding and DNA chip technology offer great potentials for screening of DNA and are emerging as new developments for future identification of species. The DNA barcode is a short DNA sequence from a standard part of genome used to identify species. To standardize the international use of DNA barcodes, the scientific community has made considerable efforts searching for suitable DNA regions to barcode every species (Kress *et al.*, 2005; CBOL Plant Working group, 2009; Yao *et al.*, 2010). In recent years, DNA-barcoding of global plant species using four standard barcodes (rbcL, matK, trnH-psbA and ITS) has been a major focus in the fields of biodiversity and conservation. In addition to the standard DNA barcodes, many DNA sequences have been used for the identification of herb medicinal materials. These include the chloroplast trnL-trnF intergenic spacer and the nuclear 5S rDNA intergenic spacer (Li *et al.*, 2011).

DNA barcodes appears to be reliable tools to facilitate the identification of herbal medicinal plants for the safe use of herbs, quality control and forensic investigation. Hao *et al.* (2008) reported the cladistic analysis of the sequence of five chloroplast (mat K, rbcL, trnL, trnL-trn-F and psbA-trnH spacer) and one nuclear (ITS) molecular marker of two conifer families, Taxaceae

and Cephalotaxaceae confirming both to be monophyletic. Evaluation of five DNA barcodes of rbcL, matK, psbA-trnH, ITS1 and ITS2 was done in order to distinguish *Taxillus chinensis* from adulterants of the five barcodes, amplification and sequencing efficiencies of rbcL and trnH-psbA were found 100% (Li *et al.*, 2010). Thus various DNA based molecular marker techniques are meanwhile applied and their application is remarkably increasing for species characterization (Shaw *et al.*, 2002; Zhang *et al.*, 2007; Sucher and Carles, 2008; Shaw *et al.*, 2009) especially useful in case of those taxa that are frequently substituted or adulterated with other species or varieties that are morphologically almost indistinguishable.

Therefore, use of molecular markers including DNA-barcoding employs standard DNA markers from plastidal, mitochondrial and nuclear regions to facilitate a correct taxonomic identification of species and has become a basic tool for DNA chip technology synthesizing corresponding probes for distinct identified DNA sequence which are immobilized in a regular pattern on a microarray by fixation on glass slides, silicon or nylon membranes (Chase *et al.*, 2005; Ratnasingham and Hebert, 2007)

### 2.9.3 Molecular Systematics/ molecular phylogeny / Evolutionary Biology / Phylogeography / Molecular Ecology

Molecular DNA based data sets are the most important resource for phylogenetic reconstruction. Among the various marker systems, which were introduced and optimized within the last decade, coding sequences played an important role especially when molecular clock approaches and multi-gene datasets were assembled. However, non-coding sequences were also popular in construction of molecular phylogeny of which nuclear encoded ITS and the plastidic trnLF region (trnL intron, trnLF intergenic spacer) are widely used (Calonje *et al.*, 2009). Phylogenetic study seeks to trace the ancestry of modern organisms (plants, animals, microbes) as far back as possible seeing how they developed from sometime less sophisticated and less adaptive progenitors disclosing the evolutionary biology.

The popularity of ITS in phylogeny derived from several merits such as biparental inheritance, universality, simplicity, intragenic uniformity, intergenomic variability, low functional constraints and high copy number (Alvarez and Wendel. 2003; Biffin *et al.*, 2007). ITS of nrDNA has been widely employed for the derivation of evolutionary/phylogenetic relationships (Lanoue *et al.*, 1996; Shrestha *et al.*, 2003; Li *et al.*, 2009; Joshi, 2011).When both cpDNA and ITS sequencing failed in resolving phylogenies, the AFLP approach has the potential to solve such difficulties,

particularly among closely related species, or at the intraspecific level (Hodkinson *et al.*, 2000; Koopman *et al.*, 2001; Sun, 2001; Zhang *et al.*., 2001; Beardsley *et al.*, 2003).

Molecular phylogenetics is main component of the field of molecular ecology that are closely related to population genetics. This branch of biology studies sharing of genetic code between organisms, inter-relation between the species and influence of environmental/ecological factors in distribution of shared genetic code called alleles between individuals (Internet visit [5]). Intraspecific phylogeography covers a wide spectrum of activities with phylogenetics (the evolutionary genetics of species-level relationships) at one end of the scale and genealogy (the genetic relationships among individuals) at the other. Phylogeography has proved dramatically successful in explaining how plant distributions have been influenced by historical events extending back millions of years (Beebee and Rowe, 2008).

### 2.9.4 Gene mapping / QTL Mapping / Molecular breeding (Marker Assisted Selection, MAS) / Genetic Engineering

Genetic engineering and biotechnology hold great potential for plant breeding as it promises to expedite the time taken to produce crop varieties with desirable characters. With the use of molecular techniques it would now be possible to hasten the transfer of desirable genes among varieties and introgression of novel genes from related wild species. Polygenic characters which were previously very difficult to analyze using traditional plant breeding methods, would now be easily tagged using molecular markers. It would also be possible to establish genetic relationships between sexually incompatible crop plants. Molecular markers techniques such as RAPDs, ,RFLPs, Microsatellites, SCARs, STS, AFLP etc are particularly promising in assisting selection for desirable characters using F2 and back-cross populations, near isogenic lines, double haploids and recombinant inbred lines (Mohan *et al.*, 1997).

Progress has been made in mapping and tagging many agriculturally important genes with molecular markers which forms the foundation for marker assisted selection (MAS) in crop plants and others. Molecular markers offer great scope for improving the efficiency of conventional plant breeding by carrying out selection not directly on the trait of interest but on molecular markers linked to that trait. Besides, these markers are not environmentally regulated and are detectable in all stages of plant growth. High-resolution linkage maps are being developed for many plants that would help to elucidate gene function, gene regulation and their expression. Among the various molecular markers developed, RFLPs were the first to be used in human genome mapping (Botstein, 1980) and later they were adopted for plant genome mapping (Weber and Helentjaris, 1989; Chawla, 2009). Newer approaches based on PCR eg. RAPD (Welsh

ad McClelland, 1990; Williams *et al.*, 1990) are relatively simple. Since then, many new modifications of the PCR based molecular marker techniques have been used widely in gene mapping and molecular breeding.

AFLP techniques is now widely used for developing polymorphism markers. Lin *et al.* (1996) have compared three different DNA mapping techniques *viz.* RFLP, RAPD and AFLP in detecting polymorphism in soyabean and found that AFLP is the most efficient technique in detecting polymorphism in soyabean determining linkages by analyzing individuals from segregating populations. SSRs and STSs have also been successfully been used to identify Quantitative Trait Loci (QTLs) for various traits.

Molecular breeding is used to describe plant-breeding programs supported by the use of DNA-based markers. It is also called as MAS which is breeding strategy in which selection of desired gene is based on molecular markers closely linked to the gene of interest. Markers are used to monitor the incorporation of desirable allele from the donor source (Singh, 2005). With MAS it is now possible for the breeder to conduct many rounds of selection in a year. Molecular marker technology is now integrated into existing plant breeding programmes all over the world in order to allow researchers to access, transfer and combine genes at a rate and with a precision not previously possible.

## 2.10 Statistical Parameters and Estimators for Genetic Diversity assessment and Population Genetic Analysis

### 2.10.1 Polymorphic Information Content (PIC)

PIC estimates the degree of polymorphism of marker, which essentially is the proportion of individuals that are heterozygous for a marker. PIC is a good measure of the heteozygosity. High PIC value indicates rich heterozygosity which in turn is associated with a high degree of polymorphism (Arya *et al.*, 2011). A PIC value of less than 0.25 indicates low polymorphism, a value between 0.25 and 0.5 indicate average polymorphism and a value higher than 0.5 indicates a highly polymorphic locus (Botstein *et al.*, 1980). The marker with highest values is best used to distinguish variety (Luce *et al.*, 2001).

### 2.10.2 Primer Resolving Power (Rp)

The capacity of each RAPD primer to distinguish the varieties can be studied by calculating resolving power (Rp), which is based on the allocation of alleles within the sampled genotypes (Prevost and Wilkinson, 1999). The resolving power is based on the distribution of detected

bands within the sampled accessions i.e. band informativeness. It is used as an index for good Primers (Azizi *et al.*, 2009).

### 2.10.3 Similarity and Dissimilarity indices for RAPD data

The binary data matrix generated based on the presence and absence of RAPD fragment is the qualitative data. Thus for the RAPD data analysis, similarity indices should be calculated on the basis of Similarity for Qualitative data (SIMQUAL in NTSYS statistical package) computational algorithm. The proper choice of a similarity coefficient, 'S' between the Operational Taxonomical Units (OTUs) is important and depends on various factors. According to Rief *et al.* (2005), the choice of a coefficient for studying divergence depends on the marker system properties involved, on the germplasm genealogy, on the operational taxonomical operational unit involved, on the study objectives and on the conditions required for a multivariate analysis. In general, the similarity coefficients are based on comparisons between the occurrence of common bands (indicated by one in common data matrix), different bands (indicated by one and zero or zero and one), while other coefficients also consider the occurrence of zeros in common.

Rief *et al.* (2005) examined 10 similarity coefficients widely used in germplasm surverys. They suggested that when the marker data are "allelic non-informative" the estimates of coefficients between OTUs under consideration can be calculated by one of three coefficients, based on the absence or presence of observed bands described by Sneath and Sokal (1973): Dice's similarity coefficient (D) equivalent to Nei-Li coefficient (Nei and Li, 1979); Jaccard's similarity coefficient (J) (Jaccard, 1908) and Simple matching similarity coefficient (SM) (Sokal and Michener, 1958). In contrast to SM, both J and D do not involve shared absence of DNA bands.

Along with the similarity coefficients, genetic dissimilarity coefficients are also used to explore the information provided by the molecular marker such as Roger's modified distance and Nei's distance (Nei, 1972).

### 2.10.4 Correlation Analysis using COPH (Ultrameric Cophenetic values) and MXCOMP (matrix comparison) by NTSYS for the selection of best fitted cluster analysis

Mantel (1967) developed a test for matrix correspondence that has been widely applied in geography and, more recently, in population biology and other fields due to its generality and to its computational simplicity (*e.g.*, Sokal, 1979). The program MXCOMP performs two kinds of matrix comparisons. It can take two symmetric similarity or dissimilarity matrices and plot one matrix against the other element by element (but with the diagonal values ignored). It can also linearly adjust two matrices for the effects of a third matrix and then plot them against each

other for a 3-way Mantel test. In either case it computes the product-moment correlation, *r*, and the Mantel test statistics, *Z*, to measure the degree of relationship between the two matrices.

Cophenetic value matrix (COPH matrix of ultrameric values) from a tree matrix can be used by the MXCOMP program to measure the goodness of fit of a cluster analysis to the similarity or dissimilarity matrix on which it was based. The COPH module performs three types of computations. First, it can take a hierarchical system of clusters and produce a symmetrical matrix of "cophenetic" (ultrametric) similarity or dissimilarity values. These can be used to test for the goodness of fit of a clustering to a set of data. Second, it can take a rooted or unrooted tree and produce a matrix of path length distances between all pairs of OTUs. This could be used to test the goodness of fit of an additive tree to a dissimilarity matrix. Third, it can take a rooted tree and produce a matrix giving the expected variances and covariances.

The input to this module consists of a set of nested clusters in the form of a tree matrix. The cophenetic value matrix can be used to test the goodness of fit of a cluster analysis to the data by using the MXCOMP module to compare the original similarity or dissimilarity matrix that was clustered with the cophenetic value matrix (Sokal, 1979).

### 2.10.5 Correlation Analysis using CONSEN (Consensus trees and Indices) by NTSYS

CONSEN (Consensus trees and indices) module produces a consensus tree and computes Consensus indices from two or more rooted labeled trees. A consensus tree is a tree that represents the Consensus topology of two or more trees. A consensus index is a numerical value that indicates the degree to which the consensus tree is resolved. The module can be used to compare trees produced by different clustering methods or to construct a tree that is a summary of many trees. The input to this module consists of sets of nested clusters in the form of a tree matrix - such as produced by the SAHN clustering module. The trees being compared must all have the same number of OTUs. The consensus index of a given tree measures how well resolved the tree is (Sokal, 1979).

### 2.10.6 Cluster Analysis

Cluster analysis refers to “a group of multivariate techniques whose primary purpose is to group individuals or objects based on the characteristics they possess, so that individuals with similar descriptions are mathematically gathered into the same cluster” (Hair *et al.*, 1995). There are

two main ways of analyzing the resulting similarity (or distance) matrix and displaying the results (Karp *et al.*, 1997b).One way is to produce a dendrogram (or tree-diagram) linking together in clusters samples that are more genetically similar to each other than to samples in other clusters. Such Cluster Analysis may proceed according to a range of different algorithms, but some of the more widely used ones include Unweighted Pair Group Method with Arithmetic Averages (UPGMA), Neighbour-Joining Method and Ward's Method (Karp *et al.*, 1997b).

Another approach is to use Principal Coordinate Analysis (PCO) to produce a 2- or 3-dimensional scatter plot of the samples such that the geometrical distances among samples in the plot reflect the genetic distances among them with a minimum of distortion. Aggregations of samples in such a plot will reveal sets of genetically similar material (Karp *et al.*, 1997b). 'Eigen values' define the amount of total variation displayed on the principle coordinate (PC) axes, eg. the first PC summarizes most of the variability present in the original data relative to all remaining PCs (Kovach, 2007). Both PCO and tree-diagram are so-called 'phenetic' methods in that they are based on measures of overall distance or similarity among samples.

### 2.10.7 Diversity indices for dominant data

The main problem with using dominant data, derived from multilocus screening methods, to estimate diversity statistics is that the frequency of heterozygotes is unknown, as they are indistinguishable from homozygotes. Therefore, it is not possible to assess directly whether a particular population is in Hardy-Weinberg equilibrium (states that both allele and genotype frequencies in a population remain constant—that is, they are in equilibrium—from generation to generation unless specific disturbing influences are introduced). There are two approaches to deal with this problem. The first uses methods that ignore this problem and thus limitations must be borne in mind when results are considered (eg. Nei's and Shannon's diversity indices). The second addresses the shortcomings of the data and uses specific statistics to offset these drawbacks (such as those developed by Lynch and Milligan, 1994).

#### 2.10.7.1 Shannon's index of diversity (Shannon, 1948)

Shannon's index of diversity (I) is the diversity measure widely used in ecology but applied to genetics (Lewontin, 1972). Shannon's index is generally preferred as it allows more refused statistical tests to be applied to the data. Shannon's index (I; Shannon, 1948), originally developed as a measure of entropy in information theory, has become a widely used statistic for quantifying levels of diversity (Lewontin, 1972). However, the Shannon index is suitable for RAPD data as it is insensitive to the bias (Dawson *et al.*, 1995; Gillies *et al.*, 1997; Meekins, 2001; Lowe

*et al.*, 2004; Tsuda *et al.*, 2004; Sebastian *et al.*, 2010). For each locus, Shannon's index produce values from 0-0.73 when natural log is used (Lowe *et al.*, 2004).

#### 2.10.7.2 Nei's Index (Nei, 1987)

Nei's method was originally derived for use with (codominant) proteins, and is based on a measure of expected heterozygosity. For dominant data, the concept of heterozygosity is not applicable and the estimate becomes "gene diversity" simply a measure of genetic variability but still of statistical value (Nei, 1987). The average gene diversity is the average of this quantity across all loci. The value is adjusted for variation in sample sizes (Nei, 1978) through multiplication by 2n/ (2n-1), where n = sample size. For each locus Nei's index produces values from 0-0.5 when natural log is used (Lowe *et al.*, 2004).

### 2.10.8 Genetic differentiation ($G_{ST}$) and Gene flow ($N_m$)

Total gene diversity ($H_T$), measured in terms of the total expected heterozygosity, can be divided into the proportion gene diversity of the species that is present within populations ($H_S$) and among populations ($D_{ST}$, Nei 1973), indicating the differentiation of diversity between populations, such that $H_T = D_{ST} + H_S$. $H_S$ is the mean of expected heterozygosities within each population. These diversity indices ($H_T$, $H_S$, $D_{ST}$) can be used to calculate measures of genetic differentiation. $G_{ST}$, the gene diversity between populations relative to the combined populations, is termed the coefficient of genetic differentiation (Nei, 1973). By comparison, $G_{ST}$ varies between 0 and 1. $G_{ST}$ is 0 when $H_T$ is the same as $H_S$, i.e. allele frequencies are identical across all populations. $G_{ST}$ is 1 when $H_S$ is 0, i.e. no variation within populations (Lowe *et al.*, 2004).

Gene flow refers to the movement of genes (via individuals or propagules) from one population to another. It has long been of interest to population and evolutionary biologists as it is a central parameter offsetting the combined effects of mutation and genetic drift that prevents populations from differentiating over time. $G_{ST}$ estimates were used to calculate the number of immigrants (gene flow) per generation for each locus. $N_m$ values greater than 1 imply that the gene flow between populations is at a sufficient level to counterbalance genetic drift (a force that reduces heterozygosity by the random loss of alleles, force of evolutionary change), while $N_m$ value less than 1 mean that genetic drift will result in substantial differentiation and $N_m$ value below 0.5 indicates that populations will diverge extensively as a result of drift (Slatkin, 1987; McDermott and McDonald, 1993).

### 2.10.9 AMOVA and indicator of genetic differentiation $\Phi_{PT}$

Analysis of molecular variance (AMOVA) is a method of estimating population differentiation directly from molecular data and testing hypotheses about such differentiation. A variety of molecular data – molecular marker data (for example, RAPD, ISSR or AFLP), direct sequence data, or phylogenetic trees based on such molecular data may be analyzed using this method, that is, a matrix of 1's and 0's, 1 indicating presence and 0 absence of a marker. A marker could be a nucleotide base, a base sequence, a restriction fragment, amplified fragment or a mutational event (Excoffier, *et al.* 1992). AMOVA can be performed using GENALEX 6 (genetic analysis in excel, Peakall and Smouse, 2006), Arlequin, WINAMOVA in RAPD, ISSR and AFLP to partition the total molecular variance between and within populations. AMOVA is an important, relatively recent statistical procedure that allows the hierarchical partitioning of genetic variation among populations and regions and the estimation of the widely used F-statistics and/or their analogues. The AMOVA framework is important, because it allows such analysis for many types of genetic marker, and it offers statistical testing by random permutation. $\phi_{pt}$ is the estimate of population genetic differentiation provided by GenAlEx when binary or haploid data are analysed for comparative studies (Peakall and Smouse, 2001).

Although AMOVA was originally designed for haploid mitochondrial DNA data (Excoffier *et al.*, 1992), it has been successfully applied in many RAPD analyses (eg. Tsuda *et al.*, 2004; Deng *et al.*, 2006; Hu *et al.*, 2008; Kelley, 2009; Alam *et al.*, 2009; Domyati *et al.*, 2011).

## 2.11 Application of RAPD Technology

The RAPD method developed by Williams *et al.* (1990) has become one of the most widely used molecular marker techniques for detecting genetic variation within and among plant populations, cultivars and species. This technique has several advantages over other molecular methods; such as technical simplicity, cost effectiveness, no requirement of prior DNA sequence information (Williams *et al.*, 1990; Dowling *et al.*, 1996) and possibility of random sampling of the whole genome. Moreover, RAPDs were found to be particularly appropriate for studies involving small sample size, especially for outbreeders because a large number of poloymorphic loci can be generated than allozyme assay with faster and easier analysis than microsatellites (Nybom, 2004; Zhang *et al.*, 2007). This is of relevance to conservation studies that often assess the genetic status of rare and endangered taxa, which can be represented by very few individuals (Cardoso *et al.*, 1998; Zhang *et al.*, 2005). However, reproducibility of RAPD profiles is sensitive, a proper optimization is required to generate reliable results. Although dominant marker, such as RAPD is not suitable for genetic analyses requiring information on allele frequencies, they are

appropriate in this case because we are not concerned with allele frequencies but instead with the number of bands shared among populations and across regions (Zhang *et al.*, 2007). RAPD, AFLP and microsatellites should not be considered appropriate for phylogenetic analyses above the species level. These markers are undoubtedly valuable tools for addressing population genetics and plant breeding issues (Arif *et al.*, 2010).

### 2.11.1 RAPD Technique in Genetic Diversity Analysis/ Population Genetics Studies and Conservation Genetics

RAPD has been widely employed successfully for assessing the genetic diversity and taxonomic relationships in many plants. The genetic diversity analysis of ten accessions of *Cassia occidentalis*, an ayurvedic medicinal plant collected from different districts of Haryana, India has been reported to be highly polymorphic (71.17%) using twelve random primers (Arya *et al.,* 2011). Jaccard similarity coefficient ranged from 0.54 to 0.73. A dendogram constructed based on UPGMA clustering revealed two major clusters. Khan and Pankajaksan (2010) subjected commercial varieties of *Anthurium andreanum* to RAPD marker analysis and examined among twelve accessions of A*nthurium* varieties with a hybrid as an out group check. A high degree of polymorphism was observed and UPGMA cluster analysis of genetic similarity indices revealed two major clusters.

In the another study done by Teklewold and Becker (2005), 43 accessions of Ethiopian mustard (*Brassica carinata*) including 14 exotic accessions were analysed for genetic diversity using a set of 50 RAPD primers that resulted into 275 polymorphic bands with PIC varied ranging from 0.05 to 0.40, Band Informativeness ($I_B$) from 0.05 to 0.65 and $R_P$ from 0.15 to 6.83 for different primers used. Jaccard's coefficient ranged from 0.44 to 0.87 indicating high level of genetic diversity in the Ethiopian accessions. Principle Coordinate analysis (PCO) revealed the inclination towards geographic differentiation of accessions.

Muthusamy *et al.* (2008) checked efficiency of RAPD and ISSR markers system in accessing genetic variation of rice bean (*Vigna umbellata*) landraces and observed RAPD fingerprinting detected more polymorphic loci (70.30%) than the ISSR fingerprinting (61.79%). Mean PIC suggested that both the marker system were equally effective in determining polymorphisms. Dendograms constructed too were found highly correlated with each other as revealed by high Mantel correlation (r = 0.95). Thirty-two accessions of *Sorghum bicolor* L. Moench were fingerprinted using 64 RAPD decamer primers to give 97.4 % polymorphism (Jeya Prakash *et al.*, 2006). Cluster analysis showed the two major clusters

Genetic diversity and differentiation among natural populations of *Dalbergia sisoo* were examined for the first time using RAPD marker (Wang *et al.*, 2011). High level of genetic diversity was observed both at the species level (% of polymorphic bands = 89.11%, Shannon's diversity Index, I= 0.2730, Nei's gene diversity, H= 0.4180) and the population level (% of polymorphic bands = 68.7%, I= 0.239, H = 0.358) along with low degree of differentiation among populations ($G_{ST}$ = 0.13, AMOVA = 14.69%). Strong gene flow (Nm) among populations was estimated to be 3.31. Informations are useful for introduction, conservation and further studies of *D. sissoo* and related species. Similar study was done in important medicinal plant, *Podophyllum hexandrum* from different geographical regions (from North West Himalaya) using RAPD markers. High genetic diversity observed among the genotypes (% polymorphic band =92.37% and Shannon information index, I = 0.50) with the mean coefficient of gene differentiation ($G_{ST}$) of 0.69 and limited gene flow among the genotypes ($N_m$ = 0.22). Based on observed genetic variations, *in situ* conservation was recommended (Alam *et al.*, 2009).

Sesli and Yegenoglu (2010) compared various combinations of similarity coefficient (Jaccard, Dice, Simple Matching) and clustering methods (UPGMA, WPGMA, single linkage and complete linkage) for olives (*Olea europaea sativa*). Closest and distant landraces were revealed. The Mantel test showed that the correlation between Jaccard and Dice similarity matrices was high and significant (0.9971). The results obtained from Consensus indices shown that consensus fork index was found high ($CI_C$ = 0.9) in Jaccard and Dice coefficients. Simple matching coefficient had very low values (0.1) with the Dice and Jaccard's coefficients. The study suggested using Dice and Jaccard genetic coefficients with UPGMA clustering method for the determination of genetic relations of olive and in addition it was concluded that Simple Matching coefficient is not suitable for the studies with RAPD since it causes change in the results due to negative co-occurrences.

Similar successful uses of RAPD markers have been reported in genetic diversity analysis of *Sesamum* (Bhat *et al.*, 1999); *Sporobolus* sp. (Shrestha *et al.*, 2005); *Capsicum* (Da Costa *et al.*, 2006); *Jatropha curcas* L. (Ranade *et al.*, 2008); *Pachyrhizus,* African yam bean (Moyib *et al.*, 2008); *Anisodus tanguticus* (Zheng *et al.*, 2008); *Rhododendron* sp. (Lanying *et al.*, 2009); *Trigonella foenum-graecum*. (Sundaram and Purwar, 2010); *Pisum sativum* (Ahmad *et al.*, 2010); *Tylophora rotundifolia* (Sebastian *et al.*, 2010); *Vigna mungo* (Srivastava *et al.*, 2011); *Gossypium* spp (Khan *et al.*, 2011).

