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# Genetic diversity of *Epimedium elatum* (Morren & Decne) revealed by RAPD characterization

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## ABSTRACT

*Epimedium elatum* (Morren & Decne) is a rare perennial monotypic medicinal herb of Berberidaceae family, endemic to high altitude shady coniferous forests of Northwestern Himalayas in India. Traditionally, it has been used in local health care system for treatment of many ailments. In the present study, a total of ten RAPD markers were used for assessment of genetic diversity in twenty accessions of *E. elatum*, collected from diverse ecozones in Jammu and Kashmir. Ten primers amplified a total of 100 polymorphic bands with average of 10 bands per assay unit. Primer OPO-07 generated maximum number of bands (15) & produced highest polymorphic information content (0.39), marker index (5.96) and resolving power (8.9), thereby distinguishing *E. elatum* genotypes. The study showed moderate level of genetic polymorphism in twenty accessions of *E. elatum*. RAPD data set revealed association between genetic diversity and eco-geographic distribution of most *E. elatum* genotypes, and thereby proved useful tool for their genetic/molecular characterization. There is an urgent need for developing quick conservation strategies to save its natural germplasm from extinction in the Northwestern Himalayas.

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**KEYWORDS:** *Epimedium elatum*, icariin, accession, northwestern himalayas, genetic diversity

## INTRODUCTION

*Epimedium elatum* (Morren & Decne) is a perennial medicinal herb (Fig.1), endemic to high altitude shady coniferous forests of Northwestern Himalayas in India and Pakistan [1-3]. This species was first reported from Kashmir Himalayas by European botanists [4-7] during their botanical expeditions in India. The plant has characteristic 'three branches and nine leaves' morphological pattern, common to most Epimedium species. Traditionally, it has been used in treating many ailments like toothaches, bone-joint and kidney disorders [8]. *E. elatum* contains significant amount of key phytochemicals (Fig. 2) like epimedin B, epimedin C, icariin and icariside-I [8-11], which are known to possess key pharmaceutical properties like phosphodiesterase-5 inhibition (aphrodisiac role), anti-osteoporosis, anti-oxidant and anticancer activities [12]. Previously, extracts or chemical components from *E. elatum* have been assessed for antioxidant [3 and 10] antimicrobial and anti-diabetic activities [13]. However, genetic diversity of *E. elatum* was unreported and determination of same is essential for conserving rare medicinal plants [14-17].

Genetic diversity assessments bring forth genetic polymorphism existing in a population. It may or may not bring about changes in the morphological, biochemical, physiological or DNA sequences [14]. These genetic changes may be monitored by the presence or absence of an allele at a locus. New alleles can appear at each generation by spontaneous mutation due to DNA replication errors or mutagen-induced DNA damage or recombination events [15]. Knowledge of genetic diversity in a population helps in better adaptability of a species. For assessing, preserving and cataloguing a population, its genetic assessment is pivotal [16]. An understanding about genetic diversity patterns is important in estimating evolutionary pathways and in predicting future risk [17]. The process becomes more pertinent in case of endangered and rare species, as it helps in prioritization of sites and man-agement choices for future conservation programmes. Molecular markers are important in assessing genetic diversity at the gene level in determining allele frequencies to nucleotide

level [18]. Literature reports that RAPD molecular markers have been used for determining the genetic diversity in *Epimedium* species [19-23]. Assessment of genetic diversity in *E. elatum* was hitherto unreported and hence present study investigated same for identification of its elite genotypes and their subsequent conservation by concerned stakeholders involved in medicinal plant research in North India.

