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# Genetic diversity and population structure analysis of *Paris polyphylla* Sm. revealed by SSR marker

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ABSTRACT

*Paris polyphylla* Sm. is a vulnerable medicinal plant distributed in the Himalayan countries. This plant has numerous pharmacological benefits, including anticancer, anti-inflammatory, analgesic, and antipyretic properties. The distribution, conservation status, and traditional usage of this species are fairly known in Nepal. However, its diversity and population structure at the molecular level are unexplored. This study analyzes, the genetic diversity and population structure of 32 *P. polyphylla* germplasms collected from Central, Eastern and Western regions of Nepal using 15 simple sequence repeat (SSR) markers. All the SSR primers were polymorphic and amplified 60 alleles ranging from 50 bp to 900 bp. The polymorphic information content (PIC) value ranged from 0 to 0.75. The average value of the observed heterozygosity (Ho), expected heterozygosity (He), Shannon's information index (I), and total heterozygosity (Ht) were 0.63, 0.53, 0.92 and 0.32, respectively. The analysis of molecular variance (AMOVA), showed a maximum variation of 74% within the individual in a population and only 26% variation among the population. In the population STRUCTURE analysis two clusters were formed where Eastern germplasms (EN) were separated far from the Central and Western germplasms (CWN), this clustering was in complete correspondence to the unweighted pair group method based on arithmetic average (UPGMA) and principle coordinate analysis (PCoA). Furthermore, in the UPGMA and PCoA, germplasms collected from the same or relatively similar geographic origin were closer. These findings are critical for developing conservation policies, facilitating evolutionary research, sustainable utilization and commercial cultivation of this pharmacologically important and threatened species.

## 1. Introduction

*Paris polyphylla* Sm. (family Melanthiaceae), commonly called Love Apple (Satuwa in Nepali), is an important medicinal herb native to the temperate forests of China, the Indian subcontinent, including Nepal, and the Indochina region [1–4]. In Nepal, *P. polyphylla* is mainly distributed in an altitudinal range of 1500–4500 m above sea level from east to west Nepal [3,5,6]. Many secondary compounds including Paris saponins I, II, V, VI, VII, H, dioscin, polyphyllin-D, daucosterol, β-ecdysterone, Paris yunnanosides G-J, oligosaccharides, 20-hydroxyecdyson saponins, heptasaccharide, octasaccharide, trigofoenoside A, formosanin C (polyphyllin B), pinnatasterone, protogracillin, padelaoside B, and gracillin have been isolated and characterized from various plant parts (rhizome, root, leaf, stem) of

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*P. polyphylla* [7–11]. The rhizome is the main economic part and has been widely used in traditional and modern medicines for various medicinal properties, including antioxidant, cytotoxic, antitumor, anthelmintic, antimicrobial, antiangiogenic, immunostimulating, contractile, hemostatic, anti-inflammatory, wound healing, and immune-enhancing activities [8,12–18]. Steroidal saponins, including polyphyllins (I, II, D, VI, VII) in extracts from *P. polyphylla* rhizomes, have properties that work against many types of cancers, including breast, cervical, lung, ovarian, and gastric cancers, as well as osteosarcoma [9,19–26]. Species with thick rhizomes have been used as a medicinal herbs in China, and more than 40 commercial drugs and health products have been developed [27]. The commercial products are also listed and made available to the consumer through the e-commerce platforms; for example, Cunningham et al. [28] reported 'alibaba.com', the popular e-commerce platform, listed *P. polyphylla* items including crude drug (dried whole or cut rhizomes) offered by various Asian suppliers, from China, Nepal, India, Pakistan, Bhutan, Hong Kong, Thailand, and Vietnam.

The trend of unmanaged and excessive injudicious harvesting (unscientific use, illegal/cross-border trade of rhizomes) of *P. polyphylla* has rapidly increased. With the consequent habitat destruction and a decreased natural population, this species has come under the vulnerable category [5,10]. *P. polyphylla* was treated in the International Union for Conservation of Nature's (IUCN) Red List of Threatened Species in 2020 and listed as vulnerable by IUCN and the Conservation Assessment and Management Plan (CAMP) [2,6, 29]. Genetic information is an important tool for analyzing the genetic structure of a population as it is used to draw the evolutionary history, present status and future prediction of any species [30]. A species' genetic diversity depends on the nucleotide sequence variations caused by mutation, gene flow, natural selection and genetic drift [31–33]. Genetic diversification varies among species and within genomes and has important implications for the evolution and conservation of species [34]. Various molecular markers, including random amplified polymorphic DNA marker (RAPD) [35], inter-simple sequence repeat marker (ISSR) [36], sequence-related amplified polymorphism (SRAP), codon targeted (SCoT) polymorphism marker [37], simple sequence repeat marker (SSR) [38] and expressed sequence tag-derived simple sequence repeat markers (EST-SSRs) [39,40], have been used for analyzing genetic diversity of *P. polyphylla* and its related species in many countries, including China, India and Vietnam. Compared to other molecular markers, the SSR marker, a codominant marker type, is more reliable and reproducible and yields more accurate estimates of genetic diversity [4,41–43].

Previous studies have accessed the ecological and traditional knowledge of *P. polyphylla*, reported habitat decline and distribution changes, and predicted the risk of extinction of this species, highlighting its conservation need [6,44–48]. However, genetic diversity and population structure of *P. polyphylla* are unexplored in Nepal. To address this gap, we analyzed the population genetic diversity and structure of *P. polyphylla* germplasms at the molecular level using SSR markers, which will help understand the genetic dynamics, including diversity, structure and evolutionary history of the species. Furthermore, this will also highlight the prioritization of sites and management choices for future conservation programs.