### 2.11.2 RAPD Technique in Genetic Mapping/Plant Breeding

Locating and identifying genes in a genetic map is called genetic mapping. RAPD is popularly employed as relatively simple polymorphism based genetic mapping technique since its

innovation (Williams *et al.*, 1990) as oppose to the RFLP analysis which is labor intensive, usually requires radioactivity and time consuming (Rafalski *et al.*, 1991). Paran and Michelmore (1993) and Nair *et al.* (1995; 1996) were able to increase the reliability of RAPD markers by converting them to SCARs which could be used in a PCR reaction to amplify the RAPD fragments linked to a insect resistance gene, specifically and reliably. The technical ease of RAPD markers and the facility of their application to new species had led their employment in many organisms including forest trees, crop as well as medicinal plants and lower plants for genetic linkage mapping (Laucou *et al.*, 1998; Hoi-Shan and Hai-Lou, 2002; Carneiro *et al.*, 2002; Mehlenbacher *et al.*, 2006; Zhang *et al.*, 2010). An integrated genetic linkage map of the medicinal and ornamental plant *Catharanthus roseus*, based on different types of molecular (RAPD, ISSR, SSR) and morphological markers was constructed (Gupta *et al.*, 2007). Similar use of RAPD marker for the linkage mapping has been reported in various plants such as Eikorn wheat and pea (Kojima *et al.*, 1998; Laucou *et al.*, 1998 etc.). Salem *et al.* (2007) pointed out that RAPD is ideal for genetic mapping, plant and animal breeding programs and DNA fingerprinting.

### 2.11.3 RAPD Technique in Major Valued Plant Identification/ Authentication and Fingerprinting

An attempt made by Transue *et al.*, (1994) for the species identification of grain *Amaranthus* genetic resources by RAPD analysis was found efficient where RAPD markers were intended to classify accessions by species. In similar attempt ascended in identification and characterization of genotypes, Arif *et al.* (2010) utilized the RAPD method for the genetic relationship and fingerprinting of 11 medicinal plant species of desert origin from Saudi Arabia. In the study done by Khan *et al.* (2011), RAPD molecular marker was employed for the identification of medicinal plants *Senna angustifolia, Senna acutifolia, Senna tora* and *Senna sephera.* In order to develop convenient and reproducible methods for the identification of ginseng drugs at a DNA level, RAPD and PCR-RFLP analysis were applied within *Panax* species (Um *et al.*, 2001). To authenticate *Panax ginseng* among ginseng populations, RAPD fingerprints can be used produced by 20 mer-random primers.

RAPD technique was used to create a series of genetic markers that could positively identify the five major weeds from the other less damaging weedy and native *Sporobolus* species. Twelve genetic markers were found that, when used in combination, could consistently identify the five weedy species from all others. Of these 12 markers, the most diagnostic revealed were $UBC51_{490}$ for *S. pyramidalis* and *S. natalensis*; $UBC43_{310, 2000, 2100}$ for *S. fertilis* and *S. africanus* and OPA $29_{850}$ and $UBC43_{470}$ for *S. jacquemonti* (Shrestha *et al*., 2005). Khan *et al.* (2010) employed RAPD to develop reproducible RAPD markers for fingerprinting and authentication of a small aromatic

shrub *Ruta graveolens,* which has been used medicinally. Since ancient times its discrimination from its adulterant *Euphorbia dracunculoides* had been a problem. Forty-two RAPD decamer primers were screened against genuine and adulterant samples using DNA isolated from the dried leafs, seed and stems, among which 12 gave species-specific reproducible unique bands.

Similarly, RAPD analysis has been widely used for the differentiation of a large number of medicinal species from their close relatives or adulterants such as: *Echinaceae* species (Nieri *et al.*, 2004); Turmeric (Sasikumar *et al.*, 2004); *Astragali radix* (Na *et al.*, 2004); *Typhonium* species (Acharya *et al.*, 2005); *Dendrobium officinale* (Ding *et al.*, 2005); *Dendrobium* species and its products (Zhang *et al.*,2005); *Rehmannia glutinosa* cultivars and varieties (Qi *et al.*, 2008); *Tinospora cordifolia* (Rout, 2006); *Glycyrrhizea glabra* and its adulterant (Khan *et al.*, 2009); *Desmodium* species (Irshad *et al.*, 2009); *Piper nigrum* (Khan *et al.*, 2010); *Cuscuta reflexa* and *Cuscuta chinensis* (Khan *et al.*, 2010);.

Correct species identification is carried in case of medicinal plants as it has got direct relationship with human health. There can be a significant application of molecular marker tools such as RAPDs in case of those major plants that are frequently substituted or adulterated with other species or varieties that are morphologically and/or phytochemically indistinguishable (Joshi *et al.*, 2004).

## 2.12 Variation Analysis of *Swertia* Spp.

*Swertia* shows wide range of morphological variation within and among the species. Rijal (2009) had carried out taxonomic study of some medicinally important species of *Swertia* L. (Gentianaceae) in Nepal which aimed to provide the most important identifying characters of eight species of *Swertia* traded from Nepal *viz. S. angustifolia, S. chirayita, S. ciliata, S. cuneata, S. dilatata, S. nervosa, S. paniculata* and *S. racemosa*. The investigation reported that the petal colour, number of nectarines (glands) in petal (1 or 2), characteristics of flap and presence of cilia are the key characters for the delimitation of various *Swertia* species.

Pant *et al.*, (2004) analysed genetic diversity in seven *Swertia* species (Gentianaceae) from three different regions of Nepal and evaluated their morphological traits which revealed the diversity existed between and within *Swertia* species. Evaluating isozyme variability by assessing four enzyme systems namely: Peroxidase, Malate dehydrogenase, Acid phosphatase and Estarase, they observed low level of genetic variation upto 14.5% only.

Phenotypic variation of *Swertia chirayita* have been investigated by Raskoti (2004) in 10 natural population of Nepal consisting 12 plants from each population and phenotypic characters were assessed in the sample plant such as height of plant, branch number, node number, leaf length, leaf width etc and he observed morphological trait of *S. chirayita* showed variation within and among populations. He reported existence of phenotypic variation in populations of *S. chirayita* may be due to environmentally or genetically controlled attributes, the verification of which can be made by progeny testing and research at molecular level (Raskoti, 2004).

Morphological characters have long been used to identify species, families, genera as well as study of population genetics in diversity analysis. But with the advent of molecular markers (DNA-based), number of limitation encountered with morphological and biochemical markers have overcome. Limited molecular researches have been carried out in *Swertia* spp. of the world. Chassot *et al.*, (2001) have studied the DNA sequencing of some Nepalese species of *Swertia* (*S. bimaculata, S. chirayita, S. ciliata, S. cordata, S. pseudo hookeri*). In a previous study involving ISSR markers, 98.7% polymorphism was found among 19 genotypes of *Swertia* species (13 of *S. chirayita* and 2 each of *S. cordata, S. paniculata* and *S. purpurascens* which were used as outliers in the study). This was reduced to 42.5% when the outliers were excluded (Joshi and Dhawan, 2007b).

In an investigation of genetic variation pattern carried out in an endangered endemic species, *Swertia przewalskii* of the Qinghai-Tibet plateau using RAPD and ISSR analysis, Zhang *et al.* (2007) observed the significant genetic differentiation based on different measures including AMOVA (52% for RAPD and 56% for ISSR) although sexual reproduction and gene flow between populations of *S. przewalskii* are very limited. In connection with the existing demand of an authentication system for *Swertia spp.* in the herbal drug industry, as well as to enable their commercial use as genuine phytoceuticals, Misra *et al.* (2010) used amplified fragment length polymorphism (AFLP) to produce DNA fingerprints for *Swertia* species. Nineteen accessions (2 of *S. chirayita*, 3 of *S. angustifolia*, 2 of *S. bimaculata*, 5 of *S. ciliata*, 5 of *S. cordata*, and 2 of *S. alata*) from India were used in the study employing 46 selected AFLP primer pairs. Species-specifc markers were identifed for all six *Swertia* species 131 for *S. chirayita*, 19 for *S. angustifolia*, 181 for *S. bimaculata*, 47 for *S. ciliata*, 94 for *S. cordata*, and 272 for *S. alata*) which could be used to authenticate drugs in pharmacopoeia. Also the study revealed polymorphism upto 99% among various species under study.

In context of Nepal, Initiation of the molecular characterization of *Swertia* spp. have been reported with the phylogeny and molecular differentiation of 11 Nepalese *Swertia* species: *S.*

*angustifolia, S. chirayita, S. ciliata, S. dilatata, S. lurida, S. macrosperma, S. multicaulis, S. nervosa, S. paniculata, S. pedicilleta* and *S. racemosa* investigated by Joshi (2008, 2011) using standard molecular techniques (PCR and DNA sequencing). The data obtained from both ITS and Chloroplast (trnL-F) regions were analysed together with Distance, Parasimony and Bayesian analyses illustrating in phylogenetic trees. The result indicated that each of the commonly used species has unique sequences and the ITS fragment can be used as a barcoding marker for *Swertia* in the local medicinal market. The study indicated that *Swertia* is higly paraphyletic. The results from ITS region of the nrDNA are generally congruent with those of the chloroplast *trnL-F* sequences. Both ITS and *trnL-F* data support the close relationship of *S. lurida* with *S. chirayita*. With an objective of studying the specific genetic diversity in *S. chirayita* population in Nepal, optimization of the RAPD-PCR conditions has been accomplished (Shrestha *et al.*, 2010).

# 3. MATERIALS AND METHODS

## 3.1 Materials

## 3.1.1 Reagents and Extraction buffers

### *(50 X) TAE Stock buffer preparation (Tris – Acetate – EDTA)*

To prepare (50 X) TAE stock buffer, Tris base (242gm; Qualigens Fine Chemicals, Mumbai) was dissolved in approximately 750 ml deionized water. To this solution, glacial acetic acid (57.1mL; Qualigens Fine Chemicals, Mumbai) was added followed by 0.5 M EDTA (pH 8.0, 100mL; Promega Corporation, USA). The final volume was made up to 1L.This stock solution can be stored at room temperature (Sambrook and Russel, 2001).

The working solution of 1X TAE buffer was made by simply diluting the stock solution by (50X) in deionized water or dd$H_2O$. Final solute concentrations were 40 mM Tris-acetate and 1mM EDTA. The buffer is now ready for use in running an agarose gel.

### *(5X) Gel loading buffer (GLB)*

Sucrose (2.5 g) was dissolved in deionized water (7ml) in which bromophenol blue (25mg, Fermentas Life Sciences, Canada) was added and the final volume made up to 10ml. The prepared gel loading buffer (GLB) was added to the sample in proportions as 1 (GLB) to 1 (DNA sample) and 1 (GLB) to 4 (PCR product) by volume, during electrophoresis.

### *Agarose gel (1.5%)*

Agarose (1.5gm; Promega, Product of Spain) was dissolved in TAE buffer (100mL, 1X) in the microwave. It was then cooled to approximately $55^0$C and poured on to the gel casting tray with an appropriate comb (12 to 20 toothed) fixed in place for well formation.

### *Tris Buffer (1M, pH 8.0 and pH 7.5)*

Tris Buffer (1M, pH 8.0 and pH 7.5) stock solutions were prepared by adding Tris base (60.55 g, Qualigens Fine Chemicals, Mumbai) to deionized water (400 mL). The pH was adjusted to 8.5 or 7.5 by the addition of concentrated HCl. The final volume was then made up to 500 mL, autoclaved and stored at room temperature until needed for preparation of extraction buffers (Sambrook and Russel, 2001).

### *EDTA (0.5M, pH 8.0)*

Disodium ethylene-diaminetetra-acetate.2$H_2O$ (EDTA, 93.05 g, Promega, Product of Spain) was added to a Schott bottle containing deionized water (400 mL), 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 500 mL with deionized water, autoclaved and stored at room temperature until needed.

### *NaCl (4M)*

Sodium Chloride (NaCl, 117 g, Qualigens Fine Chemicals, Mumbai) was added to schott bottle containing deionized water (400 mL), mixed on a magnetic stirrer. The final volume was adjusted up to 500 mL with deionized water, autoclaved and stored at room temperature.

### *Graham's CTAB (Hexadecyl Trimethyl Ammonium Bromide) extraction buffer (2% CTAB, 1.4 M NaCl, 0.1 M EDTA, 0.1 M Tris HCl, pH 8.0)*(Graham et al., 1994a)

For the preparation of CTAB extraction buffer, Tris buffer (100mL of 1M solution, pH 8.0) was dispensed in a 1L Schott bottle (Sterile) and EDTA (200 mL of 0.5M solution, pH 8.0), NaCl (350 mL of 4M solution) and CTAB (20g, Loba Chemie, India) were mixed and the final volume was made up to 1L with deionized water.

### *Doyle and Doyle extraction buffer (100mM Tris HCl, 20mM Na-EDTA, 1.4 M NaCl, 2% CTAB, 0.2% β- mercaptoethanol, 1.5% Poly Vinyl Pyrrolidone)*(Doyle and Doyle, 1990)

Tris buffer (1mL of 1M stock; pH 8.0) was pipetted in Schott bottle and Na-EDTA (0.4mL of 0.5M stock), NaCl (3.5mL of 4M stock), CTAB (0.2 g, Loba Chemie, India), PVP (1.5mL) were added and the final volume was made 10mL with deionized water. Finally, β- mercaptoethanol (20µL) was added in freshly prepared extraction buffer inside the fumehood.

### *TE buffer (TRIS-EDTA; 10mM Tris HCl, 1mM EDTA) with RNAse*

EDTA (1mL of 0.5M stock; pH 8.0) was added to a bottle containing Tris buffer (5 mL of 1M solution) and the final volume made up to 500 mL. This was autoclaved and stored at room temperature. RNase A was added to Tris-EDTA buffer (200 µl of 5 mg/ml RNAse A) in 200 mL TE buffer (to make final concentration of 10µg/mL) in sterile Schott bottle for fresh use.

## 3.1.2 Primers, dNTPs and DNA Dilution

Primers were available in lyophilized form and were diluted to required concentration 10µM (the working concentration) using sterile distilled water.

Commercially supplied dNTP mix (10mM each, Fermentas Life Sciences) was used for PCR reaction in research investigation. It was stored at -20$^{o}$C until use. Genomic DNA dilution to required concentration (25ng) was carried out by initial estimation of concentration of DNA via Biophotometer (Eppendorff, Germany).

### 3.1.3 Plant Materials

A total of 34 accessions of *S. chirayita* and six other related accessions (different species) were collected in silica gel from their natural habitat from five populations districtwise: Sankhhuwasabha, Terathum, Kathmandu, Lalitpur and Kaski representing Eastern, Central and Western development regions of Nepal.


**Figure 3.1** *S. chirayita* sampling sites in the present investigation (highlighted)

Each plant was designated with accession code with respect to the site of collection. Collected samples were brought to the NAST, Molecular Biotechnology Laboratory for the DNA extraction and subsequent molecular analysis. Sample details of this study are shown (Table 3.1).

**Table 3.1** Sample details of *S.chirayita* and outlier species used in present investigation

| S. N | District/ Locality | *Swertia chirayita* | outliers | Regions of Nepal | No. collected | Altitude (meters) | Plant Accession Code |
|---|---|---|---|---|---|---|---|
| **1** | Lalitpur/ Phulchowki, Godavari | ✓ | | Central | 9 | 2150 | $F_1$, $F_2$, $F_3$, $F_4$, $F_5$, $F_6$, $F_7$, $F_8$ and $F_9$. |
| **2** | Kaski/ Sikles | ✓ | | Western | 4 | 2000-2500 | $K_{501}$, $K_{505}$, $K_{506}$ and $K_{509}$. |
| **3** | Kaski/ Sikles | | ✓ | Western | 4 | 2000-2500 | $K_{511}$, $K_{517}$, $K_{512}$ and $K_{516}$ |
| **4** | Kathmandu/ Nagarjun, Jamacho | ✓ | | Central | 7 | 2050-2100 | $N_7$, $N_8$ $N_{12}$, $N_{14}$, $N_{15}$, $N_{16}$ and $N_{20}$ |
| **5** | Sankhuwasabha/ Lamapokhari, Shreemane, Manlabre and Chauki | ✓ | | Eastern | 6 | 2600-2950 | $S_1$, $S_2$, $S_4$, $S_5$, $S_7$ and $S_8$ |
| **6** | Sankhuwasabha/ lamapokhari | | ✓ | Eastern | 2 | 2600-2950 | SA and SB |
| **7** | Terathum/ Tirikhimti to Guphapokhari | ✓ | | Eastern | 8 | 1500-2800 | $T_2$, $T_3$, $T_4$, $T_5$, $T_6$, $T_7$,$T_9$ and $T_{10}$ |

## 3.2 Methodology

### 3.2.1 DNA Extraction

Two major genomic DNA extraction techniques were assessed to select the best technique to be used in RAPD profiling of *S. chirayita.*

#### 3.2.1.1 CTAB Extraction Method (Graham *et al.*, 1994a)

Approximately 200mg of freshly harvested young leaf samples preserved in silica gel were ground in sterilized mortar and pestle (sterilization done with 2% sodium Hypochlorite and then autoclaved at 121$^{o}$C and 15lbs pressure) in presence of liquid Nitrogen. The ground samples were treated with 1000 µl Graham's CTAB buffer and transferred to sterile eppendroff tubes (1.5 mL). The mixtures were then incubated at 55$^{o}$C for 15 minutes. Following this, the tubes were centrifuged at 11000 rpm (~13000g) for 5 minutes and the supernatants were transferred to a clean sterile microfuge tubes and equal volumes (~500µl) of chloroform: isoamyl alcohol (24:1) were added. The solutions were mixed gently by inversions several times. Tubes were then centrifuged at 11000 rpm for 1 minute at 25$^{o}$C and the upper aqueous phase were transferred to clean eppendroff tubes and re-extracted with equal volumes of Chloroform isomyl alcohol (24:1). Tubes were centrifuged for 1 minute at 11000 rpm and the upper aqueous layers were transferred to new tubes. Each sample was then treated with 1/10$^{th}$ volume (approximately 50µl) of Ammonium acetate (7.5M) followed by addition of equal volume (i.e. 500µl) of ice-cold (-20$^{o}$C) absolute ethanol. Then the tubes were placed overnight at -20$^{o}$C to allow precipitation of DNA. After overnight precipitation, the tubes were centrifuged at 11000 rpm for 10-15 minutes at 4$^{o}$C. The supernatants were discarded and the DNA pellets were quickly washed twice with ice-cold 70% ethanol (stored at -20$^{o}$C; ~300 µL). In order to get rid of unwanted salts, samples were briefly spinned at 11000 rpm for 1 minute and excess alcohol pipette off. Pellets were then dried in air for 5 minutes after centrifugation. Finally, pellets were resuspended in TE buffer (with RNase) and stored at -20$^{o}$C.

#### 3.2.1.2 Modified CTAB extraction method (Doyle and Doyle, 1990)

In this method, approximately 200mg young leaf samples were ground in sterilized mortar and pestle with liquid Nitrogen and ground samples were treated with 1000µl of freshly prepared Doyle and Doyle extraction buffer and incubated at 65°C for an hour. Samples were then centrifuged at 11,000 rpm for 5 mins and supernatants transferred to fresh tubes. Supernatants were extracted twice with 700µl Chloroform- isoamyl alcohol (24:1) and then centrifuged at 12000 rpm for 10 minutes. The supernatants were transferred to clean microfuge tubes and mixed with 0.6$^{th}$ volume of ice-cold isopropanol. The tubes were placed at -20$^{o}$C for 1 hour to allow precipitation of DNA. DNA was collected as a pellet by centrifugation at 12000 rpm for 15

minutes, washed with 70% ethanol twice, dried and resuspended in TE buffer (with RNase) and stored at -20$^{o}$C for subsequent use.

DNA quality and quantity were determined using 1.5% agarose gel electrophoresis and spectrophotometer (Biophotometer, Eppendorf, Germany) respectively.

### 3.2.2 DNA quantification

DNA quantification is the major step in molecular work. The yield of DNA per gram of leaf tissue extracted was measured using spectrophotometric method (Biophotometer, Eppendorf-AG22331, Germany).

Each DNA sample was quantified using the Biophotometer for its concentration as well as purity assessment. Determination of DNA concentration and purity were also carried out electrophoresing the samples in 1.5% agarose gel and comparing the intensities and dicreteness of bands with the Genes Ruler$^{TM}$ 100bp plus DNA ladder (Fermentas LIFE SCIENCES, #SM0323).

### 3.2.3 Gel Electrophoresis

The extracted DNA , as well as the amplified products of RAPD-PCR were analysed on a 1.5% Agarose gel in TAE buffer (1X) at 50 V (8.47 V/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 loaded in well was 15µl [9µl TAE +3µl GLB (5X) +3µl DNA for DNA analysis and 12µl PCR product +3µl GLB (5X) for product analysis]. The gel after run were stained in TAE buffer containing 35µl of Ethidium bromide (10mg/ml; Promega) for 45 minutes and destained for 15 minutes in water. Then, the gels were visualized on a gel documentation system (IN GENIUS, Syngene Bio-imaging, UK).

### 3.2.4 RAPD-PCR Optimization

All experiments related to RAPD optimization and profiling were carried out in thermal cycler (BIOER, China). The RAPD-PCR reaction conditions were optimized by varying several parameters of the assay. These factors includes: DNA extraction procedure, PCR Cycling conditions, $MgCl_2$ concentration, DNA concentration, *Taq* polymerase concentration, dNTPs concentration and primer concentrations.

CTAB method (Grahm *et al.*, 1994a) and Doyle and Doyle extraction method (Doyle and Doyle, 1990) were the two DNA extraction techniques assessed for selection of best technique for *S. chirayita*. Two different RAPD-PCR programs were assessed for the selection of the best program. PCR Program 1 consisted of initial denaturation of 1 minute at 94$^{\circ}$C, followed by 35 cycles of 10 seconds at 94$^{\circ}$C, 30 seconds at 38$^{\circ}$C, extension at 72$^{\circ}$C for 60 seconds and hold temperature of 20$^{\circ}$C (Yu and Pauls, 1992) and PCR Program 2 with initial denaturation at 95$^{\circ}$C for 2 minutes,

followed by 45 cycles of denaturation at 95°C for 20 seconds, annealing at 37°C for 60 seconds, elongation at 72°C for 60 seconds and final elongation of 10 minutes at 72°C with a hold temperature of 10°C (Edwards, 1998).

Several different ranges of concentrations of PCR-reaction parameters *viz.* $MgCl_2$, dNTPs, *Taq* Polymerase, concentration of template DNA and primer concentrations were assessed for the selection of optimized reaction conditions for *S. chirayita*. For the best optimization with productive results, a range of DNA concentrations (12.5, 25.0, 37.5, 50.0, 62.5, 75.0, 87.5 and 100.0 ng) of *Swertia chirayita* were assessed. Similarly, different $MgCl_2$ concentrations (1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 mM), *Taq* DNA polymerase concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 Units), dNTP concentrations (0.1, 0.2, 0.3, 0.4 and 0.5 mM) and primer concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5 and 1.6 µM) were assessed for the selection of best and optimum concentrations for RAPD-PCR amplification of *S. chirayita* DNA templates.

### 3.2.5 Primer Screening

Using the optimized and further refined RAPD-PCR reaction and cycling conditions, 100 different decanucleotide random primers (from a UBC set 1, University of British Columbia, Oligonucleotides Synthesis Laboratory, Vancouver, British Columbia, Canada) were screened against genomic DNA of *Swertia* from Kaski district. From one hundred primers, 28 primers were initially selected on the basis of bands visualization. Out of 28 primers again, 26 primers (*viz.* Table 4.3) that gave multiple, crispy and separable amplified bands were finally selected to be used in RAPD-PCR analysis involving all 40 accessions of *Swertia* under study. The experiments were repeated twice for the confirmation of the reproducibility of RAPD amplifications.

### 3.2.6 RAPD-PCR profiling

DNA from leaf samples of all 34 *S. chirayita* accessions and its six outlier accessions in the present investigation were tested with each of 26 primers in an attempt to study genetic diversity, identify the polymorphic markers and to find population specific polymorphic markers for *S. chirayita* from 5 different populations using the optimized RAPD-PCR conditions.

#### 3.2.6.1 Marker confirmation

According to the RAPD band profiles observed for the selected 26 primers, the profiles generated by each primer were assessed for the detection of degree of polymorphism between the individual species of *S. chirayita* as well as to find population specific markers for *S. chirayita.*

#### 3.2.6.2 Genetic Diversity Assessment based on RAPD Profiling

A polymorphic fragment is defined as an amplified DNA fragment that was present in the DNA samples of at least one accession and was absent in the DNA samples of at least one accession.