## MATERIALS AND METHODS

### Study Area and Experimenting Material

The climate of Kashmir Himalayas in Jammu & Kashmir State of India ranges between temperate and sub-alpine and it lies between 33°20–34°54'N latitudes and 73°55'–75°3' E longitudes in Northwestern Himalayas. It is home to rich and diverse medicinal plants, known globally for their pharmaceutical properties. Based on reported locations in floras, herbarium records, research articles and internet searches, extensive investigation was made in Kashmir Himalayas. The planting material was collected from twenty different ecozones and kept in ziplock bags at low temperature in laboratory for DNA extraction. The extracted DNA was stored at -80°C until PCR optimization with RAPD markers. All twenty accessions were identified by taxonomists at Kashmir University herbarium (KASH) & CSIR-IIIM Jammu herbarium (RRLH) respectively. The altitude of the representative sites ranged from 1800 m to 3400 m asl and geographical distance ranged between 5.5 km to 250 km (Table.1).

**Figure1:** *Epimedium elatum* (Morr & Decne.), unexplored medicinal herb of Northwestern Himalayas in India

### DNA Extraction for Molecular Characterization

Fresh young leaves were subjected to the several DNA extraction protocols for high quality DNA. Two extraction protocols with some modifications influenced the yield and quality of genomic DNA from the leaves of *E. elatum* viz; CTAB based method of Doyle & Doyle (1987) and Porebski et al (2009) [24, 25]. The quality of isolated DNA was checked on agar-ose gel and quantified spectroscopically by measuring light absorption at 260 nm. Nucleic acid purity was checked by determining absorption at a wavelength of 230 and 280 nm, respectively and concentration of DNA was determined in terms of ng/$\mu$l for twenty accessions (Table 2). The deviation from the value of 1.80 at A260/A280 nm absorbance ratio was used as an indicator of RNA and protein contamination in the extracted genomic DNA.

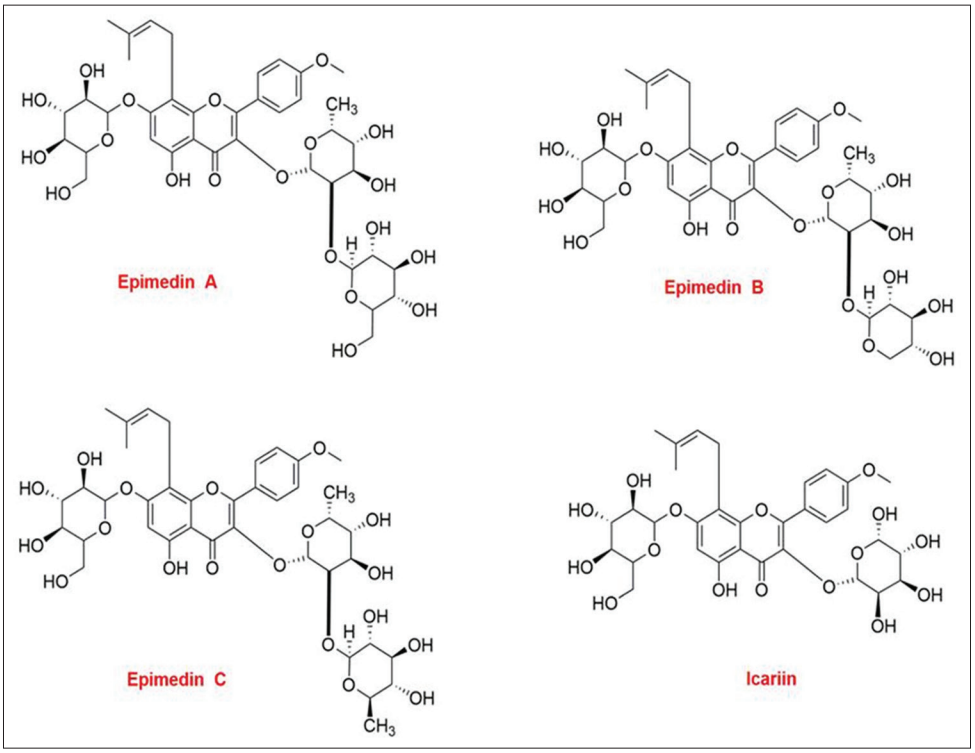


**Figure 2:** Four major chemotaxonomic markers found in *E. elatum*

Table 1: Passport data of ***Epimedium elatum*** collected from Kashmir Himalayas

