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BRIEF COMMUNICATION

Analysis of genetic diversity among *Swertia chirayita* genotypes

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Abstract

Inter simple sequence repeat (ISSR) markers were used to analyse genetic diversity of *Swertia chirayita* genotypes collected from the temperate Himalayas of India. Allied species of *Swertia chirayita* were used in the study as outliers. Nineteen UBC primers generated a total of 315 ISSR bands, revealing 98.7 % polymorphism among the genotypes assayed. This was reduced to 42.5 % when the outliers were excluded. The results revealed a high genetic diversity within the genotypes.

Additional key words: biodiversity, inter simple sequence repeat markers, medicinal plants.

Swertia chirayita is a medicinally important herb of family *Gentianaceae*. Owing to its medicinal importance, the plant has been harvested from the wild for too long and is now considered endangered in its natural habitats, resulting in a need for conservation. Genetic erosion of the species has also been attributed to destruction of natural habitat of the species by human intervention. Therefore, there is an urgent need to catalogue and preserve the existing diversity of *Swertia chirayita*. Genetic diversity studies aid in devising pragmatic conservation strategies and in formulating core collections for endangered plant species as one is able to identify regions representing maximum diversity and hence can conserve the same with least population size (Shanker and Ganeshaiah 1997, Rao and Hodgkin 2002). In genus *Swertia,* morphological, biochemical (Karan *et al.* 1997, Bhatia *et al.* 2003) and isozyme markers (Verma and Kumar 2001) have been used for demarcating different species. However, of late, molecular markers are routinely employed for genetic diversity studies in different plant systems (Frediani and Caputo 2005, Zhang *et al.* 2005) as they are potentially the most powerful tools to attain this objective. Further, the use of molecular markers is preferred over conventional morphological and biochemical markers for genetic diversity studies, since they are not influenced by environmental effects and the developmental stages of the experimental material.

In the present study inter simple sequence repeat (ISSR) marker assay was employed because it provides the ease of rapid production of reproducible amplicons as compared to random amplified polymorphic DNA (RAPD, Wu *et al.* 2004). The effectiveness of ISSR markers in genome analysis has been reported in many plant species (Blair *et al.* 1999, McGregor *et al.* 2000).

The germplasm for our study was collected from Mandal (30° 4'N, 79° 1'E), which represents one of the few surviving natural populations of *Swertia chirayita* in temperate Himalayas of India. Each sample in the study was represented by one individual plant and is referred with a specific genotype code. The data set consisted of 13 genotypes (coded A to M) of *Swertia chirayita* collected from Mandal region of Uttaranchal along with two genotypes each of *Swertia cordata* (coded N and O), *S. paniculata* (P and Q) and *S. purpurascens* (R and S), which were used as outliers in the study. The leaves of the plant material collected were lyophilised and ground to fine powder with silica beads. DNA extraction was carried out based on the protocol by Doyle and Doyle (1990).

PCR amplifications were performed in 0.025 cm^3 volume of reaction mix containing 25 - 50 ng of total genomic DNA, 1× PCR buffer, 2 mM $MgCl_2$, 0.4 mM dNTPs, 0.4 μM primer and 1 unit *Taq* (*Biotools*, Madrid, Spain). The PCR cycle consisted of an initial denaturation step at 94 °C for 5 min, 35 cycles comprising denaturation

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Abbreviations: ISSR - inter simple sequence repeat; PCR - polymerase chain reaction.
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Table 1. List of primers used in the present study and the amplifications produced.

Primers	Primer sequence	Number of bands amplified with outliers		without outliers	
		total	polymorphic	total	polymorphic
UBC 807	AGA GAG AGA GAG AGA GT	14	13	3	1
UBC 809	AGA GAG AGA GAG AGA GG	15	15	7	3
UBC 810	GAG AGA GAG AGA GAG AT	14	14	12	8
UBC 811	GAG AGA GAG AGA GAG AC	16	16	12	7
UBC 812	GAG AGA GAG AGA GAG AA	18	18	9	6
UBC 817	CAC ACA CAC ACA CAC AA	19	19	7	1
UBC 818	CAC ACA CAC ACA CAC AG	19	19	11	4
UBC 819	GTG TGT GTG TGT GTG TA	13	13	6	4
UBC 820	GTG TGT GTG TGT GTG TC	16	16	5	4
UBC 830	TGT GTG TGT GTG TGT GG	16	16	7	3
UBC 836	AGA GAG AGA GAG AGA GYA	20	19	16	5
UBC 840	GAG AGA GAG AGA GAG AYT	21	21	10	2
UBC 841	GAG AGA GAG AGA GAG AYC	18	17	13	7
UBC 842	GAG AGA GAG AGA GAG AYC	13	13	3	0
UBC 848	CAC ACA CAC ACA CAC ARG	12	12	7	4
UBC 850	GTG TGT GTG TGT GTG TYC	21	20	11	5
UBC 857	ACA CAC ACA CAC ACA CYG	21	21	12	2
UBC 860	TGT GTG TGT GTG TGT GRA	12	12	6	0
UBC 868	GAA GAA GAA GAA GAA GAA	17	17	5	3
Total number of bands		315	311	162	69

