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Assessment of Genetic Diversity in Nepalese Populations of *Swertia chirayita* (Roxb. Ex Fleming) H. Karst Using RAPD-PCR Technique

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ABSTRACT

Owing to the high demand, *Swertia chirayita* populations in the wild are being depleted beyond its regeneration capacity. *S. chirayita* is one of the most valuable medicinal plants of Nepal in trade. Present Molecular investigation was undertaken to understand the level of genetic diversity in five *S. chirayita* populations of Nepal using Polymerase Chain Reaction (PCR)-based Random amplified polymorphic DNA (RAPD) technique. Thirty four accessions of *S. chirayita* along with six outlier accessions were analyzed using 26 arbitrary primers. Of the total 285 amplified bands scored for *S. chirayita*, 263 bands (92.28%) were polymorphic. Two major clusters were revealed in the phenogram generated from cluster analysis using NTSYS-PC software (version 2.21i) for the geographic populations under study. Principal Coordinate Analysis further substantiated the results of the phenograms. *Swertia chirayita* populations from Sankhuwasabha and Terathum were found to be genetically closest (68%, similar) whilst Nagarjun and Terathum were found to be most distant (33%, similar).The high genetic polymorphism reflected in *S. chirayita* populations indicates the good survival potentiality and adaptability in changing environmental scenario. The results thus produced might be helpful to plant breeders for elite cultivar development. The RAPD-PCR technique is found to be the rapid and effective tool for genetic diversity assessment in *S. chirayita* populations and generated insights for the formulation of conservation strategy of this vulnerable species together with its phytochemical distinctiveness.

Keywords: PCR; DNA Fingerprinting; Molecular Marker; Genetic Diversity; Polymorphism

1. Introduction

Nepal is very rich in medicinal plants and their associated traditional knowledge. Over 1950 medicinal plants have so far been reported [1]. 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. *S. chirayita* has been listed as one of the top priority medicinal plants of Nepal as well as of Asia [2].

Swertia, member genus of the family Gentianaceae is widely distributed globally and is represented by over 135 species distributed in temperate areas of Asia, Africa, Europe, North America and Madagascar [3,4]. Owing to diverse physiographic zones, climatic contrasts and altitudinal gradients, Nepal possesses a rich diversity of *Swertia* species and boasts thirty species distributed from east to west and from tropical to alpine zones ranging from 600 m to 5600 m asl [5,6].

Swertia chirayita (Roxb. Ex Fleming) H. Karst, a legendary medicinal plant species of Nepal is reported to be distributed in 54 districts of Nepal [7]. Due to its highly potent and superior medicinal properties, it is highly demanded in national and international markets and plays a dominant role in trade. Along with *S. chirayita*, eight other allied species having medicinal properties are also traded *viz*. *S. angustifolia* Buch.-Ham. Ex D. Don, *S. ciliata* (D. Don ex G. Don) B. L. Burtt, *S. dilatata* C. B. Clarke, *S. multicaulis* D. Don, *S. racemosa* (Griseb) C. B. Clark, *S. tetragona* Edgew, *S. alata* (Royle ex D. Don) C. B. Clarke and *S. nervosa* (G. Don) C. B. Clarke [8,9].

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Nepal is reported to trade more than 45% of the world's total volume of *S. chirayita* [10] of which only 1% is being used locally and the rest is being exported to India, China, Malaysia, Singapore, Germany, Italy, France, Switzerland, Sri Lanka, Bangladesh, Pakistan and USA to fulfill the demand of various medicinal plant based industries [11].

Swertia chirayita is a multifaceted medicinal plant containing diverse bioactive chemical compounds and properties. Early studies documented the presence of several chemical compounds in *S. chirayita* such as chiratinin, gentianine, amarogentin, amaroswerin, and several flavonoids, xanthones, terpenoids, iridoids and secoiridoid glycosides [12-14]. These bioactive compounds and their derivatives are reported to have anti inflammatory, antidiabetic, antihepatotoxic, antileishmanial, anticarcinogenic, antiviral and antihelmithic properties [15-18].

Due to versatile medicinal properties of *S. chirayita*, it is suffering from indiscriminate and unscientific harvesting from its natural habitats to fulfill national and international demands [2]. This has rendered *S. chirayita* to be listed as critically endangered in India and vulnerable in Nepal by CITES and IUCN [19]. Therefore, an urgent need has been felt to conserve the existing diversity of this valuable germ plasm of Nepal and protect its habitats from further destruction, for its long term and sustainable utilization. As the survival index of a species is greatly determined by the existing polymorphisms and gene flow between populations, assessing genetic diversity and natural distribution pattern are considered vital for formulating conservation strategies of vulnerable species such as *S. chirayita* [20]. For a species to survive against environmental pressures, the availability of a pool of genetic diversity is an essential prerequisite. Extinction would appear inevitable in its absence [21]. Phytochemical distinctiveness is also linked to the genetic diversity [22].