| S.No | Collection Site | Code | Latitude | Longitude | RRLH# | KASH$ | District |
|---|---|---|---|---|---|---|---|
| 1 | Gulmarg | GL | 34.02° | 74.22° | 22304 | 2094 | Baramulla |
| 2 | Babareshi | BR | 34.03° | 74.23° | 22306 | 2099 | Baramulla |
| 3 | Drang | DR | 33.55° | 74.29° | 22305 | 2105 | Baramulla |
| 4 | Dangarpora | DG | 34.05° | 74.32° | 22308 | 2103 | Baramulla |
| 5 | Boniyar | BY | 34.15° | 74.21° | 22307 | 2107 | Baramulla |
| 6 | Yusmarg | YS | 33.49° | 74.40° | 22310 | 2101 | Budgam |
| 7 | Dodipathri | DP | 33.53° | 74.34° | 22309 | 2095 | Budgam |
| 8 | Naranag | NAR | 34.21° | 74.58° | 22303 | 2102 | Ganderbal |
| 9 | Gagangir | GG | 34.17° | 75.12° | 22314 | 2109 | Ganderbal |
| 10 | Dachigam | DGM | 34.08° | 75.02° | 22312 | 2100 | Srinagar |
| 11 | Pahalgam | PGM | 34.00° | 75.18° | 22319 | 2093 | Anantnag |
| 12 | Kokernag | KNG | 33.34° | 75.17° | 22311 | 2092 | Anantnag |
| 13 | Verinag | VNG | 33.32° | 75.14° | 22320 | 2108 | Anantnag |
| 14 | Khillanmarg | KMG | 34.02° | 74.21° | 22315 | 2104 | Baramulla |
| 15 | Chaknala | CNG | 34.37° | 74.51° | 22316 | 2097 | Bandipora |
| 16 | Sheikhpora | SPG | 34.35° | 74.59° | 22317 | 2096 | Bandipora |
| 17 | Kanzalwan | KZG | 34.38° | 74.42° | 22318 | 2098 | Bandipora |
| 18 | Badwan | BDG | 33.39° | 74.46° | 22321 | 2106 | Bandipora |
| 19 | Hirpora | HP | 33.39° | 74.57° | 22322 | 2090 | Shopian |
| 20 | Aharbal | AB | 33.38° | 74.74° | 22313 | 2091 | Kulgam |

#Acronym for CSIR-IIIM Jammu herbarium, $Acronym for Kashmir University herbarium

## PCR Optimization

For the successful amplification of RAPD markers in *Epimedium elatum*, different PCR conditions and cycling parameters were tested. The concentration of various components of master mix such as primer, dNTPs, 10x Taq buffer, Taq DNA polymerase, and the template were optimized for PCR analysis. A total of 10 RAPD primers were screened based on the primer set published by the Biotechnology Laboratory, University of British Columbia, Canada (UBC) and the RAPD studies on *Podophyllum hexandrum* [26-29].

The amplifications were performed in a thermal cycler (ABI Geneamp 9700, Thermo Scientific, USA) and for RAPD fingerprinting; annealing temperature was also standardized for every primer. The amplification was carried out in a reaction volume of 20$\mu$l with standardized conditions. Each primer was amplified in triplicate to confirm reproducibility. PCR was carried out for 35 cycles, under the following conditions: 95°C for 5 min, 92°C for 60 sec, (screening) 32-34°C for 1 min, 72°C for 2 min, 72°C for 7 min and 35 cycles.

The amplified PCR product were separated on 1.4% (w/v) agarose gel with 1X TAE buffer and stained with ethidium bromide (0.5$\mu$g/ml, Sigma, USA). Electrophoresis was carried out till all the amplified DNA fragments were clearly resolved. Fermentas Gene Ruler™ (100 bp plus ladder) was used marker to determine the size of the amplified products. DNA fragments were visualized under UV light and documented with the gel documentation imaging system (GelDoc- Syngene Bio imaging System UK.).