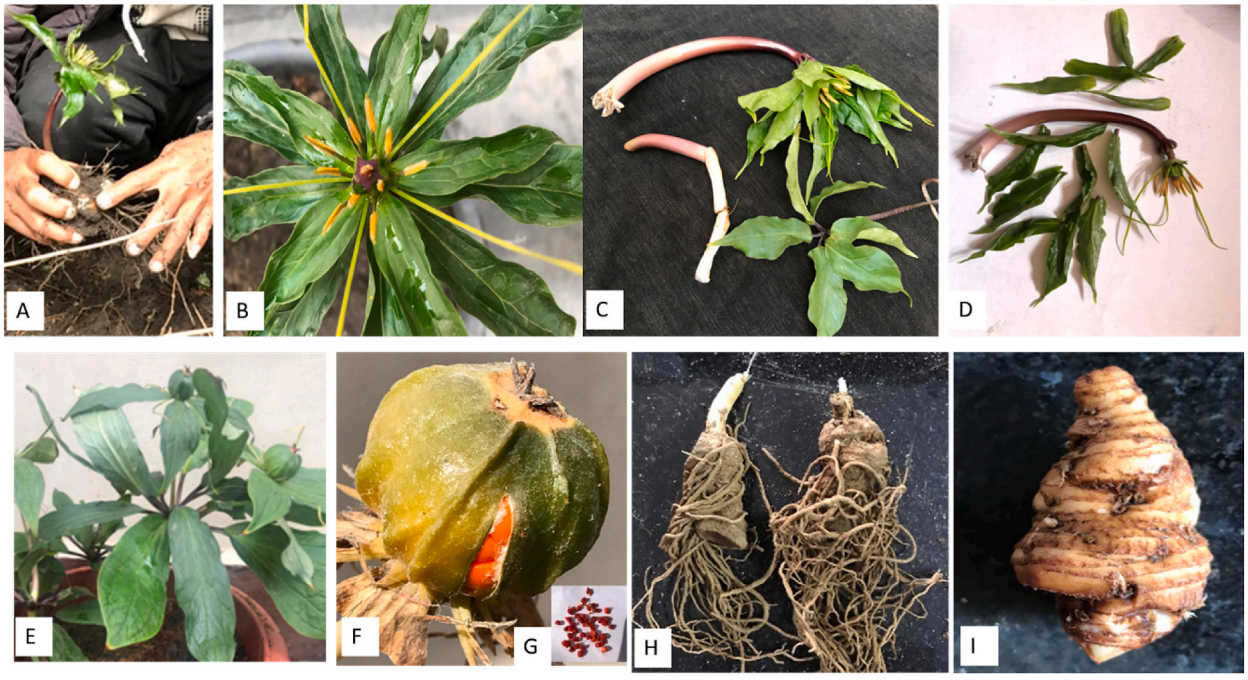


**Fig. 1.** *Paris polyphylla* Sm. representing its areal and underground parts. (A, B, C and D) a vegetative plant with Rhizome, leaf stem and flower; (E) plant at a fruiting stage; (F and G) fully matured fruit; (H) Rhizome with roots (I) matured Rhizome after removal of roots and soil.

## 2. Materials and method

### 2.1. Botanical information of P. polyphylla

*P. polyphylla* Sm. is a perennial herbaceous plant, grows in moist and shady places (such as under deciduous trees) of forests, bamboo forests, thickets, grassy or rocky slopes and stream sides of the Himalayas. The plant height ranges from 30 to 60 cm and stem 0.3–0.6 cm thick. The most important medicinal part of this plant is a rhizome, which is segmented, has grey color on the outer surface and white or light-yellow color in the inner part, and lies in the horizontal position under the ground (Fig. 1A and I). Small (1–15 cm long), thread-like roots (adventitious fibrous root) are present on the outer surface of the rhizome (Fig. 1H). The new plant arises from the rhizome bud during February and March. It has a single unbranched stem and 2 to 3 whorls of lanceolate or elliptic leaves (4–9) with pointed leaf tips, which are present on the nodes (Fig. 1A and B). Because of it, the plant is also called *tintalle banko* in some parts of Eastern and Central Nepal including Solukhumbhu, Dolakha, Ramechhap and Sindhupalchowk districts). The leaf is petiolate (purple, 1–4 cm long), 7–15 cm long and 1.5–3 cm wide, with an entire margin and smooth surface (Fig. 1C, D, and E). Only one peduncle arises between the whorled leaves and develops only one flower during April and May. The flower is bisexual. It has 8 to 12 perianths in the outer and inner layers, which are green and yellow. The stamen is long, radiating, light purple, and bears yellow stamen. The ovary is green, tetrafurcate. The fruit is 2–3.5 m in diameter and matures during September and October. One healthy fruit contains approximately 30 seeds (Fig. 1F and G). After the fruit matures, the leaf and stem dry out, and the seed spreads.