An understanding of the breeding system (self or cross) is fundamental to any genetic improvement program. Regarding *S. chirayita*, few works have so far been carried out on cytology, genetics and reproductive biology. It was reported to be a cross pollinated species [23] without any field experiment. Recent work with open and bagging experiments also showed that the probable mode of pollination is cross pollination although selfing is also present [24].

Various molecular marker (protein and nucleic acid-based) techniques have been widely used in genetic studies of wild plant populations. Such techniques enable researchers to answer questions pertinent to speciation, within and between population genetic diversity, gene flow and level of outcrossing [25]. Although co-dominant markers such as isozymes, Restriction Fragment Length Fragments (RFLPs) and Simple Sequence Repeats (SSRs) are particularly useful in wild populations, dominant markers such as RAPD and Amplified Fragment Length Polymorphism (AFLP) have also been applied widely and efficiently [26]. In the present study, RAPD marker system has been employed because of its simplicity, rapidity, low cost and non-requirement of sequence information for primer design [27,28]. RAPDs have been used in the genetic diversity analyses and diagnostic marker development for species or population of various food crops, weeds, medicinal and aromatic plants [29-33] as well as in gene tagging of disease resistant genes and Marker assisted selection [30,34].

Genetic architecture of population is generally believed to be the resultant of breeding system, gene flow within and between population, isolation mechanism and continued selection by various natural and artificial forces [35]. Genetic variation in any taxon is not uniformly distributed throughout the geographic area, where it is currently growing and populations growing in areas wide by geographical separation are expected to accumulate enormous genetic diversity. Therefore, for the assessment of genetic diversity, genotypes collected from different geographical locations have to be considered which enables capturing of all sorts of allelic diversity of a particular taxon.

So far, no molecular approaches have been employed to assess the level of genetic diversity in *S. chirayita* populations from different geographical locations of Nepal. Present investigation was undertaken to assess genetic diversity of *S. chirayita* populations representing East, Central and Western development regions of Nepal using genome-based RAPD technique.

2. Material and Methods

2.1. Plant Materials

DNA samples of 34 accessions of *S. chirayita* and 6 outlier species were collected in Silica gel from their natural habitats of five districts representing eastern, central and western development regions of Nepal (**Table 1** and **Figure 1**). Herbarium specimens of all these samples were also collected for their taxonomic identification.

Each herbarium and DNA sample was designated with accession code with respect to the site of collections and the collected samples were brought to the Molecular Biotechnology Laboratory of Nepal Academy of Science and Technology (NAST) for DNA extraction and subsequent molecular analysis.

2.2. DNA Extraction and Quantification

Genomic DNA from various samples were extracted by using Hexadecyltrimethyl Ammonium Bromide (CTAB) method [36]. Quantification and purity assessments of

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Table 1. Sample details of *S. chirayita* and other outlier species used in present investigation, locality of collection, number of samples collected, altitude of collection and plant accession codes.

S. N.	District/Locality	Region of Nepal	No. of samples collected	Altitude (meters)	Plant Accession Code
1	Lalitpur/Phulchowki, Godavari	Central	9	2100 - 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_{512}, K_{516} and K_{517}**
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}
		Total	40		

Note: Bolded sample accessions were used as outlier species in the present investigation (**K_{511} and K_{512}:** *Swertia dilatata*; **SA and K_{516}:** *Swertia nervosa*; **SB and K_{517}:** *Swertiape dicellata*).

Figure 1. Sampling sites of the present study.

extracted DNA were performed by using a UV Biophotometer (EPPENDORF AG 22331, Germany).

2.3. PCR Amplification

RAPD-PCR reaction parameters were optimized in 25 µl reaction volume by varying concentrations of template DNA, $MgCl_2$, dNTPs, primers and *Taq* DNA polymerase. Best cycling condition was chosen between the two RAPD-PCR cycling conditions described by Yu and Pauls (1992) and Edwards (1998) [37,38]. The best reaction and cycling parameters producing distinct scorable band profiles was then selected for subsequent RAPD profiling experiments. DNA amplifications were performed in BIOER Xp thermal cycler [BIOER Technology Co. LTD, Taiwan, China].

The PCR amplified RAPD fragments were separated by gel electrophoresis using 1.5% agarose in 1X TAE (Tris, Acetic acid and EDTA) buffer at 25 V (4.2 V/cm) in EMBI TEC (Santiago, CA) gel tank for one and half

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hour. The gel were then stained in TAE buffer containing 35 µL of Ethidium bromide (10 mg/mL solution, PROMEGA Co.) for 45 min followed by 15 mins destaining in water prior to gel documentation using Gel Doc system (IN GENIUS, Syngene Bioimaging, UK).

2.4. RAPD Profiling

Initially, 100 decamer primers (from UBC primer set-1, University of British Columbia, Oligonucleotide Synthesis Laboratory, Vancouver, British Columbia, Canada) were screened against fresh genomic DNA of *Swertia chirayita* from Kaski district. Twenty six UBC primers (**Table 2**) that gave reproducible and scorable amplifications were finally selected for the RAPD-PCR profiling involving all 40 accessions under study. All the accessions under study were screened with each of the 26 random primers to generate banding profiles for the genetic diversity study.