Polymorphic RAPD fragments were initially identified in amplified DNA using 26 primers from total DNA samples of the selected standard accessions. Individual accessions were then screened for the presence or absence of observed 263 polymorphic markers amplified with 26 primers. Markers were scored visually. Molecular size of the PCR products were estimated by comparing the position of bands with 100bp plus DNA ladder (Gene ruler$^{TM}$, Fermentas # SM0323). Total number of band (TNB), Number of polymorphic band (NPB) amplified by the selected primers were estimated.

During the study of genetic diversity and relationship, population specific markers (with different primers) were also investigated to find out the natural genetic distinctiveness of *S. chirayita* from the particular geographical location. The Observed results from the different statistical processes were documented and analyzed together for the study of genetic diversity of *S. chirayita* in populations of Nepal from the perspective of dominant RAPD marker system.

## 1) Estimation of Percent polymorphism, Polymorphic Information Content, Band informativeness and Resolving power

Some primer banding characteristics namely: Percent polymorphism (PP), Polymorphic Information Content (PIC), Band Informativeness ($I_B$) and Resolving Power ($R_P$) for each primer were calculated by the use of Microsoft Excel 2007.

Polymorphic Information Content (PIC) values were calculated for each RAPD primer according to the formula: PIC = 1- $\sum(P_{ij})^2$, where $P_{ij}$ is the frequency of the $i^{th}$ pattern revealed by the $j^{th}$ primer summed across all patterns revealed by the primers (Botstein *et al.* 1980). For each primer, percentage polymorphism (PP) has been calculated as NPBs/ TNBs, where NPB is number of polymorphic bands and TNB is Total number of bands amplified.

The ability of the primer combinations to differentiate between accessions was assessed by calculating their Resolving Power ($R_P$) according to Prevost and Wilkinson (1999) using Rp = $\sum I_{B;}$ Where $I_B$ is the band informativeness with $I_B$ = 1-[2 x (0.5 – P)], where P is the proportion of accessions containing the band.

## 2) Genetic Diversity analysis using similarity matrices and phenograms

The qualitative binary data matrix created for bands presence or absence obtained from the RAPD-PCR profiles, generated by twenty six primers, from 40 samples belonging to *S. chirayita* (34 species) and its outlier species (6 species) were analysed using Numerical taxonomy and multivariate system (NTSYS-PC, version 2.21i, Exeter software, Setauket, New York, USA). Every discrete RAPD fragments generated from twenty six different primers were used for the analysis.

Bands were scored as discrete variables ("1" for presence and "0" for absence). Amplification failure was scored as a "9", which was designated in the analysis procedure as an indicator of missing data (Transue *et al.*, 1994). Similarity indices were calculated using SIMQUAL (similarity for qualitative data) computational algorithm. Based on the similarity matrices, Sequential, Agglomerative, Hierarchial and Nested (SAHN) clustering was performed using the Unweighted Pair Group method of Arithmetic Average (UPGMA) algorithm (Sneath and Sokal, 1973). From these, phenograms were generated to show the relationship among the members of different populations of *S. chirayita* and its outlier species. Estimates of similarity were computed on the basis of following three different measures:

1) Simple Matching coefficient (SM) (Sokal and Michener, 1958):

   $S_{ij} = \frac{a+d}{a+b+c+d}$

2) Dice's coefficient of similarity (D) (Dice, 1945; Nei and Li, 1979):

   $S_{ij} = \frac{2a}{2a+b+c}$

3) Jaccard's Coefficient (J) (Jaccard, 1908):

   $S_{ij} = \frac{a}{a+b+c}$

   Where,

   $S_{ij}$ = the similarity between 2 individuals, i and j;

   a = the number of bands present in both i and j;

   b = the number of bands present in i and absent in j;

   c = the number of bands present in j and absent in I, and

   d = the number of bands absent from both i and j.

Cophenetic correlations were calculated by using COPH [Cophenetic values] and MXCOMP (Matrix comparision) procedures for each combination of cluster analysis using NTSYS program. In the present investigation, original matrices were compared by applying Mantel test (Mantel, 1967) in the option of MXCOMP in NTSYS-PC version 2.21i program for the comparision of original matrices by implementing Simple Matching, Dice and Jaccard Similarity coefficients.

Mantel test was applied in NTSYSPC 2.21 program through MXCOMP procedure in the comparison of original matrices and the matrices obtained from cophenetic values shows the correlation coefficients of data from original similarity matrices and cophenetic values as calculated with the Mantel test. The correlation coefficients calculated with the Mantel test enables the finding of correlation between the similarity matrix and the phenetic trees obtained as a result of cluster analysis. The correlation matrices calculated show the goodness of fit of cluster analysis in accordance with the similarity matrix (Rohlf, 2009).

Among the methods of constructing consensus trees, STRICT consensus (Sokal and Rolf, 1981) was used for computation of Consensus indices. In the present investigations, the dendogram bases of different coefficients were compared by Consensus fork index ($CI_C$). The $CI_C$ provides a relative estimate of the dendogram similarities and was calculated using NTSYS-PC version 2.21 (Rohlf, 2009). Hence, Matrix comparision, cophenetic correlation and consensus indices are used for the evaluation of best fitted matrix and tree for the analysis and further study of diversity of *Swertia chirayita*.

Using NTSYS-PC, 3D-plot of the distribution of all the *S. chirayita* accessions was constructed for the illustration of variation as compared to the dendogram using Jaccard's similarity matrix with the analysis of Eigen vector.

### 3) Principal Coordinates Analysis (PCO)

Relationship among *S. chirayita* accession were also studied using PCO according to Jaccard similarity coefficient with MVSP version 3.2 (Multi – Variate Statistical Package) program (Kovach, 2007).

## 4) Estimation of within population genetic diversity of *S. chirayita*

Using statistical package SPSS 17.0, boxplot was designed for the percent polymorphism observed with each of 26 primers for each population to clarify the polymorphism based genetic diversity of *S. chirayita* individuals within the populations under study.

## 5) Population Genetic Analysis of *S. chirayita*

Another software POPGENE version 1.32 (Population Genetic Analysis, Yeh *et al.*, 1997; University of Alberta) was used for the further analysis and estimation of standard genetic variability measures within and in between populations of *S. chirayita* using data obtained from RAPD profiling. Popgene is a user-friendly Microsoft ® windows-based computer package for the analysis of genetic variation among and within natural populations using co-dominant and dominant markers and quantitative traits using haploid and diploid data.

The estimates of NEI's (1972) standard genetic identity ($I_{xy}$) and standard genetic distance ($D_{xy}$) along with the unbiased measures (Nei, 1978) for all pair-wise comparisions of *S. chirayita* populations were calculated showing the genetic relationships between studied populations. Genetic relatedness were also illustrated with the dendrogram drawn based on UPGMA (drawn

by modified neighbor procedure of Phylip 3.5) analysis which could be compared with the results given by NTSYS-PC analysis of RAPD data. Nei's (1972) distance is calculated as given below:

$$d_{ij} = -\ln\left(\frac{\sum_k \left|x_{ki}x_{kj}\right|}{\sqrt{\sum_k x_{ki}^2 x_{kj}^2}}\right)$$

The summation indicates a summation over all loci (k) between 2 individuals, i and j.

In order to estimate the genetic variation within and among populations of *S. chirayita*, the Shannon's information index was calculated using the formula, $I = -\sum P_i \log_2 P_i$ (Lewontin, 1972), where 'I" is diversity and $P_i$ is the frequency of a particular RAPD band. Using Popgene 1.32 also, Nei's (1973) gene diversity was estimated within and between the populations as $H = 1-\sum x_i^2$ where (in the dominant case), $x_i$ is the population frequency of each allele (1 and 0) at locus i.

Various measures of heterozygosity were also calculated. Total heterozygosity (in overall population), $H_T = 2q_i (1 - q_i) + \text{var}(q_i)$ where $q_i$ is the frequency of the null allele at $i^{th}$ locus in a population. $H_T$ was calculated with Lynch and Milligan's correlation (Lynch and Milligan, 1994). In addition, Mean heterozygosity within population (Hs) was calculated as $H_S = \sum_{i=1}^{i=k} p^2$. Where p is the mean frequency of the $i^{th}$ allele (1 or 0) at the $k^{th}$ locus in each population and the value is averaged over all populations, diversity among populations ($D_{ST} = H_T - H_S$) and the coefficient of population differentiation ($G_{ST} = D_{ST}/H_T$) were also estimated. The relative magnitude of genetic differentiation among subpopulations ($G_{ST}$) was obtained according to NEI (1987). $G_{ST}$ estimates were used to calculate the Number of Immigrants ($N_m$, gene flow) per generation for each locus using the formula, $N_m = 0.5 (1 - G_{ST})/G_{ST}$ and the mean value across loci (Mcdermott and Mcdonald, 1993; Kandedmir *et al.*, 2004).

## 6) Analysis of Molecular Variance (AMOVA)

Evaluation of genetic variation was also carried out for the binary data matrix created for all RAPD loci by analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992) using the statistical program GENALEx 6.41 (Peakall and Smouse, 2001). The AMOVA was based on the Euclidean metric of Excoffier *et al.* (1992) which is given by:

$E = (\epsilon^2_{xy}) = n\,[1-2n_{xy}/2n]$, Where, $2n_{xy}$ is the number of markers shared by two individuals (x and y) and n is the total number of polymorphic markers (Tsuda *et al.*, 2004). The molecular variance within the population was calculated as an indicator of intra-population genetic variation. Estimates of the partitioning of the genetic variation among the five populations and among individuals within the populations were initially derived without considering regional differences.

However, in hierarchical analysis with AMOVA the genetic variation in *S. chirayita* was partitioned among regions (Eastern, Central and Western Nepal), as well as among populations within regions and among individuals within populations (Phulchowki, Kaski, Sankhuwasabha, Terathum and Nagarjun) along with estimation of genetic differentiation, $\phi_{PT}$. The significance of the variance components was tested by calculating their probabilities, based on 999 random permutations using the program GENALEx (Peakall and Smouse, 2001).

# 4.RESULTS

## 4.1 Collection of *S. chirayita* samples

Five different populations of *S. chirayita* representing five different geographical locations from Eastern, Central and Western development regions of Nepal were included in the present study.

### *Sankhuwasabha*

It is a high hill district of Eastern Development region of Nepal. The study area selected were Lamapokhari, Shreemane, Manlabre and Chauki at an altitude of 2600 – 2950 m asl. surrounding the latitude and longitude of 27°36.9'N and 87°8.5'E respectively. The mean annual precipitation is 2258 mm and mean temperature ranges from 10°C to 19°C. It has relative humidity of 83.21%. Vegetation of Sankhuwasabha is rich in *Alnus nepalensis*, *Rhododendron arboretum* etc. Chiraito and Lokta are popular non timber forest products of this area supporting livelihood of the local people. Population in this area is highly dominated by ethnic community of Gurung.

### *Terathum*

It is another high hill district from Eastern development region with diversified vegetation. Study area was included from Trikhimti to Guphaphokhari at an altitude of 1500m - 2800m asl. The latitude and longitude of this area are 27°11.9'N and 87°30'E respectively. The annual precipitation of the place is 2208 mm. Annual mean temperature ranges from 10.8°C to 19°C and relative humidity of 83.21%. It has very cold climate covered by dense fog during winter. Frost and snow fall meet in coldest month from December to January. Almost all the roadside is covered by Alder forest. Lokta, Chiraito, Allo are the popular non timber forest products. Gradually, people are being attracted towards cash crops in this region which are helping to improve their economic status replacing the traditional agriculture practice. Major ethnic populations there are Tamang, Rai, Gurung and others.

### *Nagarjun, Kathmandu*

In the Central development region of Nepal, the study site was selected in Shivapuri Nagarjun National Park situated on the northern most border region of Kathmandu valley. The latitude and longitude of this area are 27°48.8'N and 85°23.2'E respectively. It is 8 km far from the main city of Kathmandu. It receives annual precipitation around 1724 mm. Its mean annual temperature ranges from 11°C to 23°C. Relative humidity is 85 percent. Vegetation consists of Schima, Pine and mixed broad leaved forest. Investigation site was located at Jamacho (2050-2100m).

### *Phulchowki, Lalitpur*

In Lalitpur district investigation site was selected in the Mt. Phulchowki which is situated in south-east corner of Kathmandu at the latitude and longitude of 27°34.3'N and 85°24.3'E

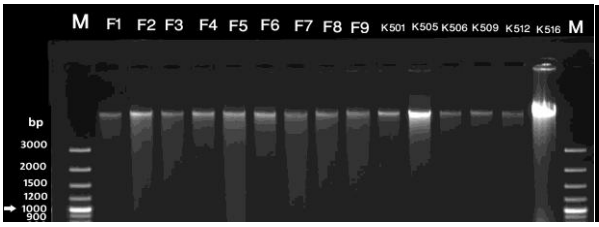
respectively. It is the highest mountain (2715m asl) in the valley. It has annual precipitation of 1881 mm and annual mean temperature ranges from 11$^{o}$C to 22$^{o}$C with relative humidity 78%. Area is covered with lush green forest constituting the diversified vegetation including the Royal Botanical Garden at Godawari established in 1962 at the base of phulchowki for the advancement of botanical research and conservation activities around this area.

### *Sikles, Kaski*

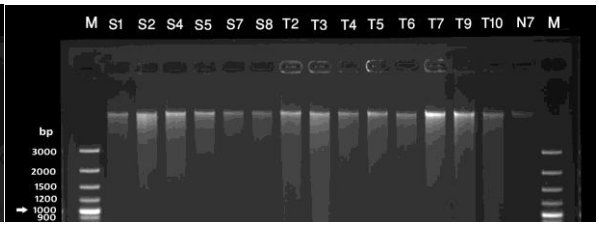
Another investigation site was selected from Western development region of Nepal at Sikles in Kaski district. Northern region with rich vegetation was chosen for study area. The latitude and longitude of this area are 28$^{o}$21.5'N and 84$^{o}$6.3'E respectively. The study site is a small part of Annapurna Conservation Area situated at 2000 – 2500 m a.s.l. The vegetation is dominated by tall and giant tress of *Abies spectabilis* and *Rhododendron barbatum*. The annual rainfall recorded was 5257 mm and the average maximum temperatures are 23.7$^{o}$C and 16.5$^{o}$C respectively.

## 4.2 Extraction of plant DNA

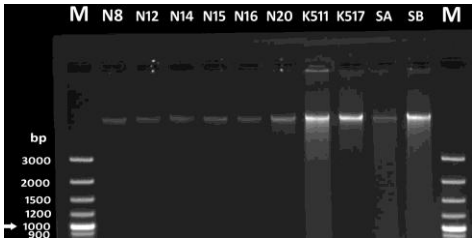
Among the two DNA extraction techniques assessed, the DNA extraction from CTAB method (Graham *et al.*, 1994) produced better bands on gel electrophoresis and hence the method was selected for subsequent extraction of DNA.




**Gel photograph: [A]**




**Gel photograph: [B]**




**Gel photograph: [C]**

**Plate 4.1** Gel photographs of DNA samples extracted using Graham's CTAB method. Lanes marked M are 100 bp plus molecular weight markers. [A] Lanes $F_1$-$F_9$ are *S. chirayita* accessions from Phulchowki, Lalitpur; $K_{501}$-$K_{509}$ are *S. chirayita* from Sikles, Kaski along with K512 and K516 (Outlier *Swertia sp.*); [B] $S_1$-$S_8$ are *S. chirayita* accessions from Sankhuwasabha; $T_2$-$T_{10}$ are *S. chirayita* accessions from Terathum; [C]

$N_7$-$N_{20}$ are *S. chirayita* accessions from Nagarjun, Kathmandu; $K_{511}$ and $K_{517}$ are *Swertia dilatata* from Kaski along with $S_A$ and $S_B$ (*Swertia sp.* from Sankhuwasabha).

## 4.3 DNA Quantification

The DNA estimation (using biophotometer (Eppendorff, Germany)) of 40 samples used in this investigation gave DNA concentrations ranging from 42.3 µg/mL to 462.6 µg/mL (Table 4.1). Majority showed the ratio of the optical density (OD), 260 nm and 280 nm ranging from 1.8-2.0.

**Table 4.1** DNA Quantification Results obtained using Biophotometer (Eppendorff, Germany)

| **Sample ID** | **Dilution Factor (**DNA:SDW**)** | **Absorbance (nm)** | | | | **Ratio 260/230** | **Ratio 260/280** | **Concentration (µg/mL)** |
|---|---|---|---|---|---|---|---|---|
| | | 230 | 260 | 280 | 320 | | | |
| F1 | 10 : 90 | 0.227 | 0.158 | 0.087 | 0.014 | 0.69 | 1.82 | 78.9 |
| F2 | 10 : 90 | 0.315 | 0.333 | 0.172 | 0.011 | 1.06 | 1.93 | 166.6 |
| F3 | 10 : 90 | 0.545 | 0.433 | 0.234 | 0.039 | 0.79 | 1.85 | 216.4 |
| F4 | 10 : 90 | 0.589 | 0.570 | 0.301 | 0.036 | 0.97 | 1.89 | 285.1 |
| F5 | 10 : 90 | 0.489 | 0.496 | 0.263 | 0.036 | 1.01 | 1.89 | 248.1 |
| F6 | 10 : 90 | 0.456 | 0.386 | 0.206 | 0.031 | 0.85 | 1.87 | 193.2 |
| F7 | 10 : 90 | 0.462 | 0.288 | 0.161 | 0.043 | 0.62 | 1.78 | 143.8 |
| F8 | 10 : 90 | 0.527 | 0.357 | 0.249 | 0.136 | 0.68 | 1.43 | 178.6 |
| F9 | 10 : 90 | 0.638 | 0.435 | 0.264 | 0.100 | 0.68 | 1.65 | 217.5 |
| K501 | 10 : 90 | 0.114 | 0.123 | 0.090 | 0.019 | 1.08 | 1.36 | 61.3 |
| K505 | 10 : 90 | 0.186 | 0.304 | 0.158 | 0.024 | 1.63 | 1.92 | 1502.0 |
| K506 | 10 : 90 | 0.059 | 0.085 | 0.041 | 0.003 | 1.43 | 2.04 | 42.3 |
| K509 | 10 : 90 | 0.199 | 0.216 | 0.120 | 0.025 | 1.09 | 1.80 | 107.8 |
| S1 | 10 : 90 | 0.171 | 0.300 | 0.155 | 0.020 | 1.76 | 1.94 | 150.0 |
| S2 | 10 : 90 | 0.165 | 0.212 | 0.112 | 0.012 | 1.28 | 1.89 | 105.8 |
| S4 | 10 : 90 | 0.146 | 0.228 | 0.119 | 0.01 | 1.56 | 1.92 | 114.0 |
| S5 | 10 : 90 | 0.156 | 0.172 | 0.096 | 0.021 | 1.10 | 1.80 | 85.9 |
| S7 | 10 : 90 | 0.147 | 0.192 | 0.102 | 0.01 | 1.31 | 1.88 | 95.9 |
| S8 | 10 : 90 | 0.164 | 0.119 | 0.112 | 0.020 | 1.21 | 1.77 | 99.5 |
| T2 | 10 : 90 | 0.226 | 0.341 | 0.189 | 0.046 | 1.50 | 1.80 | 170.3 |
| T3 | 10 : 90 | 0.258 | 0.385 | 0.210 | 0.044 | 1.49 | 1.83 | 192.5 |
| T4 | 10 : 90 | 0.291 | 0.429 | 0.243 | 0.045 | 1.47 | 1.76 | 214.5 |
| T5 | 10 : 90 | 0.323 | 0.424 | 0.224 | 0.039 | 1.31 | 1.90 | 212.2 |
| T6 | 10 : 90 | 0.232 | 0.358 | 0.189 | 0.025 | 1.54 | 1.90 | 179.2 |
| T7 | 10 : 90 | 0.232 | 0.555 | 0.281 | 0.038 | 1.72 | 1.97 | 277.5 |
| T9 | 10 : 90 | 0.242 | 0.404 | 0.203 | 0.023 | 1.67 | 1.99 | 202.1 |
| T10 | 10 : 90 | 0.186 | 0.286 | 0.148 | 0.024 | 1.54 | 1.93 | 143.1 |
| N7 | 10 : 90 | 0.260 | 0.153 | 0.085 | 0.016 | 0.59 | 1.80 | 76.4 |
| N8 | 10 : 90 | 0.209 | 0.103 | 0.057 | 0.01 | 0.49 | 1.81 | 51.5 |
| N12 | 10 : 90 | 0.215 | 0.110 | 0.063 | 0.015 | 0.51 | 1.75 | 55.2 |
| N14 | 10 : 90 | 0.235 | 0.136 | 0.080 | 0.015 | 0.58 | 1.70 | 68.0 |
| N15 | 10 : 90 | 0.210 | 0.133 | 0.07 | 0.011 | 0.64 | 1.91 | 66.7 |
| N16 | 10 : 90 | 0.217 | 0.145 | 0.075 | 0.011 | 0.67 | 1.93 | 72.5 |
| N20 | 10 : 90 | 0.269 | 0.197 | 0.104 | 0.015 | 0.73 | 1.90 | 98.7 |
| K511 | 10 : 90 | 0.845 | 0.965 | 0.685 | 0.410 | 1.14 | 1.41 | 462.6 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| K517 | 10 : 90 | 0.118 | 0.222 | 0.101 | 0.005 | 1.87 | 2.06 | 110.9 |
| K512 | 10 : 90 | 0.136 | 0.223 | 0.116 | 0.016 | 1.64 | 1.92 | 111.7 |
| K516 | 10 : 90 | 0.445 | 0.712 | 0.361 | 0.062 | 1.60 | 1.97 | 355.8 |
| SA | 10 : 90 | 0.228 | 0.296 | 0.146 | 0.021 | 1.29 | 2.03 | 147.8 |
| SB | 10 : 90 | 0.322 | 0.530 | 0.276 | 0.036 | 1.65 | 1.92 | 265.2 |

## 4.4 RAPD – PCR Optimization

### 4.4.1 RAPD-PCR Cycling conditions optimizations

Among the two cycling conditions assessed (Yu and Pauls, 1992 and Edwards, 1998), PCR program with initial denaturation of $95^0$C for 2 minutes followed by 45 cycles of denaturation at $95^0$C for 20 seconds, annealing at $37^0$C for 60 seconds, elongation at $72^0$C for 60 seconds and final elongation of 10 minutes at $72^0$C (Edwards, 1998) was found to give best RAPD banding patterns for *S. chirayita* and therefore selected for subsequent PCR amplification for RAPD profiling.

### 4.4.2 RAPD-PCR reaction conditions optimization

**Table 4.2** PCR parameters tested and selected optimized parameters

| S. N. | PCR parameters | Tested range | Optimum condition selected | Remarks |
|---|---|---|---|---|
| **1.** | DNA concentration (ng) | 12.5, 25, 37.5, 50, 62.5, 75, 87.5, 100 | 25ng | Even though most of the concentrations produced the reproducible bands, 25 ng of template DNA was selected as optimum concentration being the minimum concentration producing discernible bands.(Plate 4.2). |
| **2.** | $MgCl_2$ concentration (mM) | 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 | 3.0mM | Bands observed at 2.5 – 3.5 mM were crispy and 3.0mM $MgCl_2$ was taken as optimum concentration for further experiments (Plate 4.3). |
| **3.** | Primer concentration (µM) | 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6 | 0.4µM | The banding pattern suggested a range of primer concentration 0.4µM – 0.8µM to be good. But, 0.4µM of primer was finally selected to be used for subsequent RAPD profiling experiments (Plate 4.4). |
| **4.** | dNTPs concentration | 0.1, 0.2, 0.3, 0.4, 0.5 | 0.2mM | According to the RAPD patterns observed, 0.2 mM of dNTPs was |

| | | | | |
|---|---|---|---|---|
| | (mM) | | | selected as optimum concentration (Plate 4.5) |
| **5.** | *Taq* polymerase concentration (U) | 0.5, 1.0, 1.5, 2.0, 2.5 | 1U | Although all the concentration produced RAPD band profiles, the best banding pattern was considered for 1 unit *Taq* polymerase hence used in further RAPD experiments (Plate 4.6). |

Thus, final optimized RAPD-PCR reaction condition for *Swertia chirayita* was documented to be 25 ng of genomic DNA, 3.0mM $MgCl_2$, 2.5µL 10X PCR reaction buffer (Fermentas, 100mM Tris-HCl/pH 8.8 at $25^0$C, 500mM KCl, 0.8% Nonidet $P_{40}$), 0.2 mM dNTPs, 1U *Taq* polymerase and 0.4µM primer in a 25µL PCR reaction volume.