### 2.2. Sample information

Young healthy leaf samples of 32 *P. polyphylla* germplasms were collected from different locations (natural habitat) of Central, Eastern and Western regions of Nepal during the field visit conducted between April and July of 2021 and 2022. The plant was identified using taxonomic knowledge and comparing morphological characteristics of collected germplasms with key characteristics of *P. polyphylla* Sm. cited in three different books – "Flora of China" [49], "Flora of Langtang and Cross-section Vegetation Survey" [50] and "Plants and People of Nepal" [51] and verified with Prof. Suresh Kumar Ghimire of the Central Department of Botany, Tribhuvan University and Rajesh Tamang, the taxonomist and Assistant Scientific Officer (Section Officer Tech.) at the Ministry of Forest, Environment and Soil Conservation, Koshi Province, Biratnagar, Nepal. Of the 32 germplasms, 17 germplasms (covering 3, 2, 4, and 8 of Lamjung, Gorkha, Kaski, and Manang districts, respectively) are of Western Nepal, 9 germplasms (two each of Sindhupalchowk, Rasuwa, Nuwakot, Dolakha and one of Ramechhap districts) are of Central Nepal and 6 germplasms are of, Ilam, Eastern Nepal. The map of Nepal showing the sampling sites drawn using ArcGIS 10.5 is given in Fig. 2.

### 2.3. DNA isolation

The leaf of a single germplasm was collected in a zip-lock polybag separately and properly tagged. The collected leaf sample was wrapped in aluminium foil, frozen and stored in liquid nitrogen before the process of DNA isolation. Genomic DNA was isolated using the CTAB method [52] with some modifications. Briefly, 0.2 g of leaves were ground in liquid nitrogen in a mortar and pestle. To the fine powder, 1 ml of CTAB buffer was added and made a fine paste. Then, the pest was homogenized in the vortex at 130 rpm for 5 min (VorTemp™ 56, United States) and incubated in the water bath (WB14, Schwabach, Germany) at 65 °C for 45 min. The tubes were gently mixed during the incubation period in each 10 min interval. After 45 min, tubes were taken out from the water bath, left for 5 min to cool at room temperature, and then centrifuged at 12,000 rpm for 8 min (Mikro22 R, Tuttlingen, Germany). Then, 800 μl of the supernatant was pipetted and poured into the new 2 μl Eppendorf tube. Then an equal volume of chloroform (24): isoamyl alcohol (1) mixture was added and gently mixed at 27 rpm for 15 min (VorTemp™ 56, United States). Then, tubes were again centrifuged at 13, 000 rpm for 5 min. The upper aqueous part (600 μl) was carefully transferred to the new 1.5 ml Eppendorf tube, and to this, 1/10

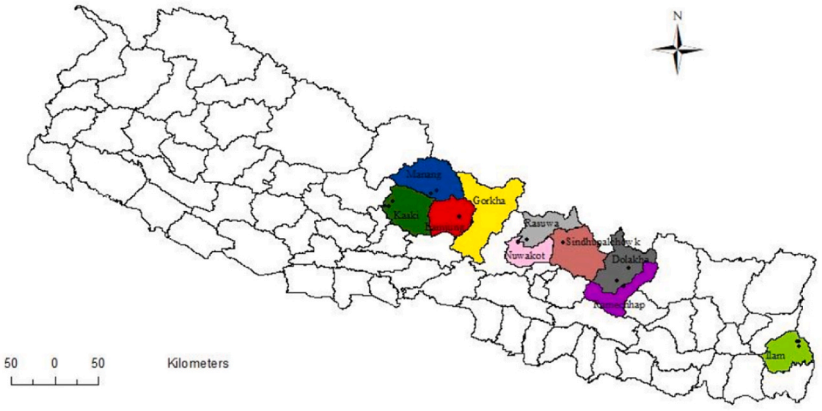


**Fig. 2.** Map of Nepal showing sampling sites of 32 germplasms of *Paris polyphylla* used in this study.

volume of 3 M sodium acetate was added followed by ice-cold absolute ethanol. It was gently mixed and centrifuged at 13,000 rpm for 2 min. After that, the upper part was discarded. The DNA of the lower precipitate was purified by washing it with 70% ice-cold ethanol and centrifuging it for 1 min. This process was repeated two times. DNA was air-dried and suspended in 200 μl of 1*X* Tris–EDTA (TE) buffer (pH-8). To this, 0.2 μl RNase A solution (1 mg/ml stock) diluted from the 10 mg/ml RNase (Thermo EN0531) was added and mixed well. The quality and quantity of the extracted DNA were examined on 0.8% agarose gel and spectrophotometer (Optizen NanoQ, Daejeon, Korea), respectively. To equalize the DNA concentration, extracted DNA was diluted to a working concentration of 30 ng/μl with 1*X* TE buffer.

### 2.4. PCR amplification

For the PCR amplification, a total of 15 SSR primers were used. Among them, eight EST-SSR primer sequences (SSRPP1, PP-1 to PP-7) were obtained from the previous study of [39], which were developed from root transcriptomic analysis of *P. polyphylla* var. *yunnanensis* and seven SSR primers (PP-19 to PP-25) were developed from a (CT)n-enriched genomic library of *P. polyphylla* var. *chinensis* and used for genetic diversity study in the Anhui Province of China [38]. PCR amplification was performed on a final volume of 25 μl containing 30 ng of genomic DNA, 11 μl of 2*X* PCR master mix with dye (including 1*X* PCR buffer, 160 μM of each of the dNTPs, 0.8 U of Taq DNA polymerase, and 6*X* loading dye (MedChem express, USA) and 1 μl (from 10 μM working concentration) of each forward and reverse primers (Macrogen, Korea). The thermal cycling condition was carried at a temperature of 95 °C for 5 min followed by 35 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 1 min, extension at 72 °C for 2 min and then final extension at 72 °C for 10 min (MG-96 MyGene™ Thermal Cycler, LongGene, UK). Then, PCR products were detected on 2% agarose gels and visualized by staining with ethidium bromide (1 mg/ml) under the bio-gel documentation system (ClinX, China). Only the clear and reproducible amplicon was considered for analysis. The binary data were scored manually in the form of 0 (for band absent) and 1 (for band present), considering the very clear and reproducible amplicon in the agarose gel image.