RAPD profiles generated by all 26 primers were used for the band scoring and binary data matrix creation. All polymorphic and monomorphic bands were scored. Markers were scored visually as discrete variables ("1" for presence and "0" for absence). Amplification failure was scored as "9", which was designated in the analysis procedure as an indicator of missing data [39]. Molecular size of the PCR products was determined by comparing the position of bands with Gene ruler^{TM} 100 bp plus DNA ladder (FERMENTAS company).

2.5. Data Analysis

The binary data matrix created was analysed using MS-Excel 2007 for the estimation of the banding characteristics namely: 1) Total number of bands (TNB), 2) number of polymorphic bands (NPB), 3) Percent Polymorphism (PP), 4) Polymorphic Information Content (PIC), 5) Band Informativeness (I_B), and 6) Resolving Power (R_P) for each primer used, which are defined by, PP = NPB/TNB generated by each primer; $PIC = 1 - \sum \left(P_{ij}\right)^2$, 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 [40]; $I_B = 1 - \left[2 \times \left(0.5 - P\right)\right]$, where P is the proportion of accessions containing the band. R_P was calculated as $R_P = \sum I_B$ [41].

Within and between population genetic diversity estimation was computed using NTSYS (Numerical Taxonomy and Multivariate System, version 2.21i, Exeter software; Setauket, New York, USA). Similarity indices were calculated applying a similarity for qualitative data (SIMQUAL) computer algorithm using NTSYS-PC. From these similarity indices, sequential, agglomerative, heirarchial and nested (SAHN) clustering was performed using the unweighted pair group method of arithmetic averages algorithm (UPGMA) [42]. Estimates of similarity were computed based on three different measures: 1) simple Matching coefficient (SM) [43], $S_{ij} = \frac{a+d}{a+b+c+d}$; 2) Dice's coefficient of similarity(D) [44], $S_{ij} = \frac{2a}{2a+b+c}$; 3) Jaccard's coefficient (J) [45], $S_{ij} = \frac{a}{a+b+c}$ where, S_{ij} = the similarity between two individuals, i and j; a = the number of bands present in both i and j; b is the number of bands present in i and absent in j; c is the number of bands present in j and absent in i, and d is the number of bands absent from both i and j.

Original matrices of SM, J and D coefficients were compared by Mantel test [46] using MXCOMP option in NTSYS program. Similarly, the correlation between the similarity matrices and its corresponding phenograms were estimated with the cophenetic correlation test. The estimated correlation matrices show the goodness of fit of cluster analysis in accordance with similarity matrix. Also, the phenograms generated from different coefficients were compared by consensus fork Index (CI_c) that estimates the dendogram similarities and measure how well resolved the tree is [47]. Best fitted coefficient of similarity matrix was employed for the diversity assessment. Relationships among the *S. chirayita* individuals were also studied using a Principal Coordinate Analysis (PCoA) using Multivariate statistical package (MVSP 3.2).

3. Results

The optimized RAPD-PCR reaction parameters consisted of DNA (25 ng), $MgCl_2$ (3.0 mM), 10X Taq polymerase reaction buffer [2.5 µl; 100 mM Tris-HCl (pH 8.8 at 25˚C), 500 mM KCl, 0.8% (v/v) Nonidet P40], *Taq* DNA polymerase (1U, MBI FERMENTAS company), dNTPs (0.2 mM each) and primer (0.4 µM) in 25 µl PCR reaction volume.

PCR cycling conditions described by Edwards (1998) produced best banding profile for *S. chirayita* and hence selected for subsequent PCR amplifications. Program consisted of an initial denaturation step at 95˚C for 2 min followed by 45 cycles of 95˚C for 20 s, 37˚C for 60 s and 72˚C for 60 s and final extension of 72˚C for 10 min.

3.1. RAPD Analysis

Primer screening involving 100 decamer primers resulted into 26 primers (**Table 2**) that produced scorable, reproducible and unambiguous polymorphic bands. The 26

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Table 2. Primer sequences, Total Number of Bands (TNB), Number of Polymorphic Bands (NPB), Percentage Polymorphism (PP), amplicon size range, PIC, I_B and R_P values of the 26 RAPD primers used to generate RAPD profiles of *Swertia chirayita* accessions.