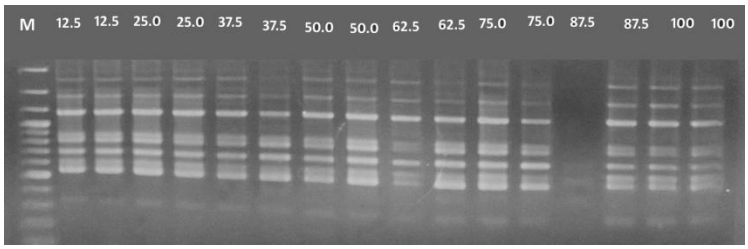



**Plate 4.2** RAPD profile generated by primer UBC 2 with a *S. chirayita* sample of Kaski with varying **template DNA concentration**. Each lane is labeled with the respective concentrations of template DNA (12.5 – 100 ng) used during RAPD-PCR. Lane labeled 'M' is 100 bp plus molecular weight marker.

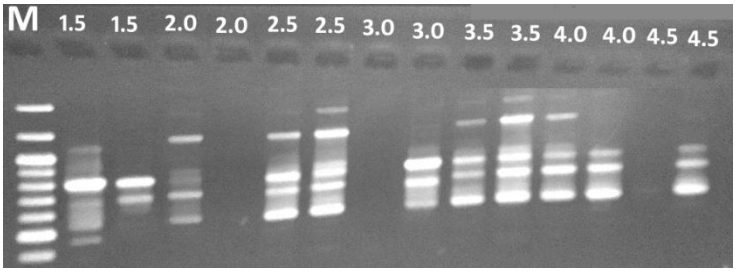



**Plate 4.3** RAPD profile generated by primer UBC 2 with the same *S. chirayita* DNA sample of Kaski and varying **$MgCl_2$ concentrations.** Each lane is marked with the respective concentration of $MgCl_2$ (1.5 - 4.5 mM) used during RAPD-PCR. Lane marked 'M' is 100 bp plus molecular weight marker.

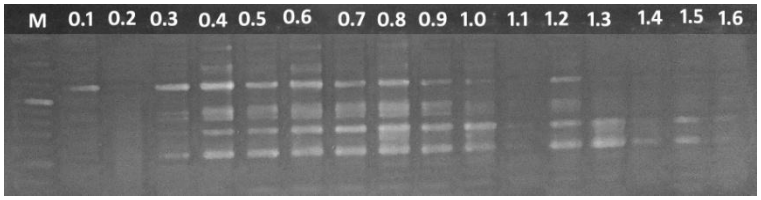



**Plate 4.4** RAPD profile generated using the *S. chirayita* tDNA of the Kaski with varying **concentration of primer** UBC2. Each lane is marked with the respective concentration of primer (UBC 2) (0.1 – 1.6 µM) used in RAPD-PCR reaction. Lane labeled 'M' is 100 bp plus molecular weight marker.

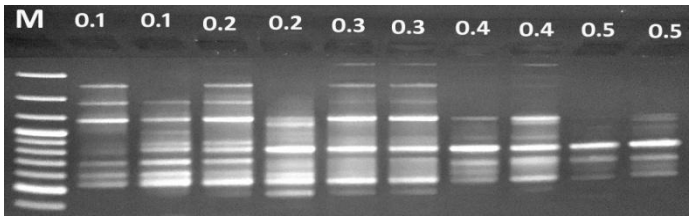



**Plate 4.5** RAPD-PCR pattern generated by primer UBC 2 with the same DNA sample from Kaski and the range of **dNTPs concentrations**. Each lane is marked with the respective concentration of dNTPs (0.1 – 0.5 mM) used during RAPD-PCR. Lane marked 'M' is 100 bp plus molecular weight marker.

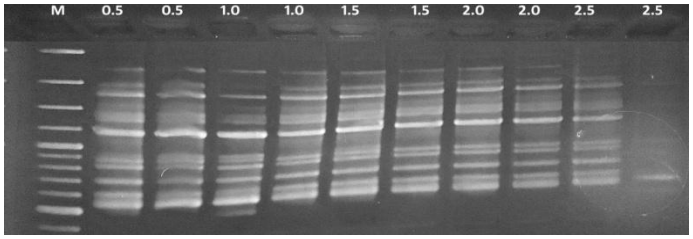
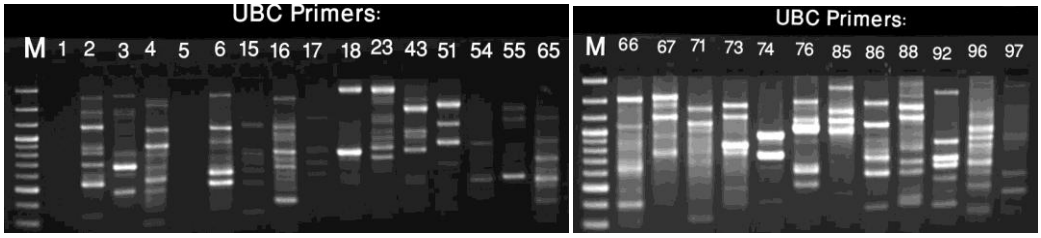



**Plate 4.6** RAPD profile generated by the use of the primer UBC 2 with the Kaski sample of *Swertia* and varying respective concentrations of ***Taq* polymerase** (0.5 – 2.5U) used during RAPD PCR. Lane marked 'M' is 100 bp plus molecular weight marker.

## 4.5 Primer Screening for RAPD Profiling

Initially, using optimized RAPD-PCR cycling conditions and reaction condition, 100 different decamer primers were screened using fresh genomic DNA of *Swertia chirayita* from Kaski. Out of 100 primers, 28 primers were selected on the basis of band visualization. Of these 28 again, 26 primers (*viz.* Table 4.3) that gave multiple and crispy bands were finally selected to be used in RAPD-PCR profiling analysis involving all the accessions of *Swertia chirayita* from different populations. Two primers, UBC 1 and UBC 5 that did not produce reproducible bandings were excluded from the study (Plate 4.7). The experiments were repeated twice for confirmation of the reproducibility of RAPD profiling.




[A] [B]

**Plate 4.7** RAPD gel pictures for the primer screening experiment using a template DNA of *Swertia chirayita* from Kaski district. Each lane in (A) and (B) corresponds to different UBC primers used in the experiment. Lane marked 'M' is 100 bp Plus DNA ladder.

**Table 4.3** Sequences and GC content of all the selected primers used in RAPD PCR profiling experiment:

| S.N. | **Primer Code** | **5' to 3' base sequences** | **GC Content (%)** |
|---|---|---|---|
| **1** | UBC2 | CCT GGG CTT G | 70 |
| **2** | UBC3 | CCT GGG CTT A | 60 |
| **3** | UBC4 | CCT GGG CTG G | 80 |
| **4** | UBC6 | CCT GGG CCT A | 70 |
| **5** | UBC15 | CCT GGG TTT G | 60 |
| **6** | UBC16 | GGT GGC GGG A | 80 |
| **7** | UBC17 | CCT GGG CCT C | 80 |
| **8** | UBC18 | GGG CCG TTT A | 60 |
| **9** | UBC23 | CCC GCC TTC C | 80 |
| **10** | UBC43 | AAA AAC GGG C | 50 |
| **11** | UBC51 | CTA CCC GTG C | 70 |
| **12** | UBC54 | GTC CCA GAG C | 70 |
| **13** | UBC55 | TCC CTC GTG C | 70 |
| **14** | UBC65 | AGG GGC GGG A | 80 |
| **15** | UBC66 | GAG GGC GTG A | 70 |
| **16** | UBC67 | GAG GGC GAG C | 80 |
| **17** | UBC71 | GAG GGC GAG G | 80 |
| **18** | UBC73 | GGG CAC GCG A | 70 |
| **19** | UBC74 | GAG CAC CTG A | 60 |
| **20** | UBC76 | GAG CAC CAG T | 60 |
| **21** | UBC85 | GTG CTC GTG C | 70 |
| **22** | UBC86 | GGG GGG AAG G | 80 |
| **23** | UBC88 | CGG GGG ATG G | 80 |
| **24** | UBC92 | CCT GGG CTT T | 60 |
| **25** | UBC96 | GGC GGC ATG G | 80 |

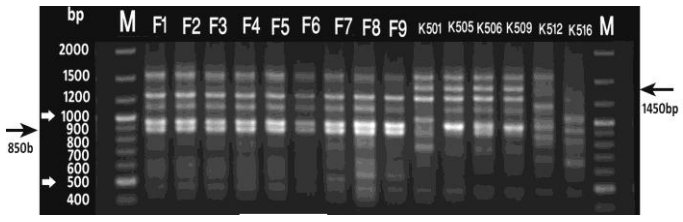
| | | | |
|---|---|---|---|
| **26** | UBC97 | ATC TGC GAG C | 60 |

## 4.6 RAPD Profiling and Identification of species specific and Population Specific RAPD Markers for *S. Chirayita*

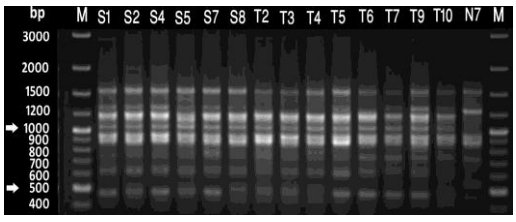
For the RAPD profile study of *S. chirayita* from 5 different populations, districtwise: Phulchowki (Lalitpur), Kaski, Nagarjun (Kathmandu), Sankhuwasabha and Terathum, the polymorphic profile of each of 26 selected UBC primers (Table 4.3) observed in electrophoresed agarose gel (1.5 %) for each of the accessions of *S. chirayita* were investigated. From the comparative analysis of RAPD profiles generated by 26 primers for the accessions from five districts, 7 primers generated 9 different population-specific polymorphic markers were obtained (Table 4.4) which can be utilized as population specific DNA fingerprints for authentication purpose. However, no such marker was revealed that was specific to *S. chirayita* species.

**Table 4.4** Population specific RAPD Markers for *S. chirayita* observed after RAPD-PCR profiling with selected primers ('+' = present and '-' = absent)

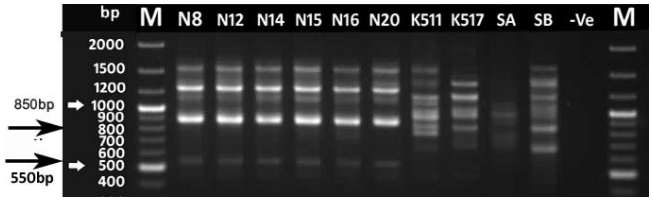
| Primer code | Marker size (bp) | *S. chirayita* population from | | | | |
|---|---|---|---|---|---|---|
| | | Phulchowki | Kaski | Sankhuwasabha | Terathum | Nagarjun |
| **UBC3** | 2800 | - | - | + | - | - |
| **UBC4** | 1350 | + | - | - | - | - |
| **UBC16** | 1350 | - | - | + | + | - |
| **UBC71** | 1050 | + | + | - | - | - |
| **UBC76** | 400 | - | - | + | + | - |
| **UBC92** | 700 | - | + | - | - | - |
| **UBC96** | 1450 | - | + | - | - | - |
| **UBC96** | 850 | - | - | - | - | + |
| **UBC96** | 550 | - | - | - | - | + |




[A]

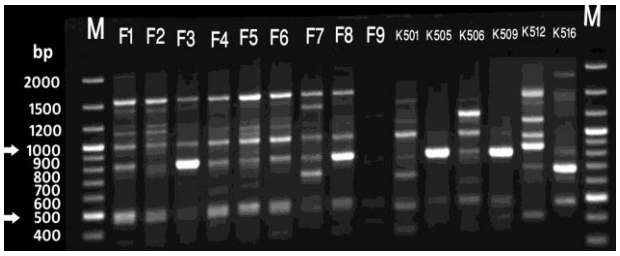



[B]

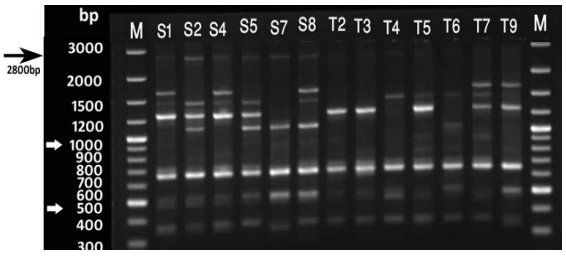



[C]

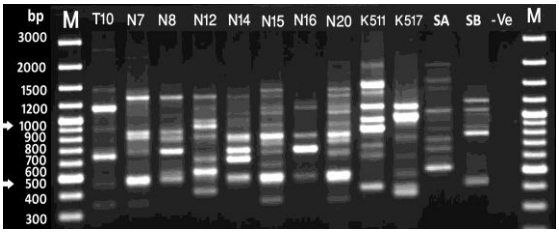
**Plate 4.8** Population Specific Markers amplified with **UBC 96**. Lanes marked with M are 100bp plus molecular weight markers. [A] Lanes $F_1$-$F_9$ are *S. chirayita* accessions from Phulchowki, $K_{501}$-$K_{509}$ are *S. chirayita* from Kaski, along with $K_{512}$ and $K_{516}$ (Outlier *Swertia sp.*); [B] $S_1$-$S_8$ are *S. chirayita* accessions from Sankhuwasbha, $T_2$-$T_{10}$ are *S. chirayita* accessions from Terathum; [C] N7-N20 are *S. chirayita* accessions from Nagarjun, K511 and K517 are *Swertia sp*. from Kaski along with SA and SB (*Swertia sp*. from Sankhuwasabha). Dark arrow indicates population specific marker for *S. chirayita* from Kaski (1450bp) and Nagarjun (550bp and 850bp).




[A]

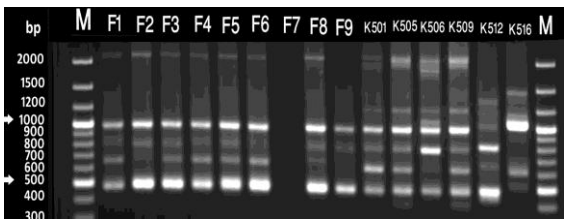



[B]

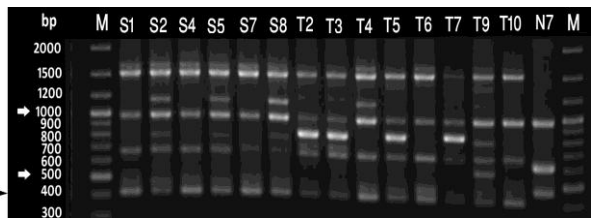



[C]

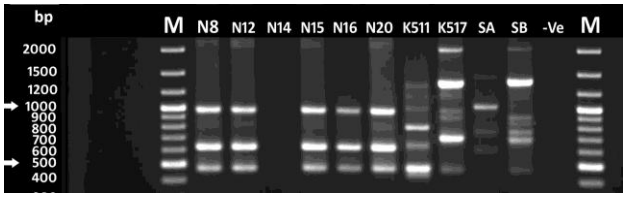
**Plate 4.9** RAPD profile produced for *S. chirayita* samples from various districts with **UBC 3**. Refer legend at plate 4.8. Dark arrows indicate population specific marker for *S.chirayita* from Sankhuwasabha (2800bp).




[A]

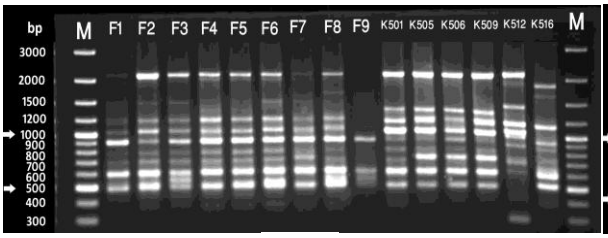



[B]

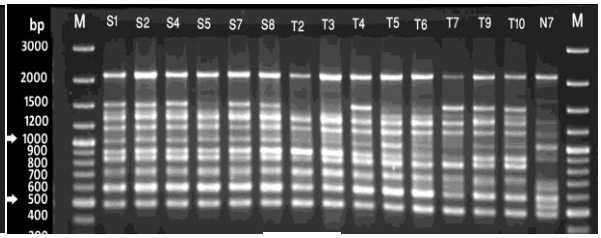



[C]

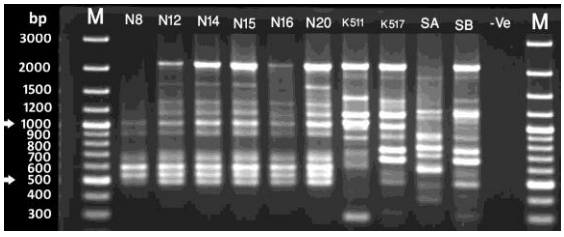
**Plate 4.10** RAPD profile produced for *S. chirayita* samples from various districts with **UBC 76**. Refer legend at plate 4.8. Black arrows indicate population specific marker for *S.chirayita* from Sankhuwasabha and Terathum (400bp).




[A]




[B]




[C]

**Plate 4.11** RAPD profile produced for *S. chirayita* samples from various districts with **UBC 6**. Refer legend at plate 4.8

## 4.7 RAPD Profiling and Assessment of Genetic Diversity

All the RAPD-PCR profiles generated by the selected 26 UBC primers were used for the assessment of genetic diversity. A total of 285 RAPD loci were amplified across the 34 *S. chirayita* accessions with an average amplification of 11 bands per primer. Of the total amplified bands, 263 (92.28%) were polymorphic whilst 22 (7.72%) bands were observed to be monomorphic. When the bands produced for the 6 outlier accessions of *S. chirayita* were also included, the total RAPD fragments were observed to be 386.

Considering band profiles of 34 *S. chirayita* accessions only, each primer produced the polymorphic band ranging from 57% to 100% individually. Sixteen out of twenty six primers used revealed 100 % polymorphism. The number of scorable band produced per primer ranged from 5 to 15 with the variation in amplicon size ranging from 200 bp to 3600 bp. The highest number of RAPD loci (15) was produced by the primer UBC 3 whereas the lowest number of RAPD loci (5) was produced by the primer UBC 17 in the total accessions tested (Table 4.5)

**Table 4.5** Primer sequences, Total number of amplified band (TNB), number of polymorphic band (NPB), Percentage polymorphism and range of amplicon size of the 26 RAPD primers used to generate RAPD profiles in 34 *Swertia chirayita* accessions.

| | Primer Sequence ( 5'-3') | TNB | NPB | Polymorphisms (%) | Amplicon size range (bp) |
|---|---|---|---|---|---|
| **UBC 2** | CCTGGGCTTG | 13 | 13 | 100.00 | 400-2500 |
| **UBC 3** | CCTGGGCTTA | 15 | 15 | 100.00 | 350-2800 |
| **UBC 6** | CCTGGGCCTA | 13 | 13 | 100.00 | 300-2100 |
| **UBC 51** | CTACCCGTGC | 7 | 4 | 57.14 | 500-2500 |
| **UBC 85** | GTGCTCGTGC | 9 | 6 | 66.67 | 450-1900 |
| **UBC 18** | GGGCCGTTTA | 9 | 9 | 100.00 | 200-2000 |
| **UBC 74** | GAGCACCTGA | 8 | 8 | 100.00 | 450-200 |
| **UBC 76** | GAGCACCAGT | 11 | 11 | 100.00 | 400-2150 |
| **UBC 23** | CCCGCCTTCC | 11 | 8 | 72.73 | 500-2400 |
| **UBC 43** | AAAACCGGG | 13 | 13 | 100.00 | 450-3600 |
| **UBC 65** | AGGGGCGGGA | 14 | 14 | 100.00 | 400-2700 |
| **UBC 97** | ATCTGCGAGC | 13 | 13 | 100.00 | 500-2000 |
| **UBC 86** | GGGGGGAAGG | 9 | 9 | 100.00 | 350-2400 |
| **UBC 4** | CCTGGGCTGG | 10 | 8 | 80.00 | 450-1900 |
| **UBC 73** | GGGCACGCGA | 10 | 10 | 100.00 | 600-2000 |
| **UBC 67** | GAGGGCGAG | 9 | 6 | 66.67 | 400-2000 |
| **UBC 15** | CCTGGGTTTG | 12 | 12 | 100.00 | 450-2500 |
| **UBC 16** | GGTGGCGGGA | 12 | 10 | 83.33 | 400-2000 |
| **UBC 54** | GTCCCAGAGC | 11 | 11 | 100.00 | 550-2600 |
| **UBC 66** | GAGGGCGTGA | 11 | 9 | 81.82 | 400-1700 |
| **UBC 71** | GAGGGCGAGG | 11 | 10 | 90.91 | 300-1800 |
| **UBC 88** | CGGGGGATGG | 10 | 9 | 90.00 | 400-3000 |
| **UBC 96** | GGCGGCATGG | 12 | 10 | 83.33 | 450-1600 |
| **UBC 92** | CCTGGGCTTT | 13 | 13 | 100.00 | 350-2700 |
| **UBC 55** | TCCCTCGTGC | 14 | 14 | 100.00 | 550-2000 |

| | | | | | |
|---|---|---|---|---|---|
| **UBC 17** | CCTGGGCCTC | 5 | 5 | 100.00 | 600-2200 |
| | **Total** | **285** | **263** | | |
| **Average polymorphic band per primer** | | | | **Avg. Pol.%** | **92.28** |
| **10.96** | | | | | |

### 4.7.1 Polymorphic Information Content, Band informativeness and Resolving power

Polymorphic Information Coefficient (PIC) score for each primer ranged from 0.67 (UBC 51) to 0.91 (UBC 6) with an average of 0.85 (Table 4.6). The primers scoring PIC value more than 0.85 were: 6, 2, 3, 92, 55, 96, 71, 76, 54, 16, 15, 86, 65 and 43 in decreasing order of their value. The average Band Informativeness ($I_B$) of the 26 primers was 0.82 and it ranged from 0.38 (for UBC 92) to 1.22 (UBC 71). The same primers that had highest PIC value also gave the highest $R_P$ score. The Resolving Power ($R_P$) of the 26 RAPD primers ranged from 2.41 for primer UBC 17 to 16.28 for primer UBC 6 with an average of 8.80. The primers furnishing high $R_P$ values more than 10 are UBC primers: 2, 3, 16, 71, 88 and 96 (Table 4.6)

**Table 4.6** Assessment of PIC, $I_B$ and $R_P$ of the 26 RAPD primers used to generate RAPD profiles in 34 *Swertia chirayita* accessions.

| Primer Code | Primer Sequence ( 5'-3') | PIC | $I_B$ range | $I_B$ Average | $R_P$ |
|---|---|---|---|---|---|
| **UBC 2** | CCTGGGCTTG | 0.90 | 0.12-1.94 | 0.95 | 13.29 |
| **UBC 3** | CCTGGGCTTA | 0.90 | 0.12-2.0 | 0.68 | 10.18 |
| **UBC 6** | CCTGGGCCTA | 0.91 | 0.18-2.0 | 1.20 | 16.28 |
| **UBC 51** | CTACCCGTGC | 0.67 | 0.06-1.94 | 1.0 | 5.0 |
| **UBC 85** | GTGCTCGTGC | 0.84 | 0.06-1.76 | 0.90 | 8.12 |
| **UBC 18** | GGGCCGTTTA | 0.84 | 0.06-1.94 | 0.96 | 8.65 |
| **UBC 74** | GAGCACCTGA | 0.83 | 0.12-1.88 | 0.99 | 7.94 |
| **UBC 76** | GAGCACCAGT | 0.89 | 0.06-1.88 | 0.79 | 9.53 |
| **UBC 23** | CCCGCCTTCC | 0.84 | 0.24-1.94 | 0.95 | 8.59 |
| **UBC 43** | AAAACCGGG | 0.85 | 0.06-1.94 | 0.74 | 9.65 |
| **UBC 65** | AGGGGCGGGA | 0.86 | 0.06-1.88 | 0.63 | 8.82 |
| **UBC 97** | ATCTGCGAGC | 0.84 | 0.06-2.0 | 0.47 | 6.06 |
| **UBC 86** | GGGGGGAAGG | 0.86 | 0.06-1.59 | 0.81 | 8.06 |
| **UBC 4** | CCTGGGCTGG | 0.84 | 0.29-2.0 | 1.10 | 9.94 |
| **UBC 73** | GGGCACGCGA | 0.81 | 0.06-1.59 | 0.55 | 5.53 |
| **UBC 67** | GAGGGCGAG | 0.81 | 0.24-1.94 | 0.94 | 7.53 |
| **UBC 15** | CCTGGGTTTG | 0.87 | 0.06-1.76 | 0.64 | 8.35 |
| **UBC 16** | GGTGGCGGGA | 0.87 | 0.12-2.0 | 1.01 | 11.06 |
| **UBC 54** | GTCCCAGAGC | 0.87 | 0.06-1.94 | 0.76 | 9.18 |

| | | | | | |
|---|---|---|---|---|---|
| **UBC 66** | GAGGGCGTGA | 0.82 | 0.06-2.0 | 0.70 | 7.65 |
| **UBC 71** | GAGGGCGAGG | 0.89 | 0.24-2.0 | 1.22 | 13.41 |
| **UBC 88** | CGGGGGATGG | 0.87 | 0.35-1.94 | 1.02 | 10.18 |
| **UBC 96** | GGCGGCATGG | 0.89 | 0.24-2.0 | 1.09 | 11.06 |
| **UBC 92** | CCTGGGCTTT | 0.90 | 0.06-0.88 | 0.38 | 4.94 |
| **UBC 55** | TCCCTCGTGC | 0.90 | 0.06-1.0 | 0.52 | 7.29 |
| **UBC 17** | CCTGGGCCTC | 0.74 | 0.06-0.88 | 0.40 | 2.41 |

## 4.7.2 Genetic diversity analysis using similarity coefficients and cluster analysis

### 4.7.2.1 Comparisons of Similarity coefficients and the phenograms

From the binary data matrix created based on presence and absence of RAPD loci, the varied range of similarity indices were obtained, using Simple Matching (SM), Jaccard's (J) and Dice (D) coefficients i.e SM (0.65-0.96), J (0.26-0.89) and D (0.42-0.94) with an average coefficient of similarity 0.81, 0.58 and 0.68 respectively (APPENDIX 3, 4 AND 5).