## Scoring of Bands and Data Analysis

The PCR-amplified fragments were scored in a binary quantitative matrix as 1 (present) and 0 (absent) for all the primers. Weak bands of negligible intensity and smeared bands were excluded. The genetic diversity parameters like percentage of polymorphism,

Table 2: Quantification of DNA concentration by UV spectrophotometry (Nanodrop)

| S.No | Accessions | Code | Conc ng/ $\mu$l | 260/280 | 260/230 |
|---|---|---|---|---|---|
| 1 | Gulmarg | GL | 1386 | 1.97 | 1.37 |
| 2 | Babareshi | BR | 514 | 1.81 | 1.42 |
| 3 | Drang | DR | 778 | 1.88 | 1.90 |
| 4 | Dangarpora | DG | 640 | 1.74 | 1.26 |
| 5 | Boniyar | BY-12 | 146 | 1.81 | 1.61 |
| 6 | Yusmarg | YS | 995 | 1.87 | 1.63 |
| 7 | Dodipathri | DP | 1779 | 1.82 | 1.32 |
| 8 | Naranag | NAR | 657 | 1.88 | 1.59 |
| 9 | Gagangir | GG | 936 | 1.80 | 1.59 |
| 10 | Dachigam | DGM | 326 | 1.98 | 1.09 |
| 11 | Pahalgam | PGM | 1609 | 1.91 | 1.63 |
| 12 | Kokernag | KNG | 827 | 1.92 | 1.69 |
| 13 | Verinag | VNG | 780 | 1.80 | 1.12 |
| 14 | Khillanmarg | KMG | 994 | 1.93 | 1.65 |
| 15 | Chaknala | CNG | 609 | 1.88 | 1.00 |
| 16 | Sheikhpora | SPG | 452 | 1.80 | 0.96 |
| 17 | Kanzalwan | KZG-3 | 219 | 1.78 | 1.15 |
| 18 | Badwan | BDG | 825 | 1.74 | 0.39 |
| 19 | Hirpora | HP | 1635 | 1.88 | 1.37 |
| 20 | Aharbal | AB | 1132 | 1.90 | 0.96 |

total number of bands (TB) amplified/primer; total numbers of polymorphic bands, total number of monomorphic bands were calculated for all the 10 primers. Besides, genotypic data was assessed for polymorphic information content [30], marker index [31] and resolving power [32].The scored binary data generated was used for the construction of dendrogram by Jaccard's similarity coefficient using NTSYS- pc version 2.02e [33].

# RESULTS AND DISCUSSION

## Collection of *Epimedium Elatum* Genotypes from Diverse Eco-regions

Distribution of *E. elatum* was poorly known in Kashmir Himalayas due to lack of research on this medicinal plant.

Surveying of the Berberidaceace accessions in herbarium of Kashmir University (KASH) provided essential clues for working out its distributional range in valley. Only two specimens of *E. elatum* were deposited by curator A.R Naqshi around 1970s. He had collected them from Harwan forests. In late 1980s, a few Indian Botanists like Gurcharan Singh reported and documented its existence in '*Forest flora of Srinagar and plants of neighbourhood*'. And some had documented its location at Pahalgam ecozone [34, 35]. But, overall, distributional data on *E. elatum* was inadequate and documentation of same was needed for developing conservational strategies. In this connection, we surveyed 30 locations in Kashmir Himalayas and collected its accessions from twenty geographical areas (Fig.3). We surveyed 8 districts out of 10 in the valley. Most of the populations of *E. elatum* were facing severe threats and had small population size [37]. Therefore, this plant needs immediate protection as there are chances of its extinction from many surveyed areas in the Kashmir Himalayas, India.

## Modified DNA Extraction Protocol

Initially, it was difficult to get good quality DNA for RAPD fingerprinting studies in *E. elatum* genotypes. We tried several DNA extraction protocols and among them, we selected two protocols [24, 25] after certain modifications to get quality DNA, prerequisite for DNA fingerprinting studies [36].