Primer code	Primer Sequence (5'-3')	TNB	NPB	PP	Amplicon size range (bp)	PIC	I_B	R_P
UBC 2	CCTGGGCTTG	13	13	100.00	400 - 2500	0.90	0.95	13.29
UBC 3	CCTGGGCTTA	15	15	100.00	350 - 2800	0.90	0.68	10.18
UBC 6	CCTGGGCCTA	13	13	100.00	300 - 2100	0.91	1.20	16.28
UBC 51	CTACCCGTGC	7	4	57.14	500 - 2500	0.67	1.0	5.0
UBC 85	GTGCTCGTGC	9	6	66.67	450 - 1900	0.84	0.90	8.12
UBC 18	GGGCCGTTTA	9	9	100.00	200 - 2000	0.84	0.96	8.65
UBC 74	GAGCACCTGA	8	8	100.00	450 - 200	0.83	0.99	7.94
UBC 76	GAGCACCAGT	11	11	100.00	400 - 2150	0.89	0.79	9.53
UBC 23	CCCGCCTTCC	11	8	72.73	500 - 2400	0.84	0.95	8.59
UBC 43	AAAACCGGG	13	13	100.00	450 - 3600	0.85	0.74	9.65
UBC 65	AGGGGCGGGA	14	14	100.00	400 - 2700	0.86	0.63	8.82
UBC 97	ATCTGCGAGC	13	13	100.00	500 - 2000	0.84	0.47	6.06
UBC 86	GGGGGGAAGG	9	9	100.00	350 - 2400	0.86	0.81	8.06
UBC 4	CCTGGGCTGG	10	8	80.00	450 - 1900	0.84	1.10	9.94
UBC 73	GGGCACGCGA	10	10	100.00	600 - 2000	0.81	0.55	5.53
UBC 67	GAGGGCGAG	9	6	66.67	400 - 2000	0.81	0.94	7.53
UBC 15	CCTGGGTTTG	12	12	100.00	450 - 2500	0.87	0.64	8.35
UBC 16	GGTGGCGGGA	12	10	83.33	400 - 2000	0.87	1.01	11.06
UBC 54	GTCCCAGAGC	11	11	100.00	550 - 2600	0.87	0.76	9.18
UBC 66	GAGGGCGTGA	11	9	81.82	400 - 1700	0.82	0.70	7.65
UBC 71	GAGGGCGAGG	11	10	90.91	300 - 1800	0.89	1.22	13.41
UBC 88	CGGGGGATGG	10	9	90.00	400 - 3000	0.87	1.02	10.18
UBC 96	GGCGGCATGG	12	10	83.33	450 - 1600	0.89	1.09	11.06
UBC 92	CCTGGGCTTT	13	13	100.00	350 - 2700	0.90	0.38	4.94
UBC 55	TCCCTCGTGC	14	14	100.00	550 - 2000	0.90	0.52	7.29
UBC 17	CCTGGGCCTC	5	5	100.00	600 - 2200	0.74	0.40	2.41
	Total	**285**	**263**		**Average**	**0.85**	**0.82**	**8.8**

primers generated a total of 285 RAPD loci across 34 *S. chirayita* accessions, with an average amplification of ≈11 bands per primer (**Figure 2**). Of these, 263 (92.28%) bands were polymorphic. Individually, polymorphic bands produced by each primer ranged from 57.14% to 100%. Sixteen out of twenty six primers furnished 100% polymerphic bands (**Table 2**). The number of scorable bands produced per primer ranged from 5 to 15 with amplicon size ranging from 200 bp to 3600 bp.

PIC score for each primer ranged from 0.67 (UBC 51) to 0.91 (UBC 6) with an average of 0.85 (**Table 2**). The primers scoring PIC value more than 0.85 were UBC: 6, 2, 3, 92, 55, 96, 71, 76, 54, 16, 15, 86, 65 and 43 in decreasing order of their values. 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 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. UBC

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primers 2, 3, 16, 71, 88 and 96 furnished R_P values more than 10 (**Table 2**).

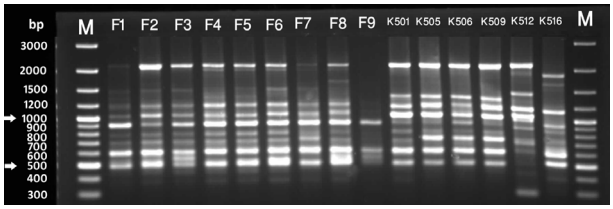
3.2. Genetic Diversity Assessment among Different Populations of *S. chirayita*

The varied ranges of similarity were obtained, using SM (0.65 - 0.96), J (0.26 - 0.89) and D (0.42 - 0.94) with an average similarity coefficient value of 0.81,0.58 and 0.68 respectively. The results from Mantel test of original matrices showed that the correlation between Jaccard and Dice similarity matrices was the highest and significant (0.92224) (**Table 3**).

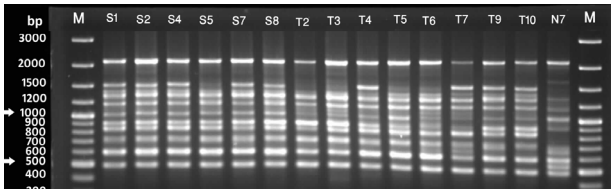
Cophenetic correlations between the genetic similarity matrices and cophenetic matrices are shown (**Table 4**). UPGMA clustering for Jaccard coefficient had the highest cophenetic correlation value of 0.85012 compared to Dice and Simple Matching coefficients. Because of the highest correlation value, Jaccard's coefficient of similarity is evaluated to be the best for deducing the genetic relationship among various *S. chirayita* accessions using UPGMA algorithm.