All the three similarity coefficients were employed for the generation of phenograms to represent the genetic relationship among all the 34 accessions of *S. chirayita* belonging to different populations under study. The topologies of the phenograms generated from all three coefficients were comparable except for few variations in range of polymorphism and slight alterations in position of few accessions within same cluster in the phenogram generated from SM coefficient (*viz*. fig 4.1, 4.2, 4.3). Two main clusters were visible in the phenogram. First cluster comprised of accessions from Phulchowki, Kaski and Nagarjun while samples from Sankhuwasabha and Terathum formed second cluster in all 3 phenograms. Within these two clusters again, distinct subclusters were visible for the samples from different geographical locations.

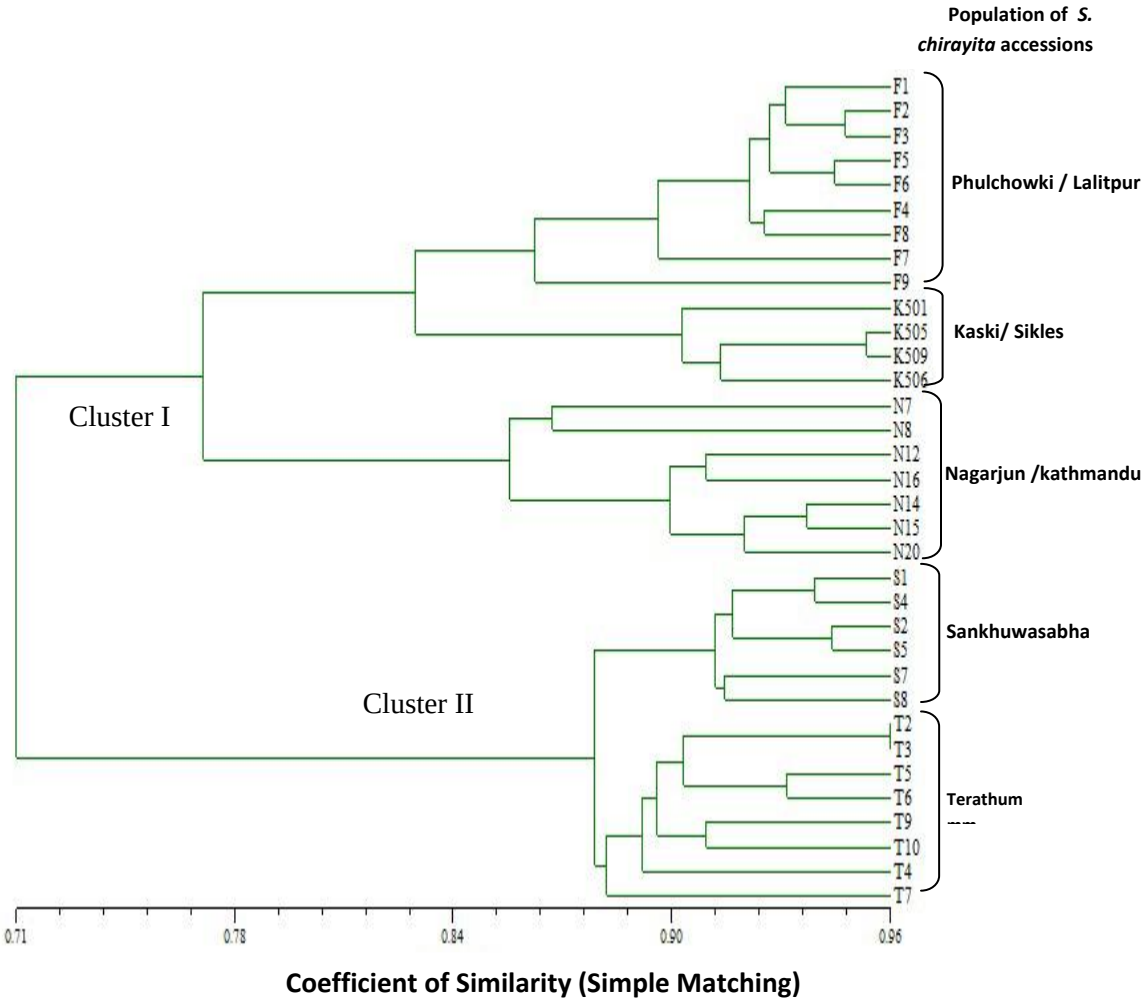



**Figure 4.1** Phenogram generated for 34 *Swertia chirayita* accessions revealed by UPGMA cluster analysis using Simple Matching coefficient of similarity calculated from 285 RAPD loci generated by 26 primers. The resulting clusters are labeled as I and II.

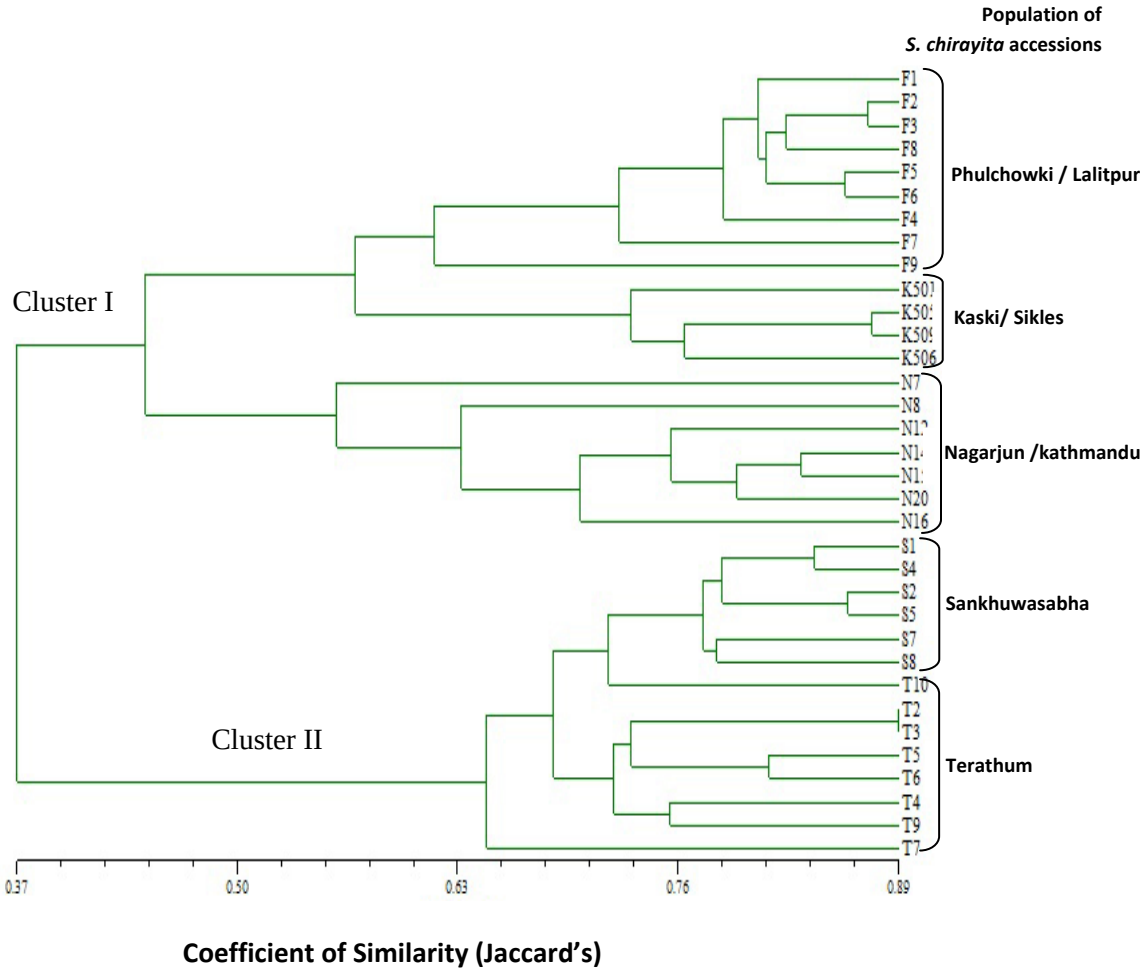



**Figure 4.2** Phenogram generated for 34 *Swertia chirayita* accessions revealed by UPGMA cluster analysis using Jaccard's coefficient of similarity calculated from 285 RAPD loci generated by 26 primers. The resulting clusters are labeled as I and II.

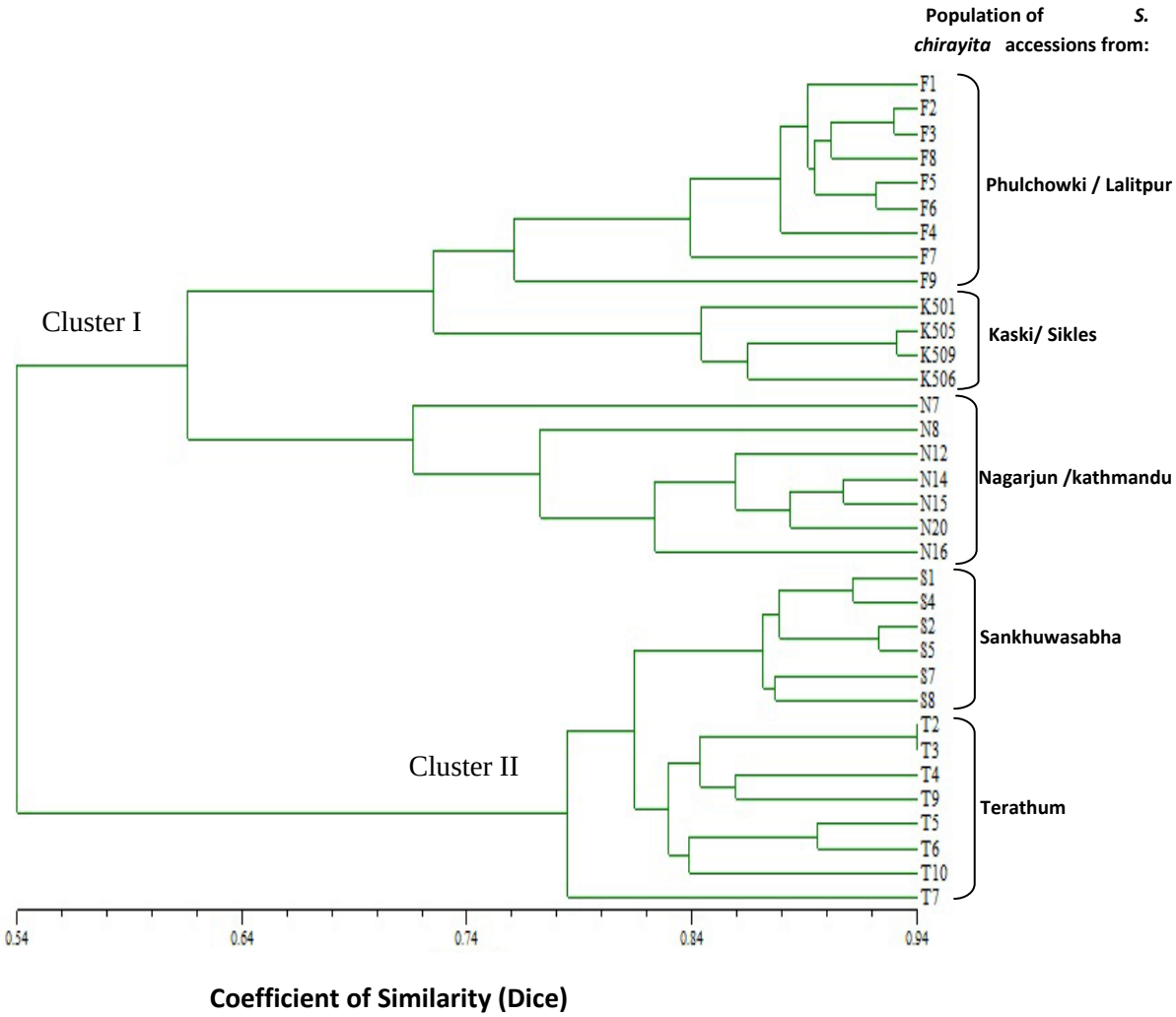



**Figure 4.3** Phenogram generated for 34 *Swertia chirayita* accessions revealed by UPGMA cluster analysis using Dice coefficient of similarity calculated from 285 RAPD loci generated by 26 primers. The resulting clusters are labeled as I and II.

The correlation values obtained from the comparison of original matrices by applying the Mantel test are shown in Table 4.7 (Rohlf, 2009). The results from Mantel test (Matrix comparison) of original matrices showed that the correlation between Jaccard and Dice similarity matrices was the highest and significant (0.92224). However, correlations between Jaccard and Simple matching and Dice and Simple Matching coefficients were low in comparison to the correlation between Jaccard and Dice.

**Table 4.7** Correlation coefficients from Mantel test (3 Way) of original similarity matrices.

| | **Simple Matching** | **Jaccard** | **Dice** |
|---|---|---|---|
| **Simple Matching** | ***** | | |
| **Jaccard** | 0.07056 | ***** | |
| **Dice** | 0.31292 | 0.92224 | ***** |

Clustering based on Unweighted Pair Group Method of Arithmetic Averages (UPGMA) for Jaccard coefficient was observed to give a highest cophenetic correlation value of 0.85012 and lowest cophenetic correlation value of 0.43580 was observed for UPGMA clustering using simple matching coefficient (Table 4.8). Because of their highest correlation rate and comparing the standard chart of goodness of fit, the value is $0.8 \leq\leq r < 0.9$ revealing Jaccard's coefficient of similarity with UPGMA clustering method as the best for deducing the genetic relationship among various *S. chirayita* accessions.

Since simple matching coefficient with UPGMA clustering method had a lowest correlation coefficient value (Table 4.8), the combination considered as least suitable for deducing the genetic relationship.

**Table 4.8** Correlation coefficients obtained for cophenetic values of similarity matrices (Simple Matching, Dice and Jaccard's coefficient) and clusters computed by UPGMA module using MXCOMP (matrix comparisons) of NTSYS.

| **Clustering modules of similarity** | **Simple Matching** | **Dice** | **Jacard** |
|---|---|---|---|
| **UPGMA** | 0.43580 | 0.84315 | 0.85012 |

In the study, Jaccard similarity with UPGMA yielded highest correlation value but the difference between Jaccard and Dice similarity coefficients with UPGMA clustering was not so far (0.85012 and 0.84315 respectively). From this test, the three coefficients can be judged in the decreasing order as J > D > SM for use in interpretation of genetic relationship in *S. chirayita*.

For the evaluation of trees constructed from UPGMA clustering by genetic similarity coefficients, consensus indices (CI) were also calculated for the each combination of coefficient and UPGMA clustering. The results from consensus indices are shown in (Table 4.9). Highest Consensus fork index of ($CI_C$ = 0.9063) was found for Jaccard and Dice coefficients. Simple matching coefficient correlated lower with the Dice and Jaccard coefficienrs ($CI_C$ = 0.7188). It supports the findings from dendograms and justify the deviation of results from the dendogram formed with simple matching coefficient as compared to the others.

**Table 4.9** Consensus fork index among the dendograms (UPGMA) produced by similarity coefficients among *S. chirayita* accessions by RAPD marker.

| | Jaccard | Dice | Simple Matching |
|---|---|---|---|
| Jaccard | ***** | 0.90625 | 0.71875 |

| | | | |
|---|---|---|---|
| Dice | | ***** | 0.71875 |
| Simple Matching | | | ***** |

From all the comparisons done for similarity coefficients, Jaccard coefficient was finally chosen to interpret the results on genetic diversity and relationships among various accessions representing different populations. On the basis of Jaccard's similarity coefficient (Appendix 4), genetic similarity (%) was estimated within and between the *S. chirayita* populations (Table 4.10). The individual genetic relatedness between the every *S. chirayita* accessions has been assessed with the pairwise comparison (Appendix 4) of Jaccard's similarity coefficient. During the pairwise comparison, *S. chirayita* accessions from Terathum: $T_2$ and $T_3$ were observed to be highly similar (89%) and the accessions from Terathum ($T_4$) and Nagarjun ($N_8$) revealed the least genetic similarity (26%). Every individual accession can be compared individually with any of other 34 *S. chirayita* sample to check out the genetic relatedness and distance. Similarly, the least genetic similarity was observed for the individuals of Nagarjun population (Table 4.10).

**Table 4.10** Percentage similarity observed for different *S. chirayita* populations based on RAPD similarity matrix (J) within and between the populations

| Pop/pop | Phulchowki | Kaski | Sankhuwasabha | Terathum | Nagarjun |
|---|---|---|---|---|---|
| **Phulchowki** | 56 - 87 | 50 - 64 | 38 - 45 | 34 - 44 | 38 - 50 |
| **Kaski** | | 71 - 87 | 35 - 41 | 35 - 40 | 38 – 49 |
| **Sankhuwasabha** | | | 73 – 86 | 61 – 77 | 30 – 49 |
| **Terathum** | | | | 62 - 89 | **26** – 37 |
| **Nagarjun** | | | | | 51 - 83 |

* Pop - Populations

### 4.7.2.2 Cluster Analysis

### Tree Phenogram

UPGMA clustering done based on the Jaccard's similarity matrix with the help of NTSYS-PC (Version 2.21i) clarified the genetic relationship of *S. chirayita* accessions from geographically diverse population. Thirty four individuals were found separated into two major subclusters at the similarity coefficient of 0.37. The first cluster contained accessions from Phulchowki, Kaski and Nagarjun populations and the second cluster contained accessions from Sankhuwasabha and Terathum populations, demarcating eastern Nepal. Nagarjun populations were found segregated from Phulchowki and Kaski populations at similarity coefficient of 0.65. The genetic relationships among *S. chirayita* accessions from different populations under study were distinct and observed

to be clustered separately according to their geographical locations that represented the genetic distinctness of the *S. chirayita* populations.

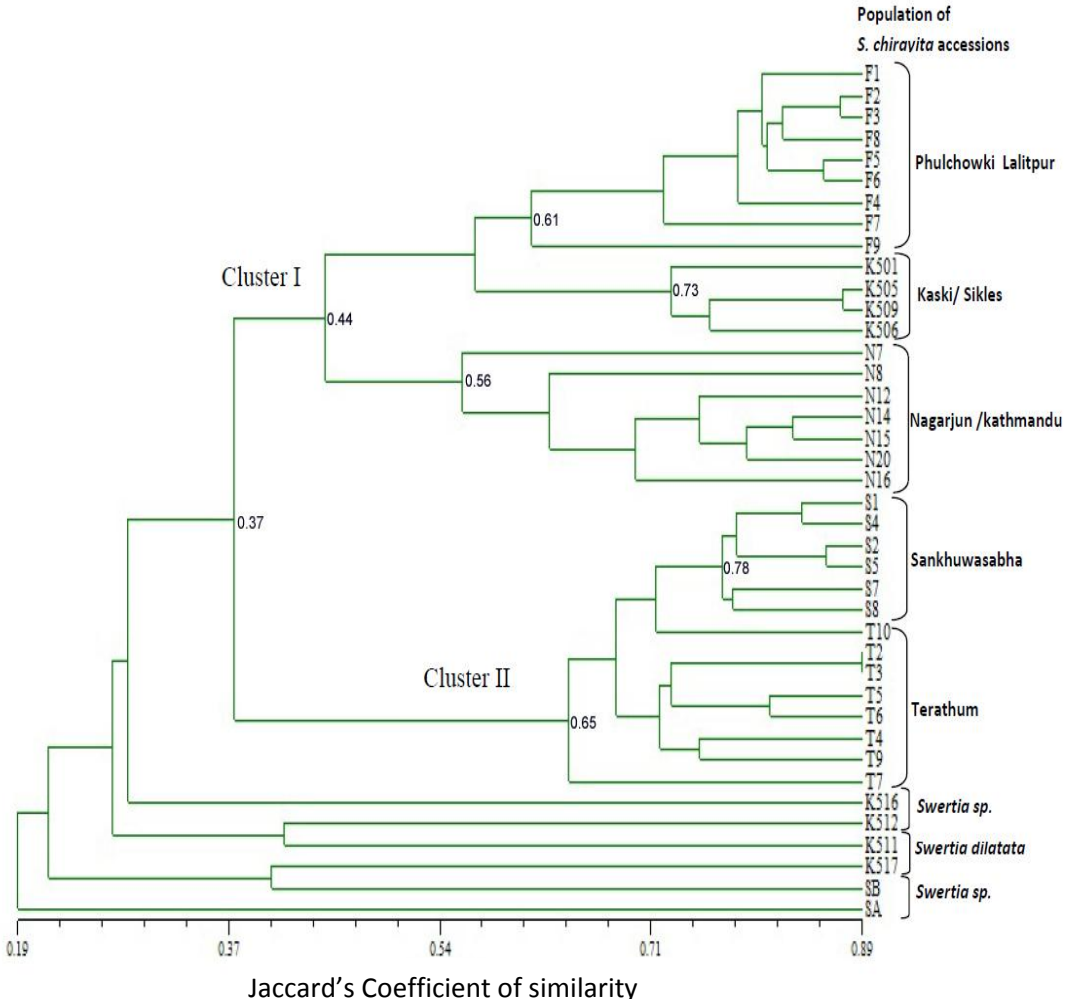



**Figure 4.4** Phenogram generated for 34 *Swertia chirayita* accessions with the six outlier species as revealed by UPGMA cluster analysis using Jaccard's coefficient of similarity calculated from 386 RAPD loci generated by 26 primers. The resulting clusters are labeled as I and II.

The individuals within a population were clustered into a group at different similarity coefficient levels, that is Phulchowki populations at 0.61, Kaski at 0.73, Nagarjun at 0.56, Sankhuwasabha at 0.78 and Terathum at 0.65. On the basis of these clusters, *S. chirayita* individuals within the population from Nagarjun were found to be most diverse and genetically distant with similarity coefficient at 0.56 (fig 4.4). From phenogram constructed including 6 other samples belonging to other spp. of *Swertia*, clear delineation of *S. chirayita* and these different spp. were visible in the phenogram (fig 4.4).