## Genetic Diversity Characterization by RAPD Markers in *E.elatum* Genotypes

In the present study, a total of ten RAPD markers were used for assessment of genetic diversity in twenty accessions of *E. elatum*. Results obtained are summarized in Table 3. These ten primers amplified a total of 100 polymorphic bands with average of 10 bands per assay unit or marker used. Size of the amplified DNA fragments scored, ranged from 450 to 1600 bp and allele size was estimated visually by comparing with 100 bp plus ladder (Fermentas) on a gel (Fig.4). The largest numbers of alleles were amplified by OPO-07 (15), whereas three primers viz; OPO-01, OPO-06 & OPO-10 produced minimum of 07 bands each. In order to detect the most effective primers that could distinguish all the cultivars either individually or in combination, three different indices like Polymorphic Information Content (PIC), Markers Index (MI) and Resolving Power (RP) were pragmatic in the present study (Table 3). The utility of a specified marker is ascertained by the level of polymorphism, and its ability to detect multiple polymorphisms [31]. Marker index is one such genetic diversity index of a marker which reveals the discriminatory power of a marker.

Among all the RAPD primers assessed, OPO-07 showed highest MI (5.96), whereas OPO-04 showed lowest MI values (2.05).

Table 3: Genetic diversity parameters for ten RAPD primers used for molecular characterization of twenty accessions of *E. elatum*

| S.No | Primers | Sequence | TNB | NMB | NPB | PPB | PIC 2fi (1-fi) | MI | Rp |
|---|---|---|---|---|---|---|---|---|---|
| 1 | OPO-01 | GGCACGTAAG | 07 | 0 | 07 | 100 | 0.29 | 2.11 | 2.8 |
| 2 | OPO-02 | ACGTAGCGTC | 10 | 0 | 10 | 100 | 0.23 | 3.67 | 4.5 |
| 3 | OPO-03 | CTGTTGCTAC | 08 | 0 | 08 | 100 | 0.28 | 2.64 | 2.7 |
| 4 | OPO-04 | AAGTCCGCTC | 11 | 0 | 11 | 100 | 0.25 | 2.11 | 4.5 |
| 5 | OPO-05 | CCCAGTCACT | 14 | 0 | 14 | 100 | 0.19 | 2.28 | 3.3 |
| 6 | OPO-06 | CCACGGGAAG | 07 | 0 | 07 | 100 | 0.29 | 2.94 | 2.5 |
| 7 | OPO-07 | CAGCACTGAC | 15 | 0 | 15 | 100 | 0.39 | 5.96 | 8.9 |
| 8 | OPO-08 | CCTCCAGTGT | 12 | 0 | 12 | 100 | 0.28 | 2.33 | 5.3 |
| 9 | OPO-09 | TCCCACGCAA | 09 | 0 | 09 | 100 | 0.29 | 2.60 | 3.2 |
| 10 | OPO-10 | TCAGAGCGCC | 07 | 0 | 07 | 100 | 0.22 | 2.39 | 2.8 |

TNB: total number of bands, NPB: number of polymorphic bands, NMB: number of monomorphic bands, PPB: percentage of polymorphic bands, PIC: polymorphism information content, MI: marker index, Rp: resolving power

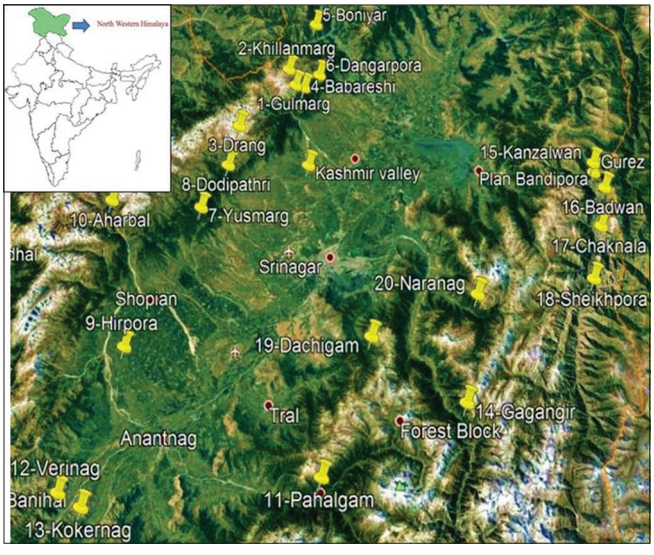


**Figure 3:** Distributional map of twenty accessions of *E.elatum* collected from diverse ecogeographical regions of Kashmir Himalayas in India