Also, the results obtained from consensus indices showed that consensus fork index was the highest (CI_C = 0.9063) for Jaccard and Dice coefficients (**Table 5**).

From all the comparisons made for similarity coefficients, Jaccard's coefficient was finally chosen as the best to interpret the results of genetic diversity among *S. chirayita* accessions. On the basis of Jaccard's similarity matrix, genetic similarities (%) were estimated between the *S. chirayita* populations. From the pairwise comparisons, *S. chirayita* accessions from Terathum: T_2 and T_3 were observed to be closest (89%, genetic similarity) and the accessions from Terathum (T_4) and Nagarjun (N_8) were revealed to be the most distant with genetic similarity value of 26%. The overall average genetic similarity value based on pairwise comparisons of 34 *S. chirayita* accessions was 49% (data not shown). *Swertia chirayita* populations from Sankhuwasabha and Terathum were found to be closest with 68% of mean genetic similarity (MGS) whilst accessions from Nagarjun and Terathum were found to be most distant with only 33% of MGS (**Table 6**).



(a)



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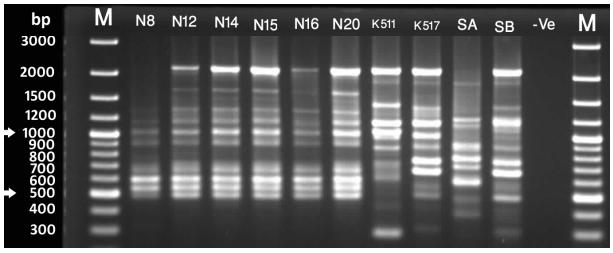

(c)

Figure 2. RAPD profile generated by primer UBC 6. 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, K_{512} is *S. dilatata* and K_{516} is *Swertia nervosa* from Kaski [B] S_1 - S_8 are *S. chirayita* accessions from Sankhuwasbha; T_2 - T_{10} are *S. chirayita* accessions from Terathum; N_7 is *S. chirayita* accession from Nagarjun [C] N_8 - N_{20} are *S. chirayita* accessions from Nagarjun; K_{511} is *S. dilatata*; K_{517} and SB are *S. pedicellata* and SA is *Swertia nervosa*.

3.3. Phenogram and PCoA Based Cluster Analysis

Figure 3 represents the phenogram constructed for 34

Table 3. Correlation coefficients from mantel test of original similarity matrices.

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

Table 4. Cophenetic correlation coefficients obtained for similarity matrices and cophenetic matrices of clusters computed by UPGMA module using MXCOMP (matrix comparisons) option of NTSYS.

Clustering modules of similarity	Simple matching	Dice	Jacard
UPGMA	0.43580	0.84315	0.85012

Table 5. Consensus fork indices among the phenograms (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			*****

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accessions of *S. chirayita* and 6 outlier accessions. Thirty four *S. chirayita* individuals were found separated into two major sub-clusters at the similarity coefficient of 0.37. The first sub-cluster contained 20 accessions from Phulchowki, Kaski and Nagarjun populations and the second sub-cluster contained 14 accessions from Sankhuwasabha and Terathum populations. In the first sub-cluster, Phulchowki and Kaski populations were shown genetically closer to each other than to Nagarjun population. Nagarjun populations were found separated from Phulchowki and Kaski populations at similarity coefficient of 0.44. In the phenogram, all five populations of *S. chirayita* under study clustered distinctly according to geographical distribution. The *S. chirayita* individuals within a population were clustered into a group at different similarity coefficient levels, *viz.* Phulchowki populations at 0.61, Kaski at 0.73, Nagarjun at 0.56, Sankhuwasabha at 0.78 and Terathum at 0.65. On the contrary, clear delineation between *S. chirayita* and different outliers were visible in the phenogram (**Figure 3**).

Table 6. Mean genetic similarity (GS) of the *S. chirayita* accessions from five different populations based on RAPD similarity matrix (J).

Populations	Phulchowki	Kaski	Sankhuwasabha	Terathum	Nagarjun
Phulchowki		57%	41%	38%	45%
Kaski			38%	38%	45%
Sankhuwasabha				68%	34%
Terathum					33%
Nagarjun					