### 2.5. Genetic diversity analysis

For each SSR markers, the polymorphic information content (PIC) was calculated using the formula $PIC = 1 - \Sigma Pi^2$, where, $Pi^2$ refers to the sum of the ith allelic frequency of each microsatellite locus to all accessions. PIC value is used to assess the informativeness of the marker developed and is usually grouped into high (PIC> 0.5), moderate (0.5> PIC> 0.25), and low (PIC< 0.25) categories [39,42,53]. For each SSR marker genetic diversity parameters, including the total number of alleles (Na), the effective number of alleles (Ne), Shannon's information index (I), observed heterozygosity (Ho), expected heterozygosity (He), total heterozygosity (Ht), genetic differentiation coefficient (Fst), gene flow (Nm) (which is also called gene migration, the transfer of genetic material from one population to another), probability in Hardy–Weinberg equilibrium (PHWE), Nei's expected heterozygosity (Nei), and Nei's genetic distance were calculated using POPGENE version 1.32 [54]. An unweighted pair group method (UPGMA) phylogenetic tree was constructed by using Nei's genetic distance matrix [55] with 1000 bootstrap replications using the MEGA 11.0 program [56]. Similarly, the principal coordinate analysis (PCoA) was also drawn using Nei's genetic distance matrix. To access the percentage distribution of genetic variation and fixation index (FST), a hierarchical analysis of molecular variance (AMOVA) was analyzed. Both PCOA and AMOVA analysis were performed by using GenAlEx 6.5 [57].

### 2.6. Population structure analysis

The Bayseian clustering method was used to infer the population structure using STRUCTURE 2.3.4 software [58]. The estimated number of populations (K) was set from 1 to 15, with 100,000 steps burning and 500,000 Markov Chain Monte Carlo iterations

**Table 1**
Primer name, forward and reverse sequence, amplification size, and the PIC value of 15 SSR primers used in 32 *paris polyphylla* germplasms.

| SN | Primer name | Forward seq. (5′ to 3′) | Reverse Seq. (5′ to 3′) | PIC | Size of amplicons (bp) |
|---|---|---|---|---|---|
| 1 | SSRPP1 | AGATACTGGCCGGAAGGAGT | GCTTCAGCATTCCACTCCAG | 0.53 | 50–200 |
| 2 | pp1 | AAAGTTCGCCTCCCTTTCTC | CCATTACCTGAGGCCTGAAA | 0.50 | 50–150 |
| 3 | pp2 | GCTGCGATGCAAAACCTTAT | GGCAACCACCACCCTACTAA | 0.59 | 50–300 |
| 4 | pp3 | CCTTTGTAGCATGGGTGGTT | GACAATTGCTCCGACTCAAAA | 0.50 | 50–170 |
| 5 | pp4 | GTATCGACGGTCGCGATTAT | AGCAGGAGATTGAACCCTCA | 0.14 | 120–450 |
| 6 | pp5 | CATTAGCCGAGAAAGGCTTG | ACTGGAGCCTCGATCACAAT | 0.00 | 200 |
| 7 | pp6 | GGAGGAAGACGATGATCGAA | GCCATGTGCAGTCTCTCAAA | 0.00 | 150 |
| 8 | pp7 | CCTCCATCACCACCTAAACC | AACTGAAGGTGGGGTCAGTG | 0.50 | 100–200 |
| 9 | pp19 | TTCTTTCAACCGCCATACCGT | TGCTTTGCTGCTTCTAACTCG | 0.54 | 50–500 |
| 10 | pp20 | AATACTCCGACGGGTGTTG | AGCGATAGCCAGAGGAAAGG | 0.50 | 100–500 |
| 11 | PP21 | CGCCATTAGGACAGGGATTCAC | TCTACTTTGCTGCGGCTCCAC | 0.65 | 100–300 |
| 12 | PP22 | ACCGTCATTTAGTTGGTGCGAG | TGTGAACGCCTTGGAACCCT | 0.52 | 100–300 |
| 13 | PP23 | GTGAGAAAACAACATGAAAGA | ACATCCCAGTATGACAGACG | 0.50 | 100–150 |
| 14 | PP24 | GACGATGAGTTCACGACGCTG | GTGTTTCGCTCACCAAGGACG | 0.75 | 200–900 |
| 15 | pp25 | AGCCTGATCGGTATTGTCGG | CCACGCCCTTTCCTTCTTTA | 0.69 | 100–700 |
| | | | | 0.46 | |

(MCMC), and 20 replications were made for each K population. The result obtained from replications for each cluster was averaged to estimate the probability values. Then, using STRUCTURE HARVESTER version 0.6.92 [60], the optimal K value was determined. In this method, the maximum value of ΔK was estimated using log-likelihood [LnP (D)] based on the rate of change in LnP (D) between successive Ks and the ΔK method [59].