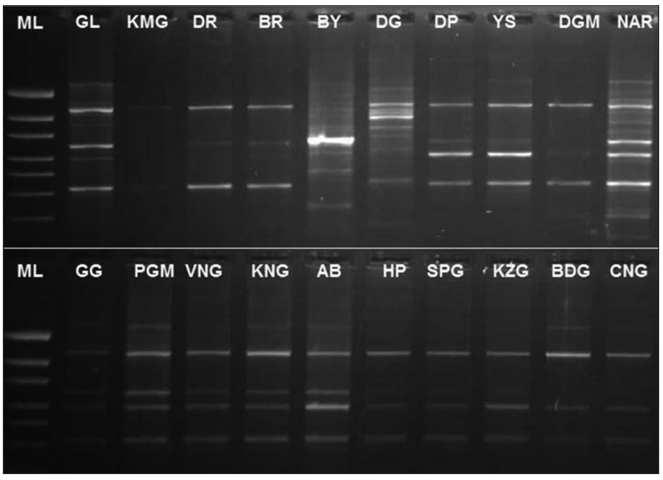


**Figure 4:** Results of PCR amplification of OPO-05 primer in twenty *E.elatum* genotypes via RAPD fingerprinting. {M: DNA ladder (100bp plus}. [Position of gel lanes (1-20) showing twenty accessions; 1-GL (Gulmarg); 2-KMG (Khillanmarg); 3-DR (Drang); 4-BR (Babareshi); 5-BY (Boniyar); 7-DG (Dangarpora); 7-; (Dodipathri) 8-YS (Yusmarg); 9-DGM (Dachigam); 10-NAR (Naranag); 11-GG (Gagangir); 12-PGM (Pahalgam); 13-VNG (Verinag); 14-KNG (Kokernag); 15-AB (Aharbal) 16-HP (Hirpora) 17-SPG (Sheikhpora); 18-KZG (Kanzalwan); 19-BDG (Badwan); 20-CNG (Checknala)]

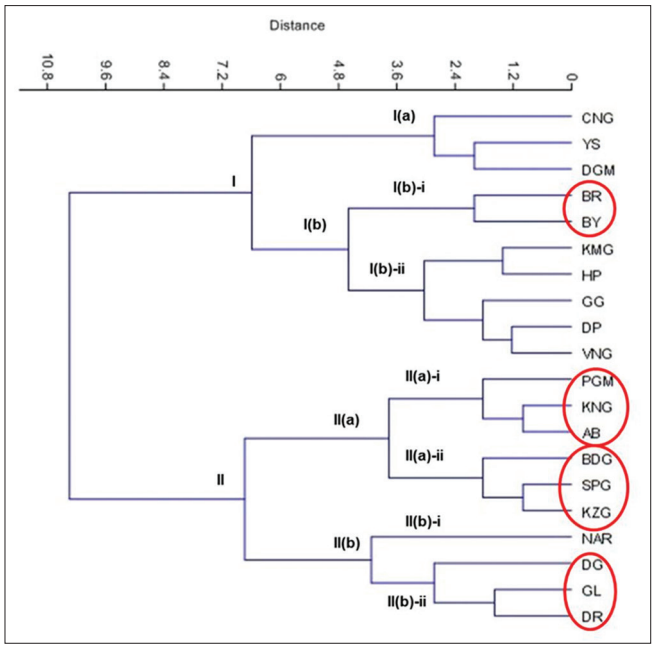


**Figure 5:** Phylogenetic tree based on RAPD data in twenty accessions of *E. elatum* using UPGMA analysis. (CNG-Checknala Gurez; YS-Yusmarg; DGM-Dachigam; BR- Babareshi BY-Boniyar; KMG-Khillanmarg; HP-Hirpora; GG-Gagangir; DP-Dodipathri; VNG-Verinag; PGM-Pahalgam; KNG-Kokernag; AB-Aharbal; BDG-Badwan Gurez; SPG-Sheikhpora Gurez; KZG-Kanzalwan Gurez;; DR-Drang; NAR-Naranag; DG-Dangarpora; GL-Gulmarg; DR-Drang)