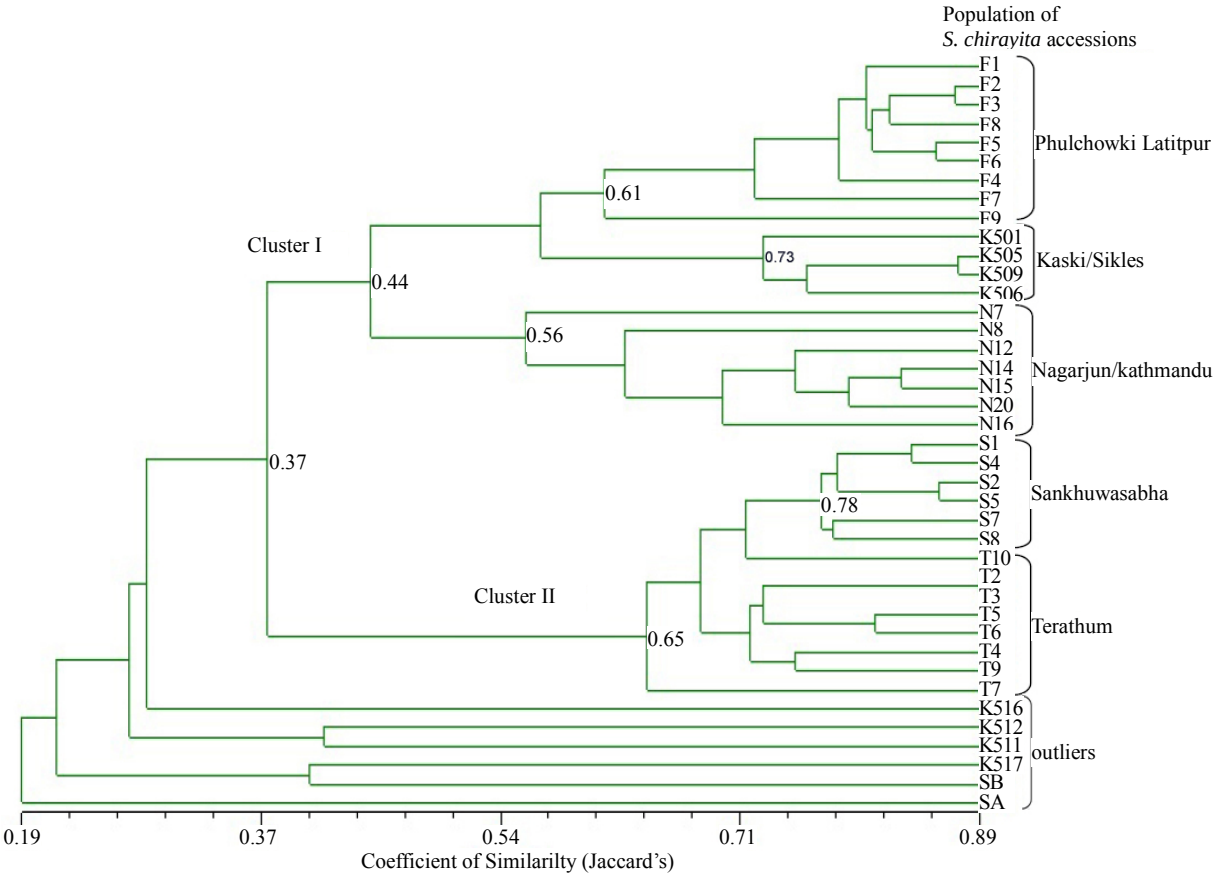

Figure 3. Phenogram generated for 34 *Swertia chirayita* accessions and six outlier accessions as revealed by UPGMA cluster analysis using Jaccard's similarity coefficient. Binary data matrix created for 386 RAPD loci generated by 26 primers was used for the analysis. Two main clusters in *S. chirayita* are labeled as I and II.

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In order to substantiate the genetic relationships among the samples collected, a PCoA analysis based on the Euclidean matrix was carried out (**Figure 4**). The first (Eigen value = 29.537; percentage of variance = 38.3%) and second (Eigen value = 11.827; percentage of variance = 15.3%) axis with a cumulative variance of 53.6% was observed. Plots of the first two coordinates were used for generating a PCoA graph (**Figure 4**) which supports the result of phenogram by clustering the individuals according to their geographical locations. *Swertia chirayita* from Sankhuwasabha and Terathum grouped together in one cluster whereas from phulchowki, Kaski and Nagarjun samples grouped into another cluster.

4. Discussion

All present day crop germplasms are products of domestication from the wild, long term selection and breeding for the improvement of desirable agronomic traits. Domestication followed by continued breedings selection for elite germplasms is an integral part of present day plant breeding [48]. Recently, developed molecular marker techniques have greatly complemented conventional breeding for crop improvement. Genetic diversity assessments using molecular tools help to analyze genetic variability among populations, select parental materials for hybridization for making new genetic recombination, select inbred parents for maximizing heterotic responses and identify materials that should be maintained for preservation of maximum genetic diversity in germplasm collections [49]. For all these applications, a simple and cost effective method for determination of genetic diversity relationships based on comparisons involving entire genomes is needed. In recent decades, a variety of molecular techniques and genetic markers have been extensively developed to estimate genetic diversity, but no single technique is universally ideal and each available technique exhibits both strengths and weaknesses. Therefore, the choice of technique is often a compromise that depends on the research question pursued and the genetic resolution needed [25]. Although it has been held, dominant marker like RAPD is not suitable for genetic analyses necessitating information on allele frequencies, they are appropriate in cases where allele frequency data are not needed. RAPD-marker system is a valuable tool for addressing genetic diversity, population genetics and plant breeding issues [50]. RAPD detects polymorphisms at DNA sequence level and has been widely applied in various disciplines [29,30,32,34]. RAPD provides a powerful tool to discriminate the closely related species even down to the level of subspecies [51].