## 3. Results

### 3.1. Genetic diversity analysis for SSR primers

All 15 SSR primers were polymorphic across 32 germplasm belonging to Nepal's Eastern, Western and Central ecological ranges. A total of 60 alleles ranging from 50 bp (SSRPP-1, PP-1, PP-2) to 900 bp (PP-24) were amplified. DNA profile of 32 germplasm of *P. polyphylla* amplified by different SSR primers are given in Supplementary Fig. 1. Among the 15 primers used, primer PP-25 amplified the maximum number (total 7) of alleles whereas, minimum number (1) of alleles were amplified by primer PP-6 and PP-7. The PIC value ranged from 0 (PP-5, and PP-6) to 0.75 (PP-24) with an average value of 0.46. A total of 12 primers has a PIC greater than 0.5 and were found to be highly effective, and the other three primers, namely PP-4, PP-5, and PP-6, with a PIC less than 0.25, were less effective for the genetic diversity study of *P. polyphylla* germplasms (Table 1).

The observed number of alleles was greater than the expected number of alleles in all loci. The observed heterozygosity was greater than expected in all the loci except PP-6 and PP-7. The value of observed heterozygosity ranged from 0 (PP-6) to 0.97 (PP-21), with an average value of 0.63. Similarly, the expected heterozygosity value ranged from 0.0 (PP-6) to 0.81 (PP-24), with an average value of 0.53. The value of total heterozygosity ranged from 0 (PP-6) to 0.48 (PP-21, PP-23), with an average value of 0.32. Similarly, the Shannon information index ranged from 0.16 (PP-4) to 1.79 (PP-24) with an average value of 0.92, and Nei ranged from 0.06 (PP-4) to 0.80 (PP-24) with an average value of 0.52, and the coefficient hierarchical Fst, estimated according to Wright, ranged from 0.18 (PP-2, PP-3, PP-23) to 0.76 (PP-5) with an average value of 0.41. Likewise, the value of gene flow detected in each locus across 32 germplasms ranged from 0 (PP-6) to 1.16 (PP-23), with an average of 0.58. The HWEP value ranged from 0.0000 to 0.09994 across the primer (Table 2).

### 3.2. Genetic differentiation and structuring

#### 3.2.1. AMOVA analysis

The AMOVA analysis conducted using a distance matrix of 32 individuals against three ecological ranges- Central (CN), Western (WN) and Eastern Nepal (EN) showed a maximum variation of 74% within the individual in a population and only 26% variation among the population (Table 3 and Supplementary Fig. 2). The value for Fst among CN, WN and EN is 0.353 (Fst >0.25), indicating a relatively high genetic differentiation among the populations, and the value of Nm is 0.458 (Nm < 1), which suggests that the genetic divergence among the population may be caused by genetic drift or migration. These values were significant at $P < 0.048$ as indicated by the randomization test (Table 3). Similarly, the AMOVA performed against the germplasms of Central and Western Nepal (CWN) and Eastern Nepal (EN) showed a 40% variation among the population and a 60% variation among individuals. The value of Fst and Nm was 0.6, and 0.2, respectively. This value indicates a more significant differentiation between CWN and EN populations (Supplementary Table 1 and Supplementary Fig. 3).

**Table 2**
Genetic diversity indices of 15 SSR primers in 32 *paris polyphylla* germplasms.

| Primer name | Na | Ne | Ho | He | I | Ht | Nei | Fst | Nm | HWEP* |
|---|---|---|---|---|---|---|---|---|---|---|
| SSRPP1 | 4.00 | 2.50 | 0.72 | 0.61 | 1.09 | 0.36 | 0.60 | 0.40 | 0.37 | 0.003155 |
| pp1 | 3.00 | 1.98 | 0.81 | 0.50 | 0.74 | 0.41 | 0.49 | 0.18 | 1.15 | 0.000610 |
| pp2 | 5.00 | 3.09 | 0.59 | 0.69 | 1.25 | 0.30 | 0.68 | 0.56 | 0.20 | 0.000295 |
| pp3 | 2.00 | 1.91 | 0.78 | 0.48 | 0.67 | 0.39 | 0.48 | 0.18 | 1.14 | 0.000577 |
| pp4 | 3.00 | 1.07 | 0.06 | 0.06 | 0.16 | 0.03 | 0.06 | 0.49 | 0.26 | 0.000396 |
| pp5 | 3.00 | 1.47 | 0.16 | 0.33 | 0.61 | 0.08 | 0.32 | 0.76 | 0.08 | 0.999444 |
| pp6 | 2.00 | 1.44 | 0.00 | 0.31 | 0.48 | 0.00 | 0.30 | 1.00 | 0.00 | 0.000000 |
| pp7 | 4.00 | 2.08 | 0.88 | 0.53 | 0.82 | 0.44 | 0.52 | 0.16 | 1.36 | 0.000000 |
| pp19 | 3.00 | 1.98 | 0.75 | 0.50 | 0.74 | 0.38 | 0.49 | 0.24 | 0.78 | 0.004895 |
| pp20 | 3.00 | 2.05 | 0.53 | 0.52 | 0.76 | 0.27 | 0.51 | 0.48 | 0.27 | 0.025094 |
| PP21 | 5.00 | 3.58 | 0.97 | 0.73 | 1.42 | 0.48 | 0.72 | 0.33 | 0.51 | 0.750158 |
| PP22 | 3.00 | 1.98 | 0.78 | 0.50 | 0.74 | 0.39 | 0.49 | 0.21 | 0.94 | 0.000010 |
| PP23 | 3.00 | 2.43 | 0.97 | 0.60 | 0.96 | 0.48 | 0.59 | 0.18 | 1.16 | 0.004407 |
| PP24 | 9.00 | 5.04 | 0.84 | 0.81 | 1.79 | 0.42 | 0.80 | 0.47 | 0.28 | 0.000001 |
| pp25 | 8.00 | 3.51 | 0.63 | 0.73 | 1.55 | 0.31 | 0.71 | 0.56 | 0.19 | 0.000000 |
| Average | 4.00 | 2.41 | 0.63 | 0.53 | 0.92 | 0.32 | 0.52 | 0.41 | 0.58 | |