### 3D-Plot

3D – plot was constructed with analysis of Eigen vector using NTSYS-PC 2.21i for all the *S. chirayita* accessions, using the Jaccard's similarity matrix showed the comparable cluster dispersion and scatterness as shown by the phenogram with the respective individual accessions grouped into a particular geographical population cluster. (Fig 4.5)

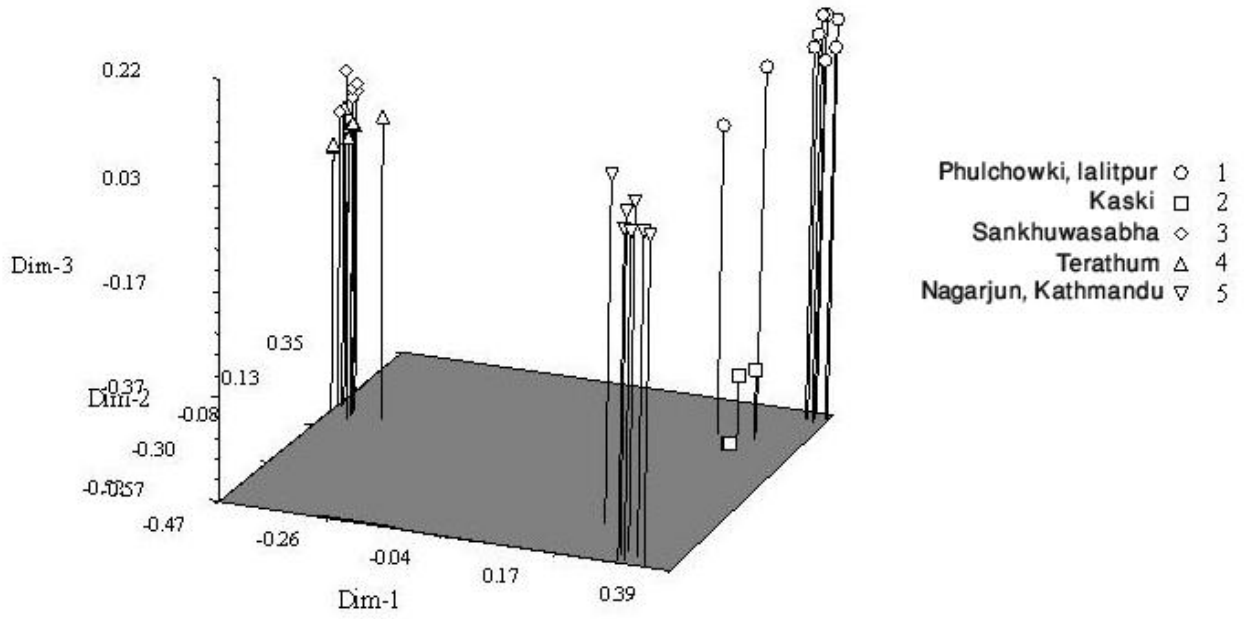



**Figure 4.5** 3D – plot constructed using NTSYS-PC 2.21i to assess dispersion of all the *S. chirayita* accessions.

## 4.7.3 Principal Coordinates Analysis (PCO)

Similarly, Eigen analysis done in Principal Coordinated analysis (PCO) of Jaccard similarity matrix by MVSP 3.2 (Multi-Variate Statistical Package) also distinctly clustered the *S. chirayita* individual accessions into the particular geographical population represented in scatterplot that is in congruence with the phenogram and 3D – plots illustration of genetic relationship of the *S. chirayita* in the present investigation (Figure 4.6). PCO also revealed the same association as revealed in the phenogram generated by NTSYS.

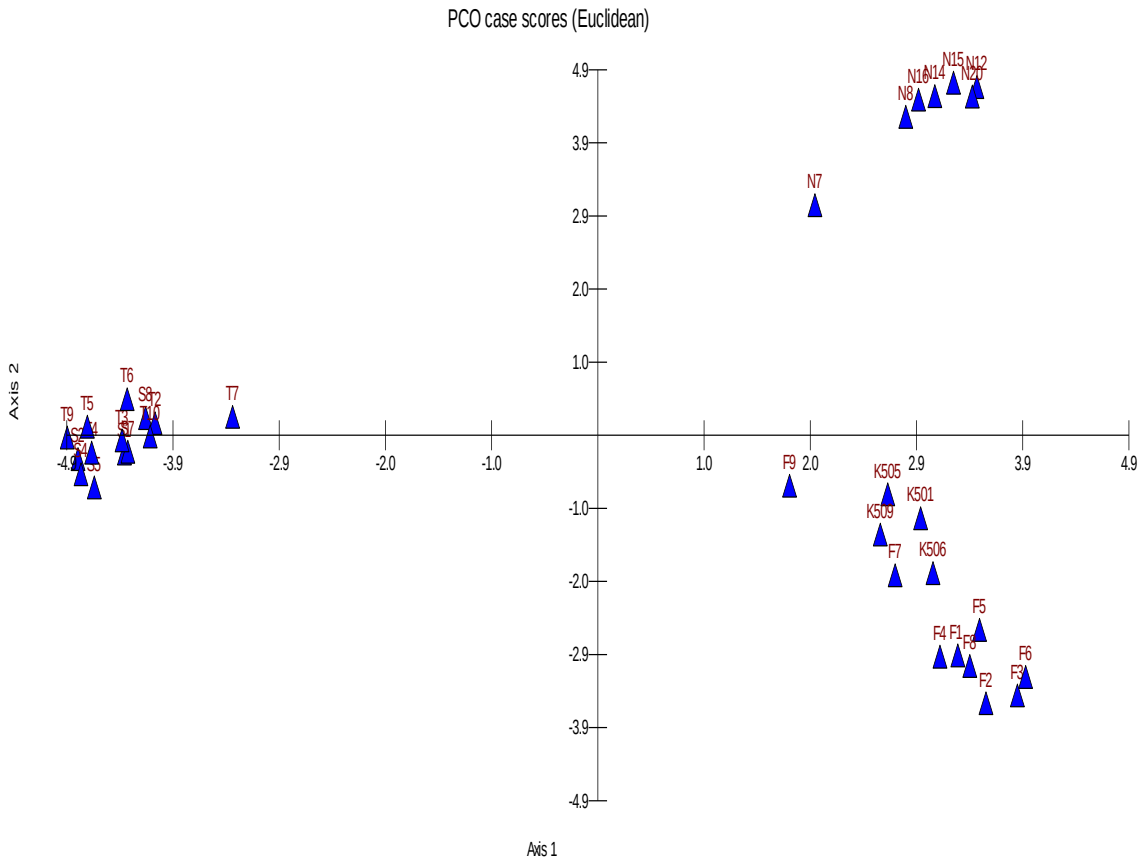



**Figure 4.6**: Assessment of Principal Coordinate Analysis (PCO) of Jaccard similarity matrix carried out with the MVSP version 3.2

## 4.7.4 Estimation of within population genetic diversity of *S. chirayita*

Following is shown the intra-population diversity of *S. chirayita* according to the polymorphism of RAPD bands. Assessing each population for the polymorphism analysis using each primer too, the most diverse accessions of *S. chirayita* have been observed for Nagarjun (64.24%) and lowest for Kaski population (38%) in correspondance to the observations made in dendogram analysis (Table 4.11). Boxplot was constructed using SPSS for the presentation of percent of polymorphisms obtained, fig 4.7).

**Table 4.11** Direct assessment of polymorphism based on monopmorphic and polymorphic bands amplified by 26 random primers.

| Pop/sample no. | Phulchowki (9) | | | Nagarjun (7) | | | Kaski (4) | | | Sankhuwasabha (6) | | | Terathum (8) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Primers** | **M** | **P** | **% POL** | **M** | **P** | **% POL** | **M** | **P** | **% POL** | **M** | **P** | **% POL** | **M** | **P** | **% POL** |
| **UBC 2** | 7 | 2 | 22.22 | 4 | 6 | 60.00 | 7 | 5 | 41.67 | 4 | 1 | 20.00 | 3 | 2 | 40.00 |
| **UBC 3** | 1 | 7 | 87.50 | 1 | 6 | 85.71 | 2 | 6 | 75.00 | 2 | 6 | 75.00 | 3 | 4 | 57.14 |
| **UBC 6** | 2 | 8 | 80.00 | 3 | 7 | 70.00 | 7 | 1 | 12.50 | 10 | 1 | 9.09 | 7 | 4 | 36.36 |
| **UBC 51** | 2 | 1 | 33.33 | 3 | 1 | 25.00 | 3 | 0 | 0.00 | 2 | 0 | 0.00 | 2 | 1 | 33.33 |
| **UBC 85** | 6 | 0 | 0.00 | 5 | 0 | 0.00 | 3 | 1 | 25.00 | 3 | 1 | 25.00 | 3 | 2 | 40.00 |
| **UBC 18** | 2 | 3 | 60.00 | 1 | 7 | 87.50 | 3 | 2 | 40.00 | 3 | 2 | 40.00 | 3 | 2 | 40.00 |
| **UBC 74** | 3 | 2 | 40.00 | 3 | 2 | 40.00 | 2 | 1 | 33.33 | 5 | 1 | 16.67 | 4 | 2 | 33.33 |
| **UBC 76** | 2 | 2 | 50.00 | 3 | 1 | 25.00 | 5 | 1 | 16.67 | 5 | 2 | 28.57 | 4 | 5 | 55.56 |
| **UBC 23** | 3 | 2 | 40.00 | 3 | 4 | 57.14 | 2 | 2 | 50.00 | 3 | 5 | 62.50 | 2 | 7 | 77.78 |
| **UBC 43** | 3 | 2 | 40.00 | 3 | 6 | 66.67 | 4 | 3 | 42.86 | 3 | 6 | 66.67 | 0 | 8 | 100.00 |
| **UBC 65** | 3 | 2 | 40.00 | 3 | 4 | 57.14 | 5 | 2 | 28.57 | 3 | 2 | 40.00 | 2 | 6 | 75.00 |
| **UBC 97** | 1 | 7 | 87.50 | 1 | 7 | 87.50 | 2 | 5 | 71.43 | 1 | 3 | 75.00 | 2 | 3 | 60.00 |
| **UBC 86** | 2 | 5 | 71.43 | 3 | 7 | 70.00 | 3 | 3 | 50.00 | 2 | 4 | 66.67 | 2 | 3 | 60.00 |
| **UBC 4** | 4 | 4 | 50.00 | 1 | 5 | 83.33 | 4 | 0 | 0.00 | 5 | 0 | 0.00 | 4 | 2 | 33.33 |
| **UBC 73** | 1 | 3 | 75.00 | 0 | 7 | 100.0 | 0 | 8 | 100.0 | 2 | 3 | 60.00 | 2 | 1 | 33.33 |
| **UBC 67** | 3 | 3 | 50.00 | 3 | 2 | 40.00 | 3 | 1 | 25.00 | 3 | 2 | 40.00 | 2 | 2 | 50.00 |
| **UBC 15** | 3 | 4 | 57.14 | 1 | 2 | 66.67 | 5 | 1 | 16.67 | 2 | 2 | 50.00 | 2 | 4 | 66.67 |
| **UBC 16** | 1 | 5 | 83.33 | 4 | 0 | 0.00 | 5 | 0 | 0.00 | 6 | 3 | 33.33 | 4 | 3 | 42.86 |
| **UBC 54** | 1 | 6 | 85.71 | 2 | 7 | 77.78 | 3 | 4 | 57.14 | 2 | 3 | 60.00 | 2 | 3 | 60.00 |
| **UBC 66** | 2 | 4 | 66.67 | 3 | 2 | 40.00 | 3 | 0 | 0.00 | 2 | 5 | 71.43 | 2 | 2 | 50.00 |
| **UBC 71** | 5 | 4 | 44.44 | 2 | 4 | 66.67 | 5 | 2 | 28.57 | 6 | 1 | 14.29 | 4 | 4 | 50.00 |
| **UBC 88** | 3 | 2 | 40.00 | 5 | 2 | 28.57 | 5 | 1 | 16.67 | 3 | 3 | 50.00 | 3 | 4 | 57.14 |
| **UBC 96** | 5 | 2 | 28.57 | 2 | 2 | 50.00 | 7 | 0 | 0.00 | 7 | 1 | 12.50 | 5 | 4 | 44.44 |
| **UBC 92** | 0 | 7 | 100.0 | 0 | 6 | 100.0 | 3 | 2 | 40.00 | 2 | 4 | 66.67 | 0 | 9 | 100.0 |
| **UBC 55** | 2 | 6 | 75.00 | 0 | 7 | 100.0 | 1 | 6 | 85.71 | 2 | 8 | 80.00 | 1 | 6 | 85.71 |
| **UBC 17** | 0 | 1 | 100.0 | 0 | 2 | 100.0 | 1 | 0 | 0.00 | 1 | 2 | 66.67 | 0 | 1 | 100.0 |
| **Total** | 67 | 94 | | 59 | 106 | | 93 | 57 | | 89 | 71 | | 68 | 94 | |
| **Average/ primer** | 2.6 | 3.6 | | 2.3 | 4.1 | | 3.6 | 2.2 | | 3.4 | 2.7 | | 2.6 | 3.6 | |
| **SD** | 1.7 | 2.2 | | 1.5 | 2.5 | | 1.9 | 2.2 | | 2.1 | 2.0 | | 1.6 | 2.1 | |
| **% POL. (Mean)** | **58.39** | | | **64.24** | | | **38.0** | | | **44.38** | | | **58.02** | | |

POP = Population Sample no. = No of sample processed P = Number of Polymorphic band M = Number of Monomorphic band % = Percentage POL = Polymorphism.

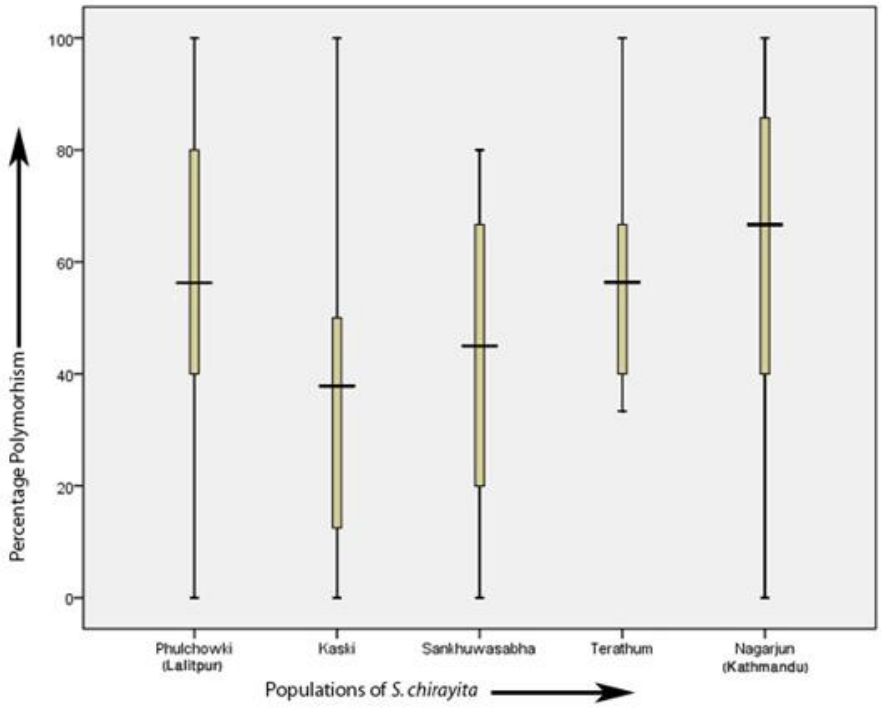



**Fig 4.7** BOXPLOT showing *S. chirayita* polymorphism within various populations as revealed by RAPD profile.

## 4.7.5 Population Genetic structure of *S. Chirayita*

### 4.7.5.1 Genetic relationship between *S. chirayita* populations

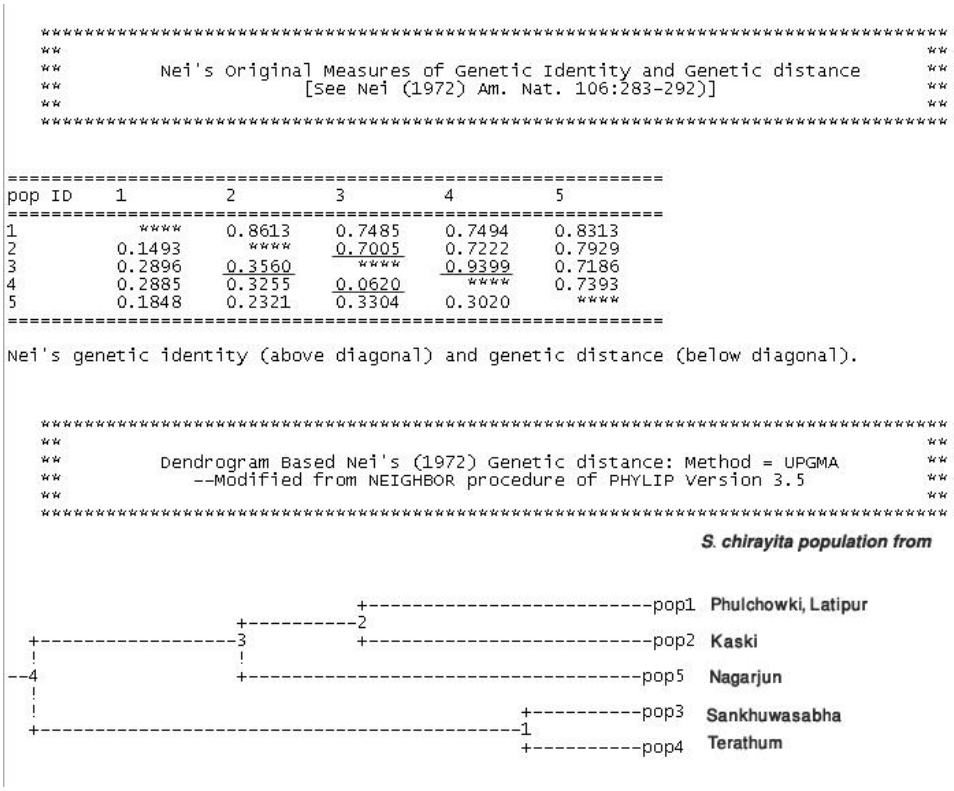
POPGENE version 1.32 was employed for the estimation of Nei's (1972) original measure of genetic identity ($I_{xy}$) and genetic distance ($D_{xy}$) along with Nei (1978) unbiased measures of genetic identity and distance illustrating interrelationship between the *S. chirayita* populations under study.

Similar observations were obtained with both original measure Nei (1972) and unbiased measures (Nei, 1978). The highest "genetic identity" was observed between the *S. chirayita* populations of Sankhuwasabha and Terathum (0.9399 as Nei's original measure and 0.9489 as Nei's unbiased measure) with lowest "genetic distance" for the same populations (0.0620 as Nei's original measure and 0.0524 as Nei's unbiased measure) (Fig 4.8 and 4.9)

Similarly, the lowest "genetic identity" was observed for the *S. chirayita* population of Kaski and Sankhuwasabha (0.7005 as Nei's original measure and 0.7078 as Nei's unbiased measure) with

highest “genetic distance” for the same populations (0.3560 as the Nei’s original measure and 0.3455 as the Nei’s unbiased measure) (Fig 4.8 and 4.9).

Also, the observations and the interrelationship between the populations were clearified by the dendograms showing two major clusters: cluster 1 with least distant (most identical) populations from Sankhuwasabha and Terathum and cluster 2 with populations from Phulchowki and Kaski (closer) together with Nagarjun (Fig 4.8 and 4.9).



```
**********************************************************************************************
**                                                                                          **
**          Nei's Original Measures of Genetic Identity and Genetic distance                **
**                        [See Nei (1972) Am. Nat. 106:283-292)]                            **
**                                                                                          **
**********************************************************************************************


============================================================
pop ID    1          2          3          4          5
============================================================
1           ****     0.8613     0.7485     0.7494     0.8313
2         0.1493       ****     0.7005     0.7222     0.7929
3         0.2896     0.3560       ****     0.9399     0.7186
4         0.2885     0.3255     0.0620       ****     0.7393
5         0.1848     0.2321     0.3304     0.3020       ****
============================================================

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).


**********************************************************************************************
**                                                                                          **
**          Dendrogram Based Nei's (1972) Genetic distance: Method = UPGMA                  **
**             --Modified from NEIGHBOR procedure of PHYLIP Version 3.5                     **
**                                                                                          **
**********************************************************************************************
                                                                        S. chirayita population from


                          +--------------------------pop1  Phulchowki, Latipur
                     +----------2
  +------------------3          +--------------------------pop2  Kaski
  !                  !
--4                  +-------------------------------------pop5  Nagarjun
  !
  !                                              +----------pop3  Sankhuwasabha
  +----------------------------------------------1
                                                 +----------pop4  Terathum
```

**Figure 4.8** Nei’s original measures of genetic identity and genetic distance with illustration in dendogram.

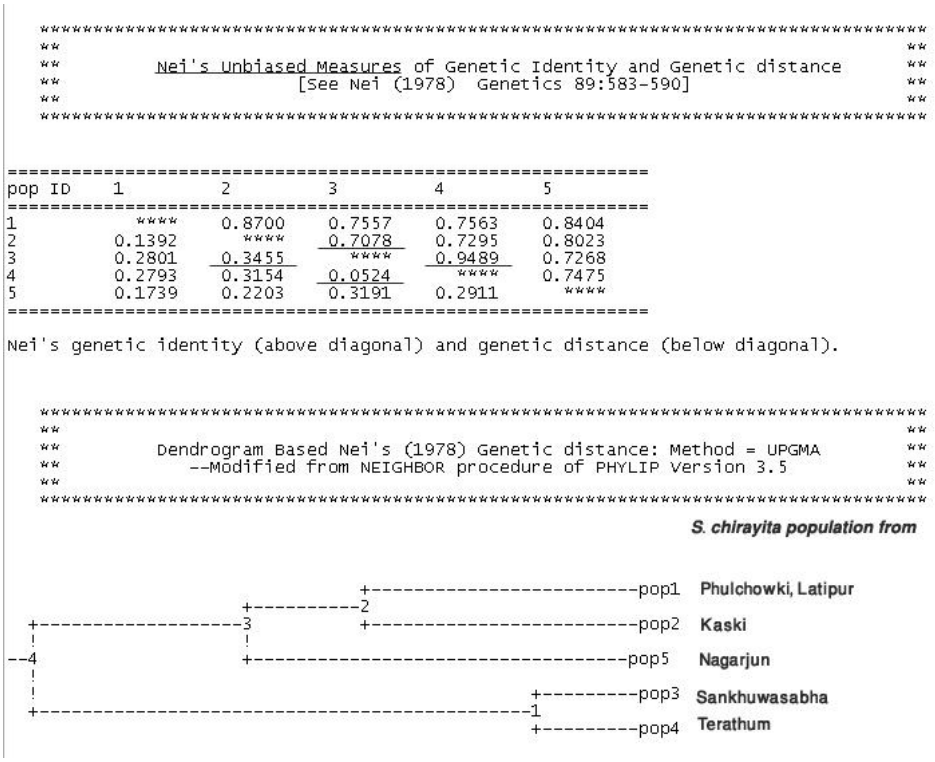



**Figure 4.9** Nei's Unbiased measures of genetic identity and genetic distance with illustration in dendogram.

### 4.7.5.2 Genetic variation within different populations of *S. chirayita* and at species level

In order to estimate the genetic variation within populations of *S. chirayita* and at species level, POPGENE Version 1.32 (Yeh *et al.*, 1997) was employed for determination of variety of indices for genetic diversity analysis (PPB, % polymorphic bands; H, Nei's index and I, Shannon's diversity index).

Of the total 285 clear and reproducible DNA fragments, 92.28% (263 in total) were observed polymorphic at species level. The percentage of polymorphic fragments for individual populations varied from 38.0 % (Kaski population) to 64.24 % (Nagarjun population) with an average of 52.61 % (Table 4.11). Genetic variation in a population is measured by the heterozygosity or the degree of polymorphism. For the conservation of a species, genetic variability is of the utmost importance. Nei's gene diversity ranged from 0.132 (for Kaski population) to 0.225 (for Nagarjun population) with average estimate of 0.192 at population level and 0.265 at the species level. Shannon's information index (I) is observed in the range of 0.199 (for Kaski population) to 0.337 (for Nagarjun population) with an average of 0.284 at the population level ($I_{pop}$) and 0.409 at the species level $I_{sp}$) (Table 4.12).

Among the five *S. chirayita* populations investigated, the *S. chirayita* population from Nagarjun, Kathmandu revealed highest variability (PPB, 64.24 %; H, 0.225; I, 0.337) whereas the *S. chirayita* population from Kaski revealed the lowest variability (PPB, 38.0 %; H, 0.132; I, 0.199) (Table 4.12).

**Table 4.12** Genetic variability within populations of *S. chirayita*, as revealed by POPGENE during the RAPD analysis.

| Population of *S. chirayita* from | Sample size | Number of polymorphic bands | PPB (%) | H | I |
|---|---|---|---|---|---|
| **Phulchowki, Lalitpur** | 9 | 94 | 58.39 | 0.224 | 0.328 |
| **Kaski** | 4 | 57 | 38.0 | 0.132 | 0.199 |
| **Sankhuwasabha** | 6 | 71 | 44.38 | 0.168 | 0.247 |
| **Terathum** | 8 | 94 | 58.02 | 0.209 | 0.311 |
| **Nagarjun, Kathmandu** | 7 | 106 | 64.24 | 0.225 | 0.337 |
| **Average** | | 84.4 | 52.61 | 0.192 | 0.284 |
| **Species level (Multipopulation)** | 34 | 263 | 92.28 | 0.265 | 0.409 |

Where, PPB, Percentage of Polymorphic Bands / loci;

H, Nei's gene diversity

I, Shannon's information index.

### 4.7.5.3 Overall Genetic Differentiation

The heterozygosity within the population ($H_S$) was observed to be 0.1078 in average with ranges from 0.0303 to 0.1656. The total diversity ranged from 0.1429 to 0.3513 with an average of 0.2647. The mean genetic differentiation ($G_{ST}$) between populations over all loci was observed to be 0.5929 and it ranged from 0.2191 to 0.8421. In contrast, the average gene flow from one population to the other generation ($N_m$) was estimated to be 0.3433 with the range of lowest estimate 0.1267 to the highest estimate 4.3694 of gene flow. Higher genetic differentiation between the populations was observed (Table 4.13).