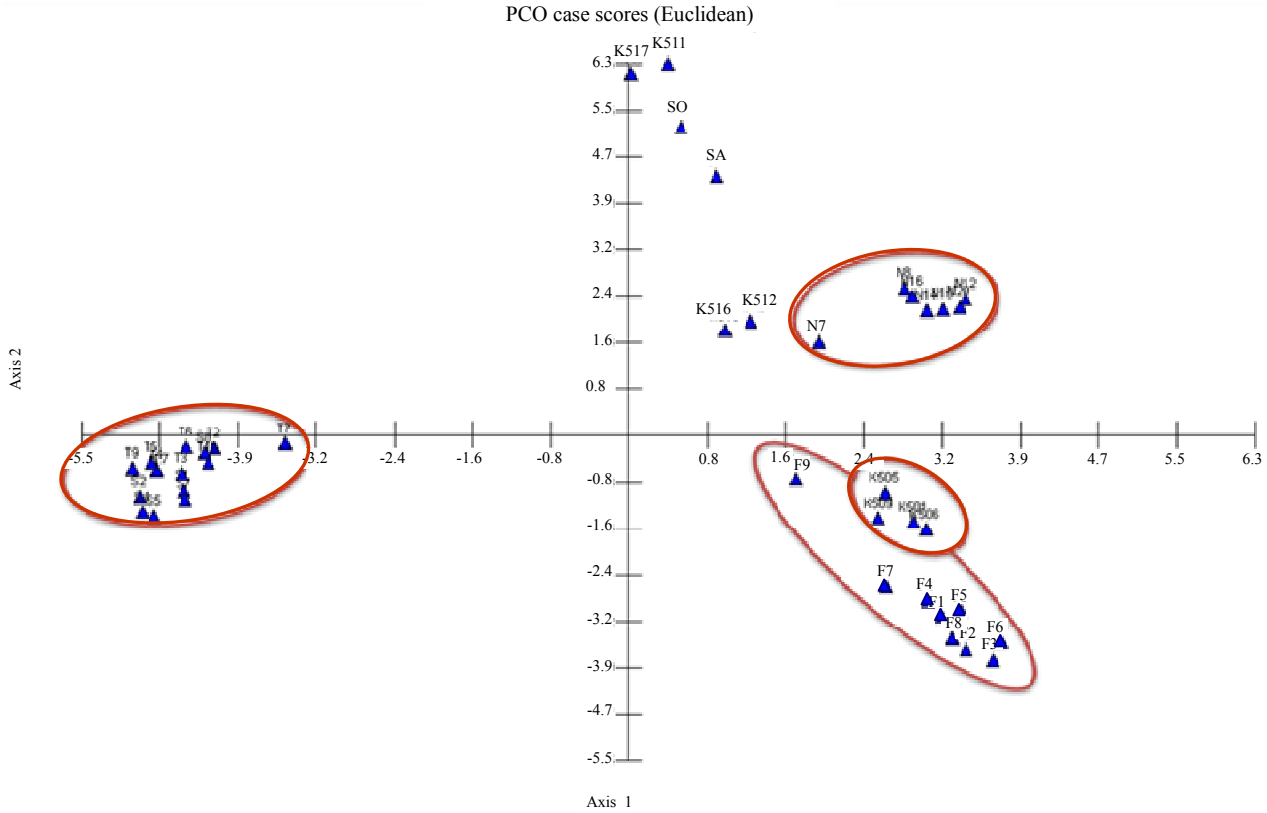

Figure 4. Principle coordinate analysis of 40 accessions of *Swertia* using MVSP 3.2.

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Swertia chirayita being one of the most important medicinal plants of Nepal having high demand in national/ international markets, natural populations of this species have become vulnerable. Although its domestication has started at various locations, scientific studies aiming at assessing genetic diversity of various populations and assessment of superior genotypes in terms of their chemical properties are still lacking. In this context, information on genetic diversity assessment of germplasm has several important applications for plant biologists, plant breeders, biotechnologists, chemists and conservationists [2,25,49, 50].

4.1. RAPD Analysis

The genetic polymorphism observed in the present RAPD investigation for *S. chirayita* is very high (*i.e*. 92.28%) in comparison to the value observed for Indian *S. chirayita* using ISSR marker. The genetic diversity study carried out in *S. chirayita* from temperate Himalaya of India involving ISSR markers indicated only 42.5% of polymorphic bands [52]. In the similar study of intra-specific genetic variation pattern in endangered endemic species, *Swertia przewalskii* of Qinghai-Tibet plateau, the genetic polymorphism was observed to be 52% for RAPD and 56% for ISSR marker [53].Genetic polymorphism shown with RAPD analysis was 56% for *Brassica carinata* [54]; 82.61% for *Anisodus tanguticus* [55] and 52.85% for *Trigonellafoenum-graecum* [56].