PIC: polymorphic information content, Na: total number of alleles, Ne: effective number of alleles, Ho: observed heterozygosity, He: expected heterozygosity, I: Shannon's information index, Ht: total heterozygosity, Nei: Nei's expected heterozygosity, Fst: fixation index, Nm: gene flow, HWEP: probability in Hardy–Weinberg equilibrium *Indicates that the p value is significant.

**Table 3**
Analysis of molecular variance (of 32 *Paris polyphylla* germplasm by ecological ranges).

| Source | Degree of freedom | Sum of square | Mean square | Estimated Variation | Variation % | Fst* | p value | Nm |
|---|---|---|---|---|---|---|---|---|
| Among Pops | 2 | 68.251 | 34.125 | 1.691 | 26% | 0.353 | <0.001 | 0.458 |
| Among Individuals | 29 | 42.343 | 1.460 | 0.000 | 0% | | | |
| Within Individuals | 32 | 151.500 | 4.734 | 4.734 | 74% | | | |
| Total | 51 | 173.481 | | 5.281 | 100% | | | |

Fst*: pairwise Fst, Nm: gene flow (calculated by (Nm) = [(1/FST) − 1]/4), .f. - degrees of freedom. *: Significance tests after 1000 permutations.

### 3.2.2. STRUCTURE analysis

Using the STRUCTURE program, SSR genotyped data of 32 *P. polyphylla* germplasms were used to perform the population structure analysis under an admixture model. The most likely number of clusters (K) was deduced by the peak ΔK found in the STRUCTURE analysis. The highest peak in ΔK revealed the best value for K = 2, which indicates the presence of two different populations (Fig. 3A). The cluster indicated by green consists of 8 germplasms collected from EN, and the cluster indicated by red consists of the rest of the 26 germplasms collected from CWN (Fig. 3B).

### 3.2.3. UPGMA phylogenetic tree

The Nei's genetic distance data obtained from SSR genotyping were applied to construct the UPGMA phylogenetic tree, where 32 germplasms were separated into two major groups: CL-I and CL-II (Fig. 4). CL-I includes germplasms collected from CWN. Six sub-clusters (CL-I1 to CL-I6) have been observed in this cluster. In the CL-I1 sub-cluster, five germplasms collected from Lamjung and Gorkha of WN were grouped. Similarly, seven germplasms collected from the Manang district of WN were grouped in CL-I2. Likewise, three germplasms – one of Manang, WN and each of Dolakha and Nuwakot of CN were gathered in CL-I3. In CL-I4, five germplasms were clustered, including two of Sindhupalchowk, each of Rasuwa, Ramechhap of CN and one germplasm of Kaski, WN. Likewise, three germplasms belonging to each of Dolakha, Nuwakot and Rasuwa of CN were included in CL-I5. In CL-I6, three germplasms of Kaski, WN were grouped. All accessions collected from EN were clustered in CL-II (Fig. 4). The clustering of germplasms in the UPGMA tree is in complete correspondence to STRUCTURE analysis (Fig. 3B).

### 3.2.4. PCoA analysis

In the PCoA analysis, the first three axes (1, 2, and 3) described the percentages of variation as 58, 10 and 8, respectively. Their corresponding cumulative percentage of variance is 58, 68, and 76. In the PCoA bi-plot, all the accessions of Eastern Nepal were separated to the right of the biplot (EN), and the accessions of Central and Western Nepal were isolated to the left of the bi-plot (CWN). In this CWN, the accessions of particular origin were again separated into two sub-clusters (CN), where germplasms of central Nepal (except 6 and 14) were gathered, and sub-cluster WN, where germplasms of the western region were clustered (Fig. 5). The distribution of germplasms in PCoA showed complete correspondence with STRUCTURE analysis (Fig. 3) and UPGMA (Fig. 4) phylogenetic tree.