**Table 4.13** Genetic differentiation and gene diversity within and between the populations of *S. chirayita* for RAPD markers.

| Primer Code | Primer Sequence ( 5'-3') | $H_t$ | $H_s$ | $G_{st}$ | $N_m$ |
|---|---|---|---|---|---|
| UBC 2 | CCTGGGCTTG | 0.2971 | 0.0945 | 0.4890 | 0.3305 |
| UBC 3 | CCTGGGCTTA | 0.2764 | 0.1429 | 0.4086 | 1.3580 |
| UBC 6 | CCTGGGCCTA | 0.3126 | 0.1210 | 0.5395 | 0.7302 |
| UBC 51 | CTACCCGTGC | 0.1429 | 0.0303 | 0.3892 | 4.3694 |

| | | | | | |
|---|---|---|---|---|---|
| UBC 85 | GTGCTCGTGC | 0.2546 | 0.0467 | 0.7256 | 0.7418 |
| UBC 18 | GGGCCGTTTA | 0.3090 | 0.1305 | 0.4553 | 1.5361 |
| UBC 74 | GAGCACCTGA | 0.3335 | 0.0708 | 0.6981 | 0.5613 |
| UBC 76 | GAGCACCAGT | 0.3288 | 0.0683 | 0.6759 | 1.2677 |
| UBC 23 | CCCGCCTTCC | 0.2494 | 0.1615 | 0.3162 | 3.5039 |
| UBC 43 | AAAACCGGG | 0.1942 | 0.1214 | 0.2410 | 3.6543 |
| UBC 65 | AGGGGCGGGA | 0.2326 | 0.0940 | 0.3845 | 2.8289 |
| UBC 97 | ATCTGCGAGC | 0.1754 | 0.1150 | 0.2191 | 4.2835 |
| UBC 86 | GGGGGGAAGG | 0.3513 | 0.1405 | 0.5677 | 1.6124 |
| UBC 4 | CCTGGGCTGG | 0.2034 | 0.1011 | 0.4732 | 0.6452 |
| UBC 73 | GGGCACGCGA | 0.2113 | 0.1365 | 0.2519 | 2.6421 |
| UBC 67 | GAGGGCGAG | 0.1691 | 0.0890 | 0.4117 | 1.088 |
| UBC 15 | CCTGGGTTTG | 0.2747 | 0.0940 | 0.5605 | 1.2050 |
| UBC 16 | GGTGGCGGGA | 0.3094 | 0.0796 | 0.6653 | 0.5770 |
| UBC 54 | GTCCCAGAGC | 0.2880 | 0.1463 | 0.4034 | 1.4833 |
| UBC 66 | GAGGGCGTGA | 0.1444 | 0.0993 | 0.2605 | 2.2603 |
| UBC 71 | GAGGGCGAGG | 0.3162 | 0.1161 | 0.5662 | 0.6655 |
| UBC 88 | CGGGGGATGG | 0.3408 | 0.1018 | 0.6201 | 0.6088 |
| UBC 96 | GGCGGCATGG | 0.3100 | 0.046 | 0.8421 | 0.1267 |
| UBC 92 | CCTGGGCTTT | 0.2422 | 0.1411 | 0.326 | 2.6709 |
| UBC 55 | TCCCTCGTGC | 0.2925 | 0.1656 | 0.3205 | 2.4237 |
| UBC 17 | CCTGGGCCTC | 0.2447 | 0.0515 | 0.5377 | 1.5570 |
| | Mean | **0.2647** | **0.1078** | **0.5929** | **0.3433** |
| | St. Dev. | 0.0285 | 0.0077 | | |

Where, $N_m$, Estimate of gene flow from $G_{st}$ $H_T$, Total heterozygosity / diversity

$H_S$, Mean heterozygosity / gene diversity within population $D_{ST} = H_T - H_S$ (Diversity among population)

$G_{ST} = D_{ST} / H_T$ (Coefficient of population differentiation) / Genetic differentiation.

## 4.7.6 Analysis of Molecular Variance (AMOVA)

Furthermore, according to the global AMOVA analysis there was no significant partitioning of the genetic variation ($p < 0.001$), with 51% occurring among populations and within population variation accounting for the remaining 49%. (Table 4.14). The among population value of the indicator of genetic differentiation, $\phi_{PT}$, for the five populations was 0.513, suggesting that genetic differentiation of *S. chirayita* was comparably higher than the genetic differentiation of *S. chirayita* within population variation accounting 49% variation (Table 4.14).

According to the hierarchial AMOVA analysis, the amount of genetic variation partitioned among regions and among populations was 31% and 24%, respectively, with the remainder (45%) occurring within – population (Table 4.14)

**Table 4.14** Analysis of molecular variance (AMOVA) for 34 individuals grouped in five populations from three regions. The degree of freedom (d.f.), sum of squares (SS), mean square (MS), Estimated variance, percent (%) and its associated significance (n = 999 permutations) are shown.

| Source of variation | d.f. | SS | MS | Estimated variance | Total variance (%) | P-value |
|---|---|---|---|---|---|---|
| **Global** | | | | | | |
| Among population | 4 | 846.430 | 211.608 | 27.694 | 51 % | <0.001 |
| Among individuals within population | 29 | 762.776 | 26.303 | 26.303 | 49% | <0.001 |
| **Hierarchial** | | | | | | |
| Among region | 2 | 587.858 | 293.929 | 18.138 | 31% | <0.001 |
| Among population within region | 2 | 258.572 | 129.286 | 13.981 | 24% | <0.001 |
| Among individuals within population | 29 | 762.776 | 26.303 | 26.303 | 45% | <0.001 |

# 5. DISCUSSIONS

Scrutinizing the studies carried out so far in the variation analysis and taxonomic identification of *S. chirayita* and other species, conventional morphology based assessments have been perceived in preponderance as compared to the molecular assessments. However, traditional morphological identification and variation analysis of folk herb *S. chirayita* has become problematic resulting into the adulteration practices with some other related species in its trade, as they are very similar to each other morphologically (Joshi, 2011). Furthermore, *S. chirayita* herbs are commercially sold in dried forms without flowers, rendering their authentication by morphological methods very difficult, if not impossible. This mal practice is depleting not only *S. chirayita* from its natural habitats but also other spp. It has been reported that nine spp. are traded in the name of chiraito (Joshi and Joshi, 2008). Also, from human health safety point of view such activities should be discouraged.

Every species on earth has its own life history of distribution and most of the times, it is impossible to trace such histories for each and every species. In case of *S. chirayita,* it has been reported to be distributed in 54 districts (Barakoti *et al*, 1999). These different populations might have been introduced at different times in the past by human beings. Being a highly valued species, this species has now become vulnerable (IUCN, 2004) due to overexploitation and other anthropogenic activities. Therefore, in this scenario, scientific research to understand existing genetic diversity in *S. chirayita* is essential in order to understand its vulnerability, to devise conservation strategy as well as for its long term sustainable utilization.

Present investigation was mainly aimed at understanding existing genetic diversity in *S. chirayita* as well as to find species specific or population specific molecular markers and DNA fingerprints for authentication. One of the major goals for the biodiversity studies is the characteristic preservation of existing genetic diversity as there could possibly be a genetic basis for species to become endangered, threatened or vulnerable. Furthermore, Survival of a species depends on the maintenance of genetic variability within and among populations that accommodate new selection pressures brought about by the environmental changes. So, the morphology and ecology based assessments (density, frequency of plants, phenotypic study) alone are not sufficient and reliable to reveal the vulnerable nature of the species. It is very important to understand the complex processes involved in the long term evolutionary history of species such as genetic drift, mutation, gene flow within and between populations (Jayanti and Mandal, 2001; Faisal *et al.*, 2007).

## 5.1 Optimization of RAPD-PCR conditions

RAPDs have often been criticized for low reproducibility, although this problem is mostly solved. However, as any PCR-based technique, the RAPD method requires stringent optimum reaction conditions to be kept constant (Weising *et al.*, 2005). Therefore, the selection of best DNA extraction protocol, identification of best PCR primer sets and maintenance of optimum RAPD-PCR reaction and cycling conditions for the individual plant species under study are pre-requisites for generating reproducible banding patterns for genetic diversity study and other applications (Lamboy, 1994; Yu and Pauls, 1994; Weising *et al.*, 2005; Padmalatha and Prasad, 2006). In the present investigation, different DNA extraction techniques together with the RAPD-PCR reaction and cycling conditions for *S. chirayita* have been optimized which were used in subsequent RAPD-PCR profiling experiments amplified with the sufficient number of RAPD primers.

### 5.1.1 Selection of DNA extraction Technique for *S. chirayita*

The DNA extracted from CTAB DNA Extraction method (Graham *et al.*, 1994) and Modified CTAB Extraction method (Doyle and Doyle, 1990) were assessed using optimized RAPD PCR reaction and cycling conditions. The CTAB DNA isolation method of Graham *et al.* (1994) produced comparatively better, suitable and reproductive RAPD profiles for *S. chirayita*.

Universal DNA extraction techniques that work for all doesnot exist as various medicinal plants contain exceptionally high levels of polysaccharides, polyphenols, tannins, hydrocolloids and many other secondary metabolites such as alkaloids, flavonoids, phenols, terpenes etc that binds with nucleic acid during DNA extraction forming complexes and can interfere with subsequent reaction (Varadarajan and Prakash, 1991). They may require certain modifications for the successful extraction of quality DNA (Padmalatha and Prasad, 2006; Shrestha *et al.*, 2010). Hence, until the expected reproducible results are obtained, numbers of techniques have to be tried. As the quality of template DNA affect greatly on the generation and resolution of amplified products during PCR, emphasis should be given to purity rather than quantity.

The extraction of DNA involves separation of DNA from naturally occurring plant cell constituents such as polysacharides and polyphenolic compounds (Pandey *et al*,. 1996; Porebski *et al.*, 1997) followed by deproteinisation of the aqueous solution containing the DNA, precipitation and purification of DNA. Polysaccharides are visually evident in DNA extractions by their viscous, glue like texture that make the DNA unmanageable in pipetting and unamplifiable in the PCR by inhibiting *Taq* polymerase (Fang *et al.*, 1992). The presence of polyphenols, a powerful oxidizing agent present in many plant species can reduce the yield and purity by binding covalently with

the extracted DNA making unreproducible during experiments (Katterman and Shattuck, 1983). Other secondary metabolites like tannins, terpenes and resins are also difficult to separate from DNA and hinder PCR amplifications (Ziegenhagan and Scholz, 1998). Although Polyvinylpyrrolidone (PVP) and beta mercaptoethanol used in Doyle and Doyle extraction method (1990) were reported to be helpful in removing polyphenols (Doyle and Doyle, 1987; Clark, 1997; Dawson and Magee, 1995) RAPD profiles with Doyle and Doyle extracted DNA were not good enough as compared with Cetyl Trimethyl Ammonium Bromide (CTAB) method of Graham et al. (1994) which contained CTAB, EDTA, NaCl and Tris in its DNA extraction buffer. CTAB may bind to polyphenolic compound during extraction by forming complex with hydrogen bonds and may help in removing impurities to some extent (Padmalatha and Prasad, 2006; Kit and Chandran 2010). Chloroform–isoamyl alcohol treatment ensures removal of chlorophyll and other colouring substances. In the plants with higher secondary metabolites like *Swertia chirayita*, CTAB/NaCl helps in the precipitation of polysaccharides and proteins, and these are eliminated through Chloroform: Isoamylalcohol treatment (Gurudeeban *et al.*, 2011). In the CTAB DNA extraction protocol, RNAse has been incorporated in the TE (Tris/EDTA) buffer which was used to re-suspend DNA pellets at the end of DNA extraction procedure. RNAse degrade RNA into small ribonucleosides preventing RNA contamination and yielding pure DNA suitable for RAPD-PCR (Padmalatha and Prasad, 2006).

### 5.1.2 Optimization of RAPD Reaction Conditions

Optimization of various reaction parameters in RAPD is crucial in order to maintain reproducibility of RAPD bands among laboratories (Weising *et al.*, 2005; Shrestha *et al.*, 2010). In the present investigation, the sensitive reaction parameters determing RAPD phenotypes during PCR, *viz.* template DNA concentration, primer concentration, $MgCl_2$ concentration, dNTPs concentrations and *Taq* polymerase concentration were optimized along with the RAPD cycling conditions for the development of standard RAPD-PCR protocol for *Swertia chirayita*.

In order to generate reproducible RAPD fingerprint profiles, quality and quantity of template DNA have been considered as one of the main factors affecting reproducibility. Hence, optimum concentration of DNA needs to be tested for good banding profile in RAPD (Micheli *et al.*, 1997). For most species of plants, good results have been achieved for RAPD using 50 to 100ng in 25-50µL PCR reaction volume (Caetano Annoles *et al.*, 1991; Padmalatha and Prasad, 2005). Thus, in the present investigation, a range of DNA concentrations from 12.5-100 ng were tested. However, identical profiles were obtained for most of the tested range of DNA concentration. The minimum DNA concentration and best banding pattern was observed for 25 ng DNA which

was thus selected and used in further experiments. Using high amount of DNA usually inhibit PCR-amplification creating hindrance for primer annealing (Micheli *et al.*, 1997) (Plate 4.2).

$MgCl_2$ is a cofactor of *Taq* polymerase enzyme, thus influences the DNA amplification process. Magnesium is an essential component of PCR-reactions affecting the quality of RAPD profiles (Munthaly *et al.*, 1992). $Mg^{++}$ is known to affect primer annealing and template denaturation, enzyme activity and fidelity together with the formation of primer-dimer artifacts (Saiki, 1988). Generally, increasing amounts of $Mg^{++}$ result in the accumulation of non-specific amplification products, and insufficient $Mg^{++}$ will reduce the yield (William *et al.*, 1993). Hence, to prevent non-specific amplification, amplification failure, low reproducibility and low product yield, it is essential to assess an optimum $MgCl_2$ concentration. The use of $MgCl_2$ concentration >1mM has been reported to be generally necessary for good levels of DNA amplification in bacterial and Plant DNAs (Bassam *et al.*, 1992). Concentrations as high as 5mM have been used successfully in RAPD analyses of plant species like Poplar (Castiglione *et al.*, 1993). In the present study, of the range of $MgCl_2$ concentration tested i.e 1.5mM-4.5mM, identical crispy profiles were obtained for $MgCl_2$ concentrations of 2.5-3.5 mM. Therefore, $MgCl_2$ concentration of 3.0 mM was taken as optimum concentration for RAPD-PCR amplification for *S. chirayita* (Plate 4.3).

Lower or higher concentration of primer leads to amplification failure and primer dimer formation respectively (Padmalatha and Prasad, 2005). So, the concentration of primer is also the matter of optimization for the reproducible results and productive amplification for RAPD-PCR. After observing banding profiles of different primer concentrations ranging from 0.1µM-1.6µM, banding pattern at 0.4µM-0.8µM were revealed to be the best concentrations, so, the low concentration with best banding pattern 0.4µM was selected as optimum and used in further PCR reactions (Plate 4.4).

The dNTP concentrations have often been reported as having little influence on the patterns of DNA amplified. 100 to 200µM of each of the four nucleotides have been quoted as being optimal for most reactions used in RAPD analyses (William *et al.*, 1990; Caetano-Anolles *et al.*, 1991). However, it is known that dNTPs chelate magnesium and thereby change the effective optimal $Mg^{++}$ concentration. Moreover, high concentration of dNTPs increase the error rate of the *Taq* polymerase, interfering its activity (occur due to less free $Mg^{++}$) (Gelfand, 1989; Padmalatha and Prasad, 2005). In the present investigation, among the tested range of dNTPs from 0.1mM-0.5mM, 0.2mM was selected as optimum dNTP concentration for RAPD profiling (Plate 4.5).

*Taq* polymerase, a thermostable polymerase plays a key role in PCR amplification. These enzymes have varying conditions for optimal performance and amplification. The choice of enzyme also depends on the requirements of the PCR experiment especially with respect to

specificity, efficiency or fidelity (reviewed by Cha and Thilly, 1993). Intensity of band increases correspondingly with increasing *Taq* polymerase concentrations. Major factor governing choice of enzymes is their availability, potential efficiency and cost (Weising, 2005). In the present investigation, of the tested range of concentrations of *Taq* polymerase ranging from 0.5U to 2.5U, best banding pattern was considered to be at 1 unit *Taq* polymerase in 25µL reaction volume of PCR (Plate 4.6).

### 5.1.3 Optimization of RAPD cycling condition

The different PCR cycling parameters, namely temperatures, durations and 'ramping' rate during denaturation, annealing and extension steps as well as the number of amplification cycles also play significant roles in attaining optimal RAPD banding patterns (Rycwik *et al.*, 1990; Weising *et al.*, 2005; Shrestha *et al.*, 2010). Of the two randomly selected RAPD-PCR programs, cycling conditions described by Edwards (1998) (ie. Program 2) produced the best RAPD profiles for *S. chirayita* in present investigation. Compared to program 1 (Yu and Pauls, 1992) denaturation temperature has been increased in program 2 (Edwards, 1998) by 1°C. But the annealing temperature was observed optimum at 37°C for 60 seconds rather 38°C/30 seconds in program 1. Yu and Pauls (1992) showed that there is an interaction between time required for primer annealing and GC content of primer. For primers having GC content of 50-80%, primer annealing time of 30 seconds appeared to be appropriate. They also observed that strand elongation time significantly affects on size of amplified fragments in the PCR reaction.

In RAPD, primers should have minimum of 40% GC content although 50-80% is generally used (Micheli, 1997). Although the reproducibility of RAPD amplification is known to be highly sensitive to experimental PCR reaction conditions (Devos and Gale, 1992; Wolf *et al.*, 1993), Weeden *et al.* (1992) concluded that the amplification process is not so influenced seriously by one or more of parameters to affect reproducibility of the technique. Therefore, standard reaction condition and cycling parameters appear to be appropriate for a wide range of plant materials with the reduction of artifacts. To reproduce the discernible banding patterns consistently, reaction parameters should be strictly maintained. Very few non-reproducibility cases were encountered during this investigation. The reproducibility was checked by repeating the doubtful as well as important experiments.

## 5.2 Primer Screening for RAPD Profiling

Twenty eight primers were selected from 100 decanucleotide primers (UBC set-1) in a preliminary primer screening experiment based on visibility of amplified bands using fresh genomic DNA of *Swertia chirayita* from Kaski district and the pre-optimized RAPD-PCR reaction

and cycling conditions. Of the 28 primers, 26 primers were finally selected (Table 4.3) that gave reproducible and scorable banding patterns in RAPD-PCR analyses involving all 40 accessions of *S. chirayita* (Plate 4.7). Experiment was repeated twice for the final confirmation of primers producing good crispy bands.

The primer screening step is important as only the best primer template combinations were suitable for the generation of scorable bands and desirable markers (Shrestha, 2001). The number of primers used in similar studies varies according to the investigators. Dharmar and Britto (2011) used six selected random primers for study of diversity analysis of medicinal plant *Withania somnifera*. In population genetic analysis of endangered medicinal plant *Tylophora rotundifolia* within and among 12 populations, 20 selected random Operon RAPD primers (OPI) were used extensively for RAPD assessments (Sebastian *et al.*, 2010). Graham *et al.*, (1994b) screened 80 Operon primers for the development of RAPD markers specific to navy bean varieties using RAPD-PCR.

## 5.3 Identification of Population specific markers for *S. chirayita*

RAPD-PCR profiles generated by selected 26 UBC primers (Table 4.5) for the 34 individual accessions of *Swertia chirayita* together with 6 outlier species of *Swertia* from 5 different populations under study were observed to be reproducible and scorable for the diversity analysis. Amplified RAPD fragments basically ranged from 200 bp to 3000 bp. Therefore, in present study, gel electrophoresis of the RAPD profiles were done in 1.5% Agarose according to Sambrook *et al.* (1989) which recommend use of 1.5% Agarose gel for running DNA of 200-3000 bp.

RAPD-method provides a powerful tool to discriminate the closely related species even down to the level of subspecies (Black, 1996). In the present investigation, while exploring the RAPD band profiles of each primer, nine population specific markers based on the RAPD loci have been observed from the overall investigation which can be employed as RAPD fingerprints for the identification of particular population of *S. chirayita* (Table 4.4). The particular RAPD fragment which was seen amplified for all the individuals of particular population and absent in others can easily be employed to distinguish individuals of particular population. Therefore, acquirement of particular population specific RAPD markers and fingerprints for *S. chirayita* from the experiments describe the characteristic differences between the genomes of *S. chirayita* between the populations with certain genetic identity between the individuals of the same population. In the present study, population specific RAPD markers have been observed for all five populations of *S. chirayita* under study (Section 4.6; Plate 4.8). However, no species specific marker for *S. chirayita* could be found were revealed from entire observations.

## 5.4 RAPD Profiling and Study of Genetic Diversity

One of the pitfalls of RAPD-marker technology is its inability to resolve heterozygous genotypes (Moodie *et al.*, 1997). RAPD marker being dominant marker, the resulting fragment may either be homozygous (AA) or heterozygous (Aa) and the absence of the fragment indicates the underlying genotype (aa) (Weising *et al.*, 2005). RAPD bands are produced by PCR using a single random primer that amplifies segments of DNA flanked by two primer-binding regions that theoretically are exactly complementary to the primer. The primer binding sites must be close enough (no more than 2.5-3.0 kb) so that the intervening region can be amplified by PCR. There are several mutations that could disrupt amplification (Black, 1996). Point mutations (Single base alteration) in the annealing sites would prevent the RAPD primer from pairing with the target DNA at one or both sites. An inversion containing one annealing site would prevent amplification as would an insertion increased the distance between the annealing sites beyond what can be extended with routine PCR. Also, deletion in the priming site may results the same (Williams *et al.*, 1990; Klein-Langhorst *et al.*, 1991; Black, 1996). Presumably when one or more of these conditions occur, the PCR reaction fails and no product appears. Thus, polymorphisms are revealed and expressed according to the presence or absence of a fragment of a particular size among individuals.

RAPD loci were scored visually in each lane corresponding to an individual for each random primer assessed. Binary data matrix was created using all the RAPD profiles generated by 26 primers on the basis of presence (1) or absence (0) of the particular RAPD loci. From the total amplification profiles for every accession with every UBC primers (Selected 26 UBC primers), monomorphic and polymorphic RAPD loci were revealed. Among the total of 285 loci amplified by 26 primers, 263 bands were observed polymorphic revealing 92.28% polymorphism in 34 accessions of *S. chirayita.* Among them, 16 primers furnished 100% polymorphism (Table 4.5).

Observed percentage of polymorphism for *S. chirayita* shows higher genetic polymorphism in comparison to the other plants analysed with RAPD [*Tylophora rotundifolia*, 46% (Sebastian *et al.*, 2010); *Cassia occidentalis*, 71.17% (Arya *et al.*, 2011); *Trigonella foenum-graecum* L., 52.85% (Sundaram and Purwar, 2010); *Withania somnifera*, 83.78% (Dharmar and Britto, 2011)]. Some other plant species have also shown extensively high genetic polymorphism than the one observed for variation in *S. chirayita* in the present study with RAPD-PCR [*Vigna mungo,* 97.68% (Srivastava *et al.*, 2011); *Sorghum bicolor* L. Moench, 97.4% (Jeya Prakash *et al.*, 2006); *Podophyllum hexandrum*, 92.37% (Alam *et al.*, 2009)]. The present investigation on genetic diversity of *S. chirayita* from representative districts of Nepal using genome based marker is the

first initiative in the field of application of molecular marker technology in the field of medicinal plants sector in Nepal.

Average PIC score of 0.85 was observed with highest PIC score of 0.91 for primer UBC 6. Value of PIC is between 0 and 1 which estimates the degree of polymorphism of marker (Arya *et al.*, 2011). The study reported that primers having comparably higher PIC score were more useful than others for distinguishing accessions (Teklewold and Becker, 2006). DNA amplification by each of the UBC primers: 6, 2, 3, 92, 55, 96, 71, 76, 54, 16, 15, 86, 65 and 43 (in order of highest PIC) gave a PIC score greater than 0.85 suggesting that these primers may be especially useful for RAPD based analyses of genetic diversity of *S. chirayita* (Table 4.6). Based on the band informativeness the primer resolving power ($R_P$) provides quantitative data allowing direct comparisons between primers (Prevost and Wilkinson, 1999). In this study, the same primers that had highest PIC value also gave the highest $R_P$ score indicating the same good primers for the RAPD-based diversity assessment of *S. chirayita*. $R_P$ of the 26 RAPD primers ranged from 2.41 for primer UBC17 to 16.28 for primer UBC 6 (Section 4.7). As compared to values reported in other plants, mean PIC and $R_P$ score in this study were high indicating higher genetic variation (Sebastian *et al.*, 2010; Teklewold and Becker, 2006; Arya *et al.*, 2011) (Table 4.6).