The study reports that some of the primers were more useful than others for distinguishing accessions. Higher the PIC, I_B and R_P values, superior is the discriminating power of primer used [54,57]. PIC value higher than 0.5 indicates a highly polymorphic locus [40]. Amplification profiles for most of the UBC primers (6, 2, 3, 92, 55, 96, 71, 76, 54, 16, 15, 86, 65 and 43) gave a PIC score greater than 0.85 suggesting that these primers are especially useful for RAPD based analyses of genetic diversity in *S. chirayita*. Band informativeness (I_B) and the primer resolving power (R_P) provide quantitative data allowing direct comparisons between primers [41]. R_P of the 26 RAPD primers ranged from 2.41 for the primer UBC 17 to 16.28 for the primer UBC 6 with average estimate of 8.8 (**Table 2**). As compared to values reported for other plants, mean PIC score (0.85) and R_P score (8.8) in this study were comparatively higher [22,54,58].

4.2. Genetic Diversity Assessment and Cluster Analysis

It was suggested when the marker data are "allelic non-informative", the estimates of coefficients between OTUs under consideration can be calculated by one of the three coefficients, based on the absence or presence of observed bands *viz.* Dice's similarity coefficient (D) is equivalent to Nei-Li coefficient [60]; Jaccard's similarity coefficient (J) [45] and Simple matching similarity coefficient (SM) [43] after examining 10 similarity coefficients are widely used in germplasm surveys [59]. In contrast to SM, both J and D do not involve shared absence of DNA bands. Thus, comparisons of these three similarity coefficient matrices and clustering methods seem imperative to find out the most fitted matrix for the discrimination of genetic diversity using RAPD profiles. Similarity matrices based on J and D were highly correlated ($r > 0.92$) according to Mantel matrix correspondence test together with the high correlation of their phenograms as given by Consensus index ($r > 0.9$). Comparing Cophenetic correlations between the genetic similarity matrices and cophenetic matrices, UPGMA clustering for J had the highest cophenetic correlation value ($r > 0.85$) than D and SM coefficients. According to Rohlf (2009), if the cophenetic correlation values, r is such that $0.8 \leq r < 0.9$, the similarity coefficient is interpreted as good fit for determination of genetic variability. Because of the highest correlation value, Jaccard's coefficient of similarity is evaluated as the best for deducing the genetic relationship among various *S. chirayita* accessions using UPGMA module and hence this has been employed for diversity assessment. In a similar study of *Sesamum indicum* L. using RAPD technique [61], presence of high genetic diversity was observed with Jaccard's coefficient ranging from 0.19 to 0.89. The present study also revealed the similar range of Jaccard's similarity coefficient of0.26 to 0.89 reflecting the high genetic diversity within *S. chirayita* accessions coming from various geographical populations. This is in contrary to the low diversity observed with ISSR marker based similarity values of Jaccard's coefficient (*i.e.* 0.68 to 0.97) among *S. chirayita* accessions from temperate Himalaya of India [52].

Among the five populations investigated, the mean genetic similarity among the *S. chirayita* populations varied from 33% (for Nagarjun and Terathum) to 68% (for Sankhuwasabha and Terathum) (**Table 6**). Lower the MGS, higher is the genetic diversity between those accessions. Higher the genetic distance, more ideal will be the accessions to be used as parents in hybridization, to develop improved new elite varieties [62]. The considerably high genetic diversity observed might be the synergistic consequences of cross pollination between the individuals in the population and act of evolutionary forces (natural selection, genetic drift and mutation) in the global climate change perspective [63]. This may be reflecting the good survival potential of the species in the existing environment and also demonstrating fine degree of adaptability and acclimatization in the new selection pressure brought about by environmental changes. In addition to disease

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resistance, greater genetic diversity leads to greater productivity in plant communities, greater nutrient retention as well as ecosystem stability [64]. The knowledge thus generated on the genetic diversity among *S. chirayita* accessions can play a crucial role towards their conservation and elite cultivar development.

In conclusion, the present molecular study based on RAPD highlighted that the high levels of genetic diversity are still maintained among *S. chirayita* accessions in different populations. Despite its vulnerable status, it has got the potential to adapt in its existing environment if the over encroachment by human is controlled. All these populations need to be judiciously conserved *ex situ* (e.g. in seed banks) as well as *in situ* in their natural habitats and sustainable harvesting practices must be followed for long term survival of this species. Furthermore, relevant laws should be strictly enforced to restrict the rampant exploitation of the species beyond its regeneration capacity that is still threatening many populations of valuable species in their natural habitats.

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 intraspecific polymorphic nature of *S. chirayita* accessions. Present investigation has opened up new avenues of academic research in the field of high value germplasm characterization, documentation, conservation and sustainable utilization in Nepal.

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