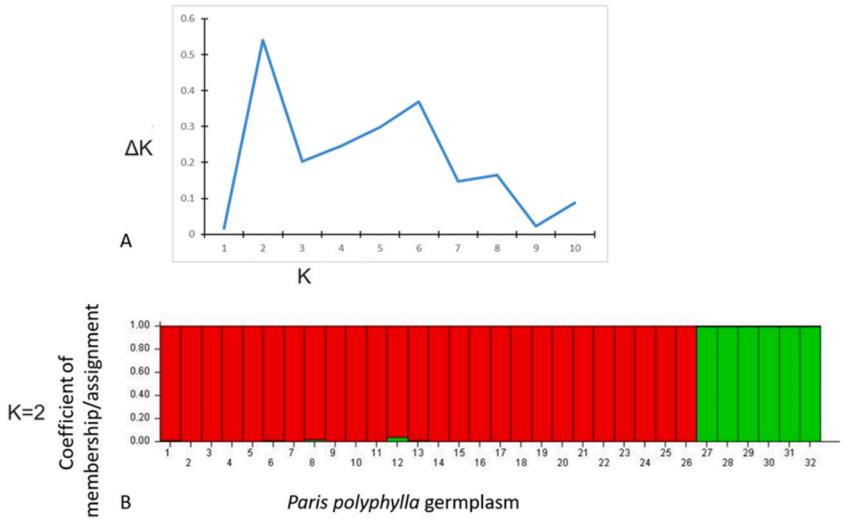


**Fig. 3.** Population structure of 32 *Paris polyphylla* germplasms representing three ecological ranges of Nepal constructed by 15 SSR genotyped data. (A) STRUCTURE plot representing subpopulation K = 2 (peak), obtained by Evano et al. (2005) method; (B) Bayesian model estimation of population structure, where, the cluster representing by red are the germplasms of Central and Western Nepal (CWN) and the cluster representing by green are the germplasms of Eastern Nepal (EN).

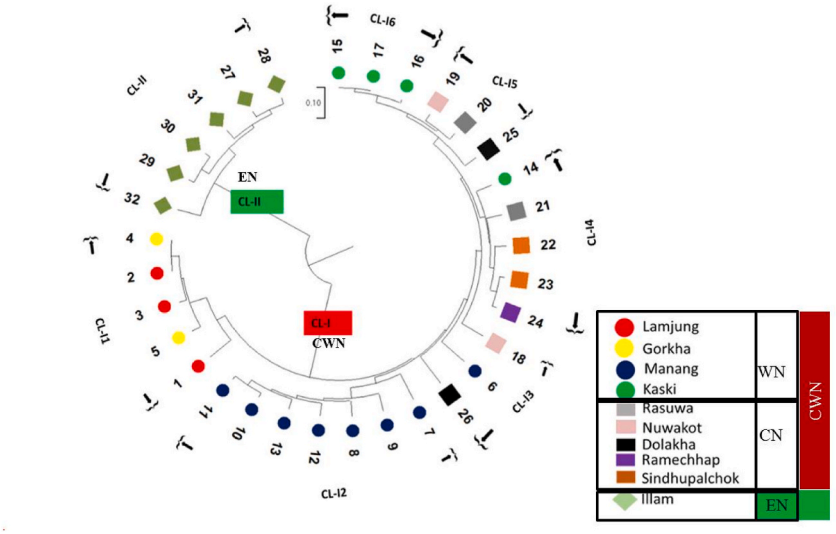


**Fig. 4.** UPGMA phylogenetic tree of 32 *P. polyphylla* germplasms constructed by using 15 SSR genotyped data.

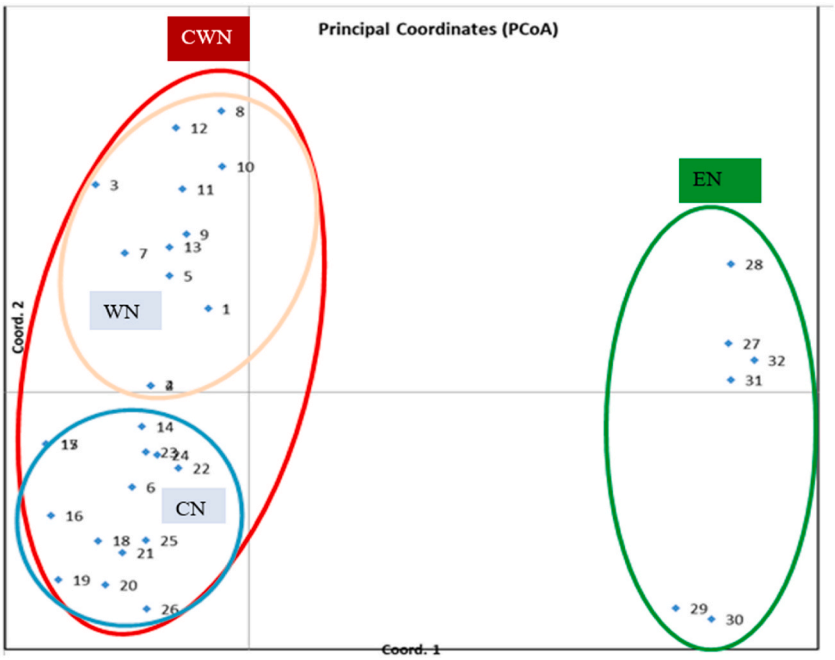


**Fig. 5.** Distribution of 32 *P. polyphylla* germplasms in separate clusters in a Principal coordinates analysis (PCoA) bi-plot constructed using Nei's genetic distance obtained from *15* SSR genotyped data.

## 4. Discussions

Study of genetic diversity and population structure is the foundation of conservation genetics and selective breeding programs. Such study at molecular level provides information on the evolutionary history, status of the species, identify populations at risk and implement conservation strategies to preserve their genetic variability. In the present study, 15 SSR primers provided reproducible polymorphic bands and a considerable level of genetic diversity and proved these SSR as a powerful and reliable molecular tool for analyzing the genetic diversity of *P. polyphylla* germplasms. The results of genetic diversity estimation and AMOVA indicated a low degree of differentiation among populations and a high degree of genetic diversity within individuals in a population. The population's genetic diversity is greatly affected by the breeding system, where outcrossing species tend to be more genetically diverse [42,61]. The genetic diversity in *P. polyphylla*, could be because of mixed mating (a combination of self and cross-pollination) characteristics [39,

62].