### 5.4.1 Genetic diversity assessment with Similarity coefficients and the phenograms

Binary data matrix created on the basis of presence or absence of RAPD bands in gel pictures were employed for the deduction of the pairwise similarity matrices by means of Simple Matching (SM), Jaccard's (J) and Dice (D) coefficient of similarity using SIMQUAL (Similarity for Qualitative data). Only those bands were considered during scoring which were visibly distinguished in agarose gel pictures. In some of the experiments with the confused RAPD bands, the experiments were repeated to verify the RAPD banding patterns. Very few samples were observed to give amplification failure with few employed UBC primers. Those experiments were also repeated to ensure the failure of amplification and scoring was done as "9" for those missing band which was designated in the analysis procedure as an indicator of missing data by NTSYS-PC (Transue *et al.*, 1994). Range of similarity coefficients given by SM, J and D individually were different [i.e. SM (0.65-0.96), J(0.26-0.89) and D(0.42-0.94)] as the estimates of similarity coefficients are different for all three similarity coefficients (Sokal and Michener, 1958; Dice, 1945; Jaccard, 1908) [Section 3.2.6.2]. The SM coefficient is the sum of the proportion of shared positive bands plus the proportion of shared negative bands where as, Jaccard's and Dice coefficient considers only the proportion of positive bands shared by sample ignoring the proportion of shared negative bands. Each similarity coefficient matrices (SM, J and D) were

employed in generating phenograms to represent the genetic relationship among the *S. chirayita* accessions under study using UPGMA with SAHN clustering module. SAHN clustering module is commonly used for constructing phenogram for both similarity and dissimilarity matrices (Day and Eddsbrunner, 1984). The most elementary distance matrix algorithm is UPGMA for clustering analysis (Weising *et al.*, 2005; Graham *et al.*, 1994b; Moodie *et al.*, 1997). Compared to Jaccard's and Dice similarity matrices and phenograms, SM coefficient has slight variation in the range of genetic variation with few accessions of *S. chirayita* showing slight alteration in position within the same cluster in the phenogram. Taking a glimpse to the phenograms, the overall topology of all three phenograms were more or less similar except variation in the one shown by SM coefficient (Fig 4.1, 4.2 and 4.3)

In the study undertaken by Kosman and Leonard (2005), for discriminating similarity coefficients for molecular markers in studies of genetic relationships between individuals of haploid, diploid and polyploid species, they determined that no suitable method can be proposed for measuring genetic similarity between diploids on the basis of dominant banding profiles so no any preferred similarity measure was recommended for dominant markers in diploid. However, for molecular marker data, inclusion of 0-0 matches as in SM similarity coefficient seems appropriate when two alleles exist at a locus and one produces a band while the other doesnot and both alleles are present in the materials being compared (Dudley, 1994). For dominant markers it is generally assumed that each band represents a different bi-allelic locus (Williams *et al.*, 1990). Preference to the use of SM coefficient to discriminate the genetic variation analysis between the accessions using RAPD have been documented less eg. Sorghum (Agrama and Tunistra, 2003); potato (Paz and Veilleux, 1997). The three most commonly used similarity coefficients with RAPD data, SM, D and J differ in the amount of bias produced by the level of artifactual bands. Considering several factors, Lamboy (1994) recommended that the Dice coefficient of similarity, to be used routinely for measuring similarities in RAPD data unless specific circumstances or needs dictate the use of the other two coefficients (Lamboy, 1994; Duarte *et al.*, 1999). Also, Jaccard's similarity index have been used chiefly for the diversity analysis of the plant species utilizing the RAPD markers (Jeya Prakash *et al.*, 2006; Teklewold and Becker, 2006; Cordeiro *et al.*, 2008; Sundaram and Purwar, 2010; Khan *et al.*, 2010; Arya *et al.*, 2011).Therefore, all three types of similarity coefficient employed in present study possess its own kind of significance according to the goodness of fit they provide. So, comparison of the three similarity coefficient matrices with their phenotypic illustrations seem to be effective to select the proficient and competent one with proper goodness of fit leading to the unbiased assessment. In the present study, comparison of these similarity coefficients were done comparing individual matrices (using MXCOMP, Mantel 3 way test), comparing consensus indices of the phenograms constructed using respective

similarity matrix and by comparing goodness of fit of a cluster analysis to the similarity matrix on which it was based.

Original matrices were compared by applying Mantel test (Mantel, 1967) in the option of MXCOMP in NTSYS-PC (version 2.21i) program for comparison of original matrices by implementing two similarity matrices at a time among Simple matching, Dice and Jaccard similarity coefficients. The obtained correlation values were then tabulated (Table 4.7). Correlations between J and SM as well as D and SM were observed significantly low (0.07056 and 0.31292 respectively) as compared to the correlation observed between J and D (i.e. 0.92224), which is significantly high. Similar results have been observed by Consensus fork index ($CI_C$) which was used to compare two rooted labeled trees (phenograms) at a time of the same three different similarity coefficients (SM, J and D) using NTSYS-PC (Rohlf, 2009). Significantly high consensus fork index of $CI_C = 0.9063$ was found for Jaccard and Dice coefficients (Table 4.9).

Also, comparing highest cophenetic correlation, Jaccard's similarity with UPGMA clustering method revealed the highest correlation value of 0.85012 followed by Dice with 0.84315 and the least for Simple Matching 0.4358. Therefore, from all the comparisons carried out, Simple matching similarity coefficient seems to be unsuitable for the discrimination of genetic variation study of *S. chirayita* where as Dice and Jaccard's Similarity coefficient with significantly high correlation values appear to show the best fitted matrix and tree comparing the standard value (Rohlf, 2009).

| Level | Interpretation |
|---|---|
| $0.9 \leq\leq r$ | Very good fit. |
| $0.8 \leq\leq r < 0.9$ | Good fit. |
| $0.7 \leq\leq r < 0.8$ | Poor fit. |
| $r < 0.7$ | Very poor fit. |

Where, 'r' is the correlation coefficient between clusters analysed in accordance with the similarity matrix.

### 5.4.2 Cluster Analysis

Further assessment of genetic relationship was carried out using Jaccard's similarity matrix and phenogram as it revealed the highest goodness of fit of the similarity matrix to the phenogram (even slightly higher than Dice similarity coefficient). Populationwise comparisons of Jaccard's similarity coefficient showed the most genetically diverse individuals present within the Nagarjun *S. chirayita* population and from interpopulation relatedness study, individuals from Terathum and Nagarjun populations were observed genetically furthest (Table 4.10). In addition to disease resistance, broadly, greater genetic diversity leads to greater productivity in plant communities,

greater nutrient retention in ecosystems as well as greater ecosystem stability (Tilman, 2000). More higher the diversity, more ideal will be the accessions to be used as parents in hybridization, to develop improved varieties (Jeya Prakash *et al.*, 2006). In support to the result derived with NTSYS, Population genetic analysis was also conducted using statistical software POPGENE (version 1.32) designed for dominant marker systems such as RAPD.

3D-Plot constructed with Eigen vector using NTSYS-PC (version, 2.21i) for all the *S. chirayita* accessions (figure 4.5) and Eigen analysis done with Principal Coordinated analysis (PCO) using MVSP 3.2 for all investigated individuals with Jaccard's similarity coefficient (figure 4.6) substantiated the same pattern of genetic relationship between the populations of *S. chirayita* as shown by UPGMA based SAHN clustering phenograms. Every population was found demarcated and clumped into separate clusters revealing less genetic contamination within the individual natural habitats of *S. chirayita*. It yielded firm geographic pattern of groupings of *S. chirayita* populations which probably reflects no disturbance at gene pool over time.

Direct assessment of polymorphism based on monomorphic and polymorphic bands amplified by 26 random primers also revealed the *S. chirayita* individuals of Nagarjun as the most diverse and polymorphic (64.24%) where as less diverse individuals were observed for Kaski population (38.0 %) (figure 4.7, Table 4.11). The results agree with the one as shown by Phenogram. As genetic variability is important factor for sustainability of the species in nature (Sebastian *et al.*, 2010), estimation of genetic variation is essential to implement the strategic plan for conservation of species according to its adaptability potential.

## 5.4.3 Population Genetic Structure of *S. chirayita*

Genetic diversity refers to the variation at the level of individual genes (polymorphism) and provides a mechanism for population to adapt to their ever-changing environment. Higher level of intraspecific genetic variation has been observed among and within the populations of *S. chirayita* investigated. The use of RAPD technique to detect genetic variation at the level of DNA was found to be sensitive and powerful in *Swertia chirayita*. POPGENE was employed for the analysis of genetic variation among and within natural populations (Population Genetic structure) of *S. chirayita* using RAPD as dominant marker.

### 5.4.3.1 Genetic relationship between *S. chirayita* populations

Using POPGENE (Version, 1.32), estimation of Nei's (1972) standard genetic identity ($I_{xy}$) and genetic distance ($D_{xy}$) for the different populations of *S. chirayita* showed the comparative outcomes in congruence to the unbiased measures (Nei, 1978) calculated together (Fig 4.8 and Fig 4.9). Among the five populations investigated, highest "genetic identity" was observed for the

*S. chirayita* populations from Sankhuwasabha and Terathum whereas the highest "genetic distance" was revealed for the *S. chirayita* populations from Kaski and Sankhuwasabha [Section 4.7.5]. The interrelationship were also illustrated with UPGMA based phenograms justifying the genetic relationship between the *S. chirayita* populations as shown in fig 4.8 and 4.9 which are in equivalence to the observation revealed from NTSYS-PC (fig 4.4) using Jaccard's similarity matrix. Although Phulchowki (Lalitpur) and Nagarjun (Kathmandu) are very close geographically (<20 km), they were observed genetically far as compared to the Kaski accessions which are approx. 200 km far geographically. It revealed the uniqueness in the genetic characteristics of the *S. chirayita* from Kathmandu (Nagarjun). From this observation, it can be concluded that individuals from kaski population might have been introduced in the past to Phulchowki or vice-versa.

Similar result in resemblance was shown by phenogram constructed using SAHN, UPGMA using Jaccard's similarity matrix in which the *S. chirayita* individuals were appeared to be clustered in characteristic geographic pattern. Individuals from a particular geographic population was observed to be grouped in a particular cluster (fig 4.4). Thus, the genetic pattern of *S. chirayita* were found to be differentiated according to the geographic demarcation and it revealed the purity of the genetic characters of the particular population of *S. chirayita* as all the individuals from different populations clustered separately under the particular group of their population (figure 4.4; figure 4.5; figure 4.6) and hence the conservation of *S. chirayita in situ* should be imperative focusing on the different populations in Nepal due to their genetic distinctness.

After assessing the genetic relatedness among every population, the study was focused on the individual species diversity within the population.

### 5.4.3.2 Genetic variation within population of *S. chirayita* and at species level

On the basis of the clusters formed by *S. chirayita* individuals within a single population, the most diverse *S. chirayita* individuals were observed within the Nagarjun population as they were found to get clustered at least similarity coefficient value of 0.56 (fig 4.4) indicating the individuals were genetically distant within Nagarjun population. For the conservation of a species, genetic variability is of the utmost importance. It provides a mechanism for individuals and populations to adapt to their varying environment. Survival of a species depends on the maintenance of genetic variability within and among populations that accomodate new selection pressures brought about by environmental changes. The more diverse is the genetic variability, more is the potentiality of the species to sustain in the changing environment which is very significant in the perspective of species existence and conservation (Jayanti and Mandal, 2001; Faisal *et al.*, 2007; Sebastian *et al.*, 2010; Dharmar and Britt, 2011). Hence, from the present study, as compared to the other 4 populations, within population genetic diversity is found higher for Nagarjun

population of *S. chirayita* revealing its better potentiality and sustainability to adapt in its existing environment.

The results of genetic diversity assessment within populations of *S. chirayita* employing POPGENE for genetic diversity analysis also agree with the one observed with NTSYS-PC showing highly diverse individuals from Nagarjun population (PPB, 64.24%; H, 0.225; I, 0.337). The most significant unbiased discriminating index, Shannon's Index (Tsuda *et al.*, 2004, Sebastian *et al.*, 2010) was also observed highest for individuals from Nagarjun together with higher Nei's gene diversity index (H, 0.225) among the populations whereas, the *S. chirayita* individuals from Kaski showed the lowest variability (PPB, 38.0 %; H, 0.132; I, 0.199). The order of priority for conservation initiatives to be taken on the basis of low genetic diversity observed from this study is Kaski> Sankhuwasabha> Terathum> Phulchowki (Lalitpur)> Nagarjun (Kathmandu). These findings are based on the molecular genetic diversity assessment using RAPD marker. However, for the more comprehensive picture, other strategies for the assessment of vulnerability (such as via ecological and other molecular marker based on co-dominant analyses should also be employed.

Estimation at species level diversity showed appreciably high level of genetic differentiation with 92.28% of PPB and high Shannon index of 0.409 signifying the enhanced degree of polymorphism in the existing populations of *S. chirayita* under study.

#### 5.4.3.3 Overall Genetic Differentiation

High levels of genetic variability have been reported for many plant species that are vulnerable and with narrow range of distribution including *Centaurea wiedemanniana* ($H_T$ = 0.278; Sozen and Ozaydin, 2010); *Gentiana atuntsiensis* ($H_T$ =0.391) and *G. striolata* ($H_T$ = 0.324) (Zhang *et al.*, 2007); *C. nivea* ($H_T$ = 0.296) (Sozen and Ozaydin, 2009); *Primula apennica* ($H_T$ =0.24) (Crema *et al.*, 2009). The genetic diversity level for *S. chirayita* as estimated by POPGENE was also found to be similar at the range of 0.1429-0.3513 with high level of total heterozygosity ($H_T$). Therefore, it is likely that the breeding characteristics and habitat heterogeinity of *S. chirayita* might be responsible for the maintenance of the high genetic diversity in this species. In a recent review on estimates of genetic diversity obtained by RAPD markers, Nybom and Bartish (2000) compiled mean $G_{ST}$ values of 0.59, 0.19 and 0.23 for selfing, mixed mating and outcrossing plant species, respectively. Compared to these values, the population differentiation of *S. chirayita* ($G_{ST}$ = 0.5929) is close to that of the selfing breeding system. High genetic differentiation in this species may suggest that the individual populations have been reproductively isolated and that there is little current gene flow between them. The result is somehow in agreement with Chakraborty *et al.* (2009) in which *S. chirayita* has been declared as a self-pollinating species. However, in

contrast the higher polymorphism and diversity observed among the *S. chirayita* individuals of different population indicates the existence of significant cross pollination among the individuals in the population. For the confirmation of its breeding system, detail study seems essential at genetic level. In plants, gene flow can occur through effective cross pollination or seed dispersal (Ellstrand, 1992). The population genetic diversity and structure of a species is affected by a number of evolutionary factors, including mating system, seed dispersal, geographical range as well as natural selection. Of these factors, the breeding system is the main factor that affects genetic diversity both among and within populations (Hamrick and Godt, 1989).

Gene flow ($N_m$) among the individuals affects the genetic characteristics of the particular population. "Gene flow" is the change due to movement of gametes, individuals, or groups of individuals from one place to another. Gene flow is often regarded as a constraining force in evolution. Natural selection will tend to adapt a population to local environmental conditions but immigrants from other populations will introduce genes adapted to other conditions. In fact, gene flow between populations may prevent them from evolving into different species. But as emphasized by Sewall Wright in 1969 in particular, gene flow can also be a creative force in evolution. The movement of individuals and even entire populations may spread superior genes and combinations of genes throughout a species once they become common in one location. What role gene flow plays in a particular species depends both on the geographic distribution of that species and on the importance of other evolutionary forces (natural selection, genetic drift, mutation, non random mating and migration) (Slatkin, 1987; McDermott and McDonald, 1993). Observed low range of gene flow (0.1267 - 4.3694) with average of 0.3433 as compared to other studies reporting higher geneflow such as *Dalbergia sissoo* ($N_m$ = 3.3125; Wang et al., 2007), *Coptis chinensis* ($N_m$ = 7.116; Shi et al., 2008), *Festuca campestris* Rydb. ($N_m$ = 10.92; Mengli et al., 2005) etc. indicates the occurrence of restricted gene flow among *S. chirayita* populations and underlies an evolutionary process for population differentiation in *S. chirayita*.

### 5.4.5 Genetic differentiation using Analysis of Molecular Variance (AMOVA)

The analysis of molecular variance (AMOVA) carried out using GENALEx substantiated the earlier declaration made on population differentiation. AMOVA will describe how RAPD variance is partitioned within and among populations (Stewart et al., 1996). During global AMOVA analysis, there was no significant partitioning of the genetic variation ($p < 0.001$), with very less difference between the genetic variation occurring among populations (51%) and within population (49%). $\phi_{PT}$, an indicator of genetic differentiation among five populations was 0.513 suggesting comparable genetic differentiation of 0.49 within *S. chirayita* populations (Sozen and Ozaydin, 2010). The observed variation among populations agree with the restricted gene flow

among *S. chirayita* populations stated earlier along with the evolutionary process for population differentiation in *S. chirayita*. These findings also agree with the inferences of the hierarchial AMOVA analysis, indicating that the among-region genetic variation was somewhat high most probably due to higher geographical distance than between populations.

When the polymorphic loci produced by the 6 other species of *Swertia* were considered during the construction of phenogram i.e taking overall 386 loci produced during RAPD amplification by the 26 primers, the other species were found to be delineated out from the major clusters of *S. chirayita* verifying that they are different from the populations of *Swertia chirayita* studied (Fig 4.4). Thus, RAPD markers can be used as potent molecular marker for the easy and economic identification of the species which can be made far more robust converting the RAPD markers to the specific SCAR markers (Melotto et al., 1996; El-Ghore et al., 2004; Park et al., 2004).

# 6. CONCLUSIONS

*Swertia chirayita* is one of the highly prized medicinal plants of Nepal occupying a major volume in medicinal plants trade. Due to its high demand in international and national markets, existing populations are in great pressure and has resulted into vulnerability of the species. Due to its high demand as well as the existing difficulty in species identification, eight other species of *Swertia* are also traded in the name of Chiraito. Therefore, present investigation was undertaken firstly to understand existing genetic diversity within and between different *S. chirayita* populations of Nepal and secondly to develop species specific and population specific molecular markers that could be utilized in future for reliable identification of *S. chirayita* and its different populations.

In the present investigation, the use of genome based RAPD technique has been employed for the assessment of existing genetic diversity and relationships among five *S. chirayita* populations representing eastern, central and western development regions of Nepal. Although, conventional approaches of morphological, anatomical, cytological and chemical analyses have aided in documenting organismal diversity. DNA based molecular tools add up a new dimension to the identification, diagnostics, phylogeny and genetic diversity studies of high value plants. In the pool of various molecular techniques, RAPD-PCR has received major attention during the past decade because of its broad merits such as technical simplicity, non requirement of prior information of the DNA sequences of experimental organism for primer designing and non requirement of DNA probes or hybridizing steps. Furthermore, it is popular for its rapidity, cost effectiveness and requirement of nanogram quantity of DNA with the feasibility of automation. Hence, RAPD is handy in generating potential markers from readily available commercial primers. It has been proven that higher levels of polymorphism can be detected with RAPD-PCR compared to that achievable with RFLPs (Williams *et al.*, 1990; Clapp, 1996; Weisings *et al.*, 2005).

The genetic diversity estimates generated from the present study on the basis of RAPD data have not only highlighted the inherent genetic relationship among five populations under study but also produced data on the polymorphic nature of each of these populations. Besides, population specific RAPD markers and fingerprints generated by various random primers can be used for species authentication purpose. However, for a more robust molecular diagnostic development, further research involving more *S. chirayita* populations from Nepal have to be profiled using more number of primers. In addition, these species-specific and population specific markers need to be converted into a co-dominant marker system called SCARs. These markers can then be used for practical application of *S. chirayita* authentication. Alternatively, more extensive research involving DNA-sequence-based molecular markers need to be conducted in order to

generate DNA-barcodes for various *Swertia* spp. of Nepal including *S. chirayita*. Reliable species-identification protocols hold great promise in herbal drug industries and pharmaceutical companies. In the meantime, genetic diversity studies furnish valuable information for conservation and sustainable utilization of valuable germplasm such as *S. chirayita*.

Survival of a species depends on the maintenance of genetic variability within and among populations that help to adapt and acclimatize the new selection pressures brought about by environment changes (Jayanti and Mandal, 2001; Sebastian *et al.*, 2010). Among the five populations investigated, within population genetic diversity was found higher and prominent for Nagarjun population of *S. chirayita* revealing its best potentiality to adapt in its existing environment if the over encroachment by human is controlled. Unwise exploitation of plant beyond its regeneration capacity for trade has been observed as one of the most serious problems in the conservation of such vulnerable species. Similarly, *S. chirayita* individuals in Kaski revealed the lowest genetic variability among the populations studied. Although all these populations need to be judiciously conserved in their natural habitats, on the basis of genetic variation based on RAPD study, priority of conservation emphasis should be in the order *viz.* Kaski> Sankhuwasabha> Terathum> Phulchowki> Nagarjun.

Therefore, RAPD marker technology is found to be very useful for *S. chirayita* germplasm evaluation by providing a plethora of information about genetic variation in the existing gene pool. Ultimately, the information generated on genetic distances among various populations can be utilized for developing new elite culativars of *S. chirayita* via conventional breeding. Present investigation has opened up new avenues of academic research in the field of germplasm characterization, documentation, conservation and sustainable utilization in Nepal.

## RECOMMENDATIONS

For the long term conservation and sustainable utilization of *S. chirayita* in Nepal following need to be done.

1) Although present molecular study based on RAPD showed sufficient genetic variability within and between different populations of *S. chirayita*, unsustainable harvesting from natural habitats may still threaten this species to extinction. Therefore, sustainable harvesting is of utmost importance for long term retension (maintenance) of *S. chirayita* populations in their natural habitats.
2) Both *in situ* and *ex situ* conservation practices (including seed conservation in seed banks) need to be practiced in Nepal. Local people need to be trained on vulnerability of the species and hence it's proper harvesting and sustainable utilization.

3) Further research aiming at molecular genetics, molecular diagnostics, molecular phylogeny and DNA-barcoding needs to be conducted employing other PCR-based and DNA-sequencing based molecular marker techniques.
4) It is highly anticipated that the development of a robust molecular tool for species identification and its practice in public/private level is needed to check adulteration problem of *S. chirayita* in trade.
5) Based on molecular genetic diversity investigations carried out among various populations, elite cultivars of *S. chirayita* having enhanced medicinal properties can be developed. This demands a consolidated effort of plant breeders and molecular geneticist/biologists.

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# APPENDICES

**Appendix 1** Commonly used different molecular markers.

| | |
|---|---|
| AFLP | Amplified Fragment Length Polymorphism |
| AP-PCR | Arbitrarily primed PCR |
| ARMS | Amplification Refractory Mutation System |
| ASAP | Arbitrary Signatures from Amplification |
| ASH | Allele-Specific Hybridization |
| ASLP | Amplified Sequence Length Polymorphism |
| ASO | Allele Specific Oligonucleotide |
| CAPS | Cleaved Amplification Polymorphic Sequence |
| CAS | Coupled Amplification and Sequencing |
| DAF | DNA Amplification Fingerprint |
| DGGE | Denaturing Gradient Gel Electrophoresis |
| GBA | Genetic Bit Analysis |
| IRAO | Inter-Retrotrasposon Amplified Polymorphism |
| ISSR | Inter-Simple Sequence Repeats |
| ISTR | Inverse Sequence-Tagged Repeats |
| MP-PCR | Microsatellite-Primed PCR |
| OLA | Oligonucleotide Ligation Assay |
| RAHM | Randomly Amplified Hybridizing Microsatellites |
| RAMPs | Randomly Amplified Microsatellite Polymorphisms |
| RAPD | Randomly Amplified Polymorphic DNA |
| RBIP | Retrotrasposon-Based Insertion Polymorphism |
| REF | Restriction Endonuclease Fingerprinting |
| REMAP | Retrotrasposon-Microsatellite Amplified Polymorphism |
| RFLP | Restriction Fragment Length Polymorphism |
| SAMPL | Selective Amplification of Polymorphic Loci |
| SCAR | Sequence Characterised Amplification Regions |
| SNP | Single Nucleotide Polymorphism |
| SPAR | Single Primer Amplification Reaction |
| SPLAT | Single Polymorphic Amplification Test |
| S-SAP | Sequence-Specific Amplification Polymorphisms |
| SSCP | Single Strand Conformation Polymorphism |
| SSLP | Single Sequence Length Polymorphism |
| SSR | Simple Sequence Repeats |
| STMS | Sequence-Tagged Microsatellite Site |
| STS | Sequence-Tagged-Site |
| TGGE | Thermal Gradient Gel Electrophoresis |
| VNTR | Variable Number Tandem Repeats |
| RAMS | Randomly Amplified Microsatellites |

***Source:*** Mondini *et al.,* 2005

***Source***: Joshi and Dhawan (2005)