OPO-07 primer showed highest PIC (0.39) values, whereas, OPO-05 produced lowest PIC value (0.19). Highest resolving power was observed for primer OPO-07 (8.9), whereas, lowest value was found in OPO-03 primer (2.7). The average values for each of these genetic diversity parametres were found to be 0.27 (PIC), 2.89 (MI) and 4.05 (Rp) in our study.

All genetic diversity values obtained were lower than our earlier results [3], employing ISSR primers for analysing genetic diversity of *E. elatum*. On comparing the two marker systems, it was observed that the average number of bands amplified by ISSR fingerprinting was 17.5/primer as compared to RAPD (10). Comparatively, ISSR analysis provided better results than RAPD characterization. However, one RAPD primer OPO-07 was able to distinguish most genotypes of *E. elatum* at genetic level effectively. The average value of PIC (0.36) and Rp (7.2) were also higher in ISSR marker system than in RAPD. Our study was in confirmation with many earlier RAPD studies on *Epimedium* species. Nakai et al. [19] had shown that *Epimedium* species can be genetically distinguished on the basis of unique RAPD fingerprint. In related studies, RAPD markers aided in solving phylogenetic relationship of *Epimedium* species by establishing unique fingerprints and consequently showing high genetic diversity [21,22]. Both RAPD & ISSR molecular markers assisted in the easy genotype characterization of *E. elatum*. These markers showed moderate to high genetic diversity existing in its different populations.

### RAPD based Genetic Relationship among Genotypes

The genetic relationship between the twenty wild *E. elatum* accessions was determined by clustering the genotypes using UPGMA based dendrogram, which phylogenetically classified twenty accessions into two broad clusters (I & II) with each cluster having ten accessions (Fig.5). Cluster I was further sub-divided into two sub-clusters I(a) & I(b), with former cluster grouping three accessions (CNG, YS & DGM) and latter clustering seven accessions in two sub-groups [I(b)-i & I(b)-ii]. Cluster II was further subdivided into II (a) and II (b) with two sub-clusters [II (b)-i &II (b)-ii]. Correlation was observed between genotype clustering & geographical distribution of eleven *E. elatum* accessions. For example, genotypes collected from Gurez clustered together in dendrogram. Our results are in line with earlier RAPD studies in *Epimedium* species, where geographic distribution was correlated with dendrogram topology [21-23]. RAPD markers used in the present study revealed representation of the genetic and geographic relationship for most of the *E. elatum* genotypes. Efficient molecular markers are needed to throw more light on overall genetic characterization of *E. elatum* in Kashmir Himalayas in India.

## CONCLUSIONS

*Epimedium elatum* was a neglected species before we initiated a bioprospection of this plant. Recent literature has shown its medicinal potential at par with the officially recognized ingredients of *Herba Epimedii*, commonly known as Herbal Viagra [38]. The plant has great potential for several future medicinal uses because of its reported pharmacological activities (9, 10, & 13). To get the best efficacy of an herbal product the plant needs to be authenticated. Both RAPD & ISSR molecular techniques have shown their potential in distinguishing and identifying *E. elatum* genotypes. They have provided useful information regarding the level of genetic polymorphism in *E. elatum* collected from geographically diverse regions. Both the marker systems have comparable accuracy in grouping genotypes according to their geographical area. This study will provide valuable evidence for decision making in choosing of markers for future work, characterization of germplasm, breeding of *E. elatum* germplasm management. This plant needs immediate *ex-situ* and *in-situ* conservation in Northwestern Himalayas in India.

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## COMPETING INTERESTS

The authors declare that they have no competing interests.

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