Many studies have been carried out about the genetic diversity of *P. polyphylla* and related species outside Nepal. EST-SSR markers that were developed from polyphyllin backbone biosynthesis of *P. polyphylla* var. yunnanensis detected a high level of genetic diversity in *P. polyphylla* var. yunnanensis that were collected from Yunnan Province, Southwest China [63]. EST-SSR primers detected a relatively high level of genetic diversity of *P. polyphylla* collected from the fields of Kunming City of Yunnan Province, China [39]. Using SSR primers, a low level (observed heterozygosity being 2 to 3 times less than the value of expected heterozygosity) of genetic diversity in *P. polyphylla* var. chinensis is reported in a sample collected from a natural population in the south of Anhui Province, China [64].

The genetic diversity obtained in the present study is greater than the diversity reported by Ref. [37] in 33 samples of *P. polyphylla* collected from Dabie Mountains using SRAP (H = 0.23, I = 0.37) and SCoT (H = 0.29, I = 0.45) primers. The study conducted using ISSR primer also finds a relatively higher genetic diversity of cultivated *P. polyphylla* var. *yunnanensis* than that of the natural population of Central and Eastern parts of Yunnan Province, China [36]. The result indicates that artificial planting was a realistic approach for preserving *P. polyphylla* var. yunnanensis germplasms. This variation in the genetic diversity of *P. polyphylla* could be accounted to the variation in the primer, cultivar, stochastic events such as genetic drift and inbreeding, anthropogenic activities, variation in the sample size, sampling distance, and the geographic origin of the sample [65]. The genetic structure in our study revealed that the germplasms distributed in Nepal's Himalayas are probably derived from two ancestral groups/gene pools. Eastern germplasms isolated from the Central and Western clusters suggest the obvious difference between the population of CWN and EN. This divergence in the population could be accounted for the spatial environmental (climate, temperature, rainfall, precipitation) heterogeneity which may impact adaptive gene frequencies and shape the genetic structure of neutral loci by affecting population dynamics [66,67].

Furthermore, between Central-Western and Eastern Nepal, many geographic barriers, such as the number of hills, mountains, and rivers, hinder gene flow by seed/pollen dispersal, wind, bird, grazers etc. These environmental and biotic factors related to reproduction, dispersal and survival are indisputably pivotal to genetic dynamics and affect the chlorotype distribution via the genetic draft, particularly for populations in Central-Western to Eastern Nepal. On the other hand, the low genetic distance and admixture of some accessions between Central and Western populations could be accounted for the proximity, overlapping geographic distribution, the possibility of migration and mixing of genetic material (by both biotic and abiotic factors), a high rate of gene flow among the populations and the sharing of large amounts of ancestral polymorphism [68].

The primary factor contributing to the apparent genetic differentiation among *P. polyphylla* populations could be the germplasm's geographic isolation, which hinders the exchange of genetic material. Moreover, it also suggests that the genotypes dispersed in a particular region maintained themselves as constant gene pools for many generations [69]. In line with our result, the geographical isolation of populations has been reported in medicinal plants *Monochasma savatieri* Franch. ex Maxim, using EST-SSR marker [70]. A narrow genetic base of the *P. polyphylla* germplasm of particular locations, including that of Manang, Illam, Lamjung, Gorkha, Kaski and Rasuwa, probably because of overharvest and destruction of the germplasms, high selection pressure for rhizome collection, over rooting of the plant before fruit maturity, the repeated use of the same origin parents for propagation and the resulting significant genetic erosion of the local *P. polyphylla* gene pool. In addition, plants synthesize metabolites to adapt to a continuously changing environment. In the case of *P. polyphylla*, the climatic and geographic variation may have affected the development of putative genes involved in the biosynthesis of novel therapeutic compounds, including polyphyllins [39,40,71]. Moreover, the various climatic conditions such as temperature, humidity, and soil may also have affected its genetic structure. The effect of the climatic factor was previously observed in the medicinal plant *Nilgirianthus ciliates* [72]. The impact of environmental factors causing a high genetic diversity of *Caragana microphylla* has also been reported [73]. The cultivars adapted to the local ecological environment could be the potent germplasm for commercializing *P. polyphylla* in that area.

## 5. Conclusion

In the present study, young leaves of *P. polyphylla* germplasms were collected from the natural habitats of Central, Eastern, and Western Nepal and genetic diversity and population structure were studied using SSR markers. This is the first report on genetic diversity and population structure analysis of *P. polyphylla* at the molecular level in Nepal. The study of the SSR primers showed a relatively high level of genetic polymorphism. The overall germplasms exhibited a low degree of differentiation among populations but maintained abundant genetic diversity among individuals. The germplasms of Eastern Nepal differ markedly from Central and Western Nepal. They indicate the presence of two gene pools and the impact of climatic and geographic attributes on the genetic diversity of this species. Furthermore, the clustering of the germplasm of closer geographic locations suggests a higher risk of extinction in the long run and the urgency of genetic resource management and conservation. The study concludes that there is an urgent need to identify the uniform and the potential *P. polyphylla* germplasm which is suitable for a particular geographic region and commercial cultivation for biomedical exploitation. This requires the stoppage of unscientific, and illigal harvesting, as well as formulation of conservation and cultivation strategies of *P. polyphylla* in Nepal.

## Author contribution statement

BKO: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper; LM: performed the experiment; BP: Conceived and designed the experiments.

## Data availability statement

Data will be made available on request.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e18230.

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