cycle at 94 °C for 30 s; extension cycle at 45 °C for 45 s and annealing at 72 °C for 1 min and a final extension step at 72 °C for 7 min. PCR amplifications were performed on a *Perkin Elmer* (Cetus, USA) DNA thermocycler. Amplification products were separated on 1.5 % agarose (*United States Biochemicals*, Cleveland, Ohio, USA) gel using 0.5× Tris-Borate EDTA buffer and stained with ethidium bromide. The gels were photographed under UV using a gel documentation system (*UVItec*, Cambridge, UK).

The ISSR amplification products within a range of 200 bp to 2.0 kb, were scored as 1 or 0 indicating the presence or absence of bands, respectively, across the samples analysed. The binary data, thus obtained, was used to estimate genetic similarity using Jaccard's coefficient (Jaccard 1908). The above-mentioned analysis was performed using *NTSYS-pc* (2.02 k version) software (Rohlf 1998). Bootstrap analysis (*WINBOOT* software, Yap and Nelson 1996) was carried out to find the statistical support for internal branches of the UPGMA tree.

A set of thirty-three UBC primers was screened with four *Swertia chirayita* samples and of these, 19 primers were finally employed. A total of 315 amplification products, averaging to 16.5 bands per primer, were obtained. This is a sufficiently good number to analyse genetic diversity as is supported by other studies in literature. Nagaoka and Ogihara (1997) scored a total of 260, 300 and 350 bands in diploid, tetraploid and hexaploid wheat germplasm, respectively, whereas Gilbert *et al.* (1999) scored 137 ISSR markers in 37 accessions of lupin germplasm. Ruas *et al.* (2003) observed that 200 RAPD markers were sufficient for dendrogram stability while assessing genetic diversity of *Coffea* species.

The total number of bands produced per primer ranged from 21 (primers UBC 840, UBC 850, UBC 857), to 7 (UBC 848 and UBC 860). Fig. 1 illustrates the representative amplification profiles obtained with primers UBC 809 and UBC 818, respectively. UBC 809 amplified a total of 15 scorable bands within the molecular range of 300 bp to 1600 bp. Of the seven bands amplified within *S. chirayita* genotypes, four bands were monomorphic. The rest of the bands were polymorphic within the *S. chirata* population and also among the outliers. UBC 818 enabled scoring of 19 bands in total (Fig. 1*B*). Eleven bands were scored for the *S. chirayita* genotypes A to M; of these seven were monomorphic.

A high percentage of polymorphic bands (98.73 %) were produced among the genotypes used. However, on exclusion of the bands obtained in outlier species, the percent polymorphism was reduced to 42.59. This indicates that the outliers contributed substantial polymorphism. Among the *Swertia chirayita* genotypes, coded A to M, primer UBC 819 generated a maximum of 80 % polymorphism. However, the primers UBC 842 and UBC 860 could not detect any polymorphism within these samples (Table 1).

Considering the prevalent view that rare species are genetically not very diverse and the fact that *S. chirayita* is a rare and endangered species, the high level of variations (42.59 %) revealed within its population is quite unexpected. This can be mainly attributed to two reasons. First, the mating model of a given plant species

is known to influence the levels of heterozygosity maintained in natural populations. Evidently, cross-pollinating species maintain higher herterozygosity as compared to self-pollinating plant species. It can be reasoned that since each individual is derived through genetic recombination, it is considered to be genotypically unique, thus contributing to the high levels of polymorphism within the population (Weising *et al.* 1995). *S. chirayita* is a cross-pollinator; bees being thepredominant pollinators of the species (Khoshoo and Tandon 1963). This explains the broad genetic base observed in the *S. chirata* population.

The high levels of polymorphism detected can also be attributed to the type of marker employed (Powell *et al.* 1996). High levels of genetic diversity have been revealed by ISSR marker assay in many plant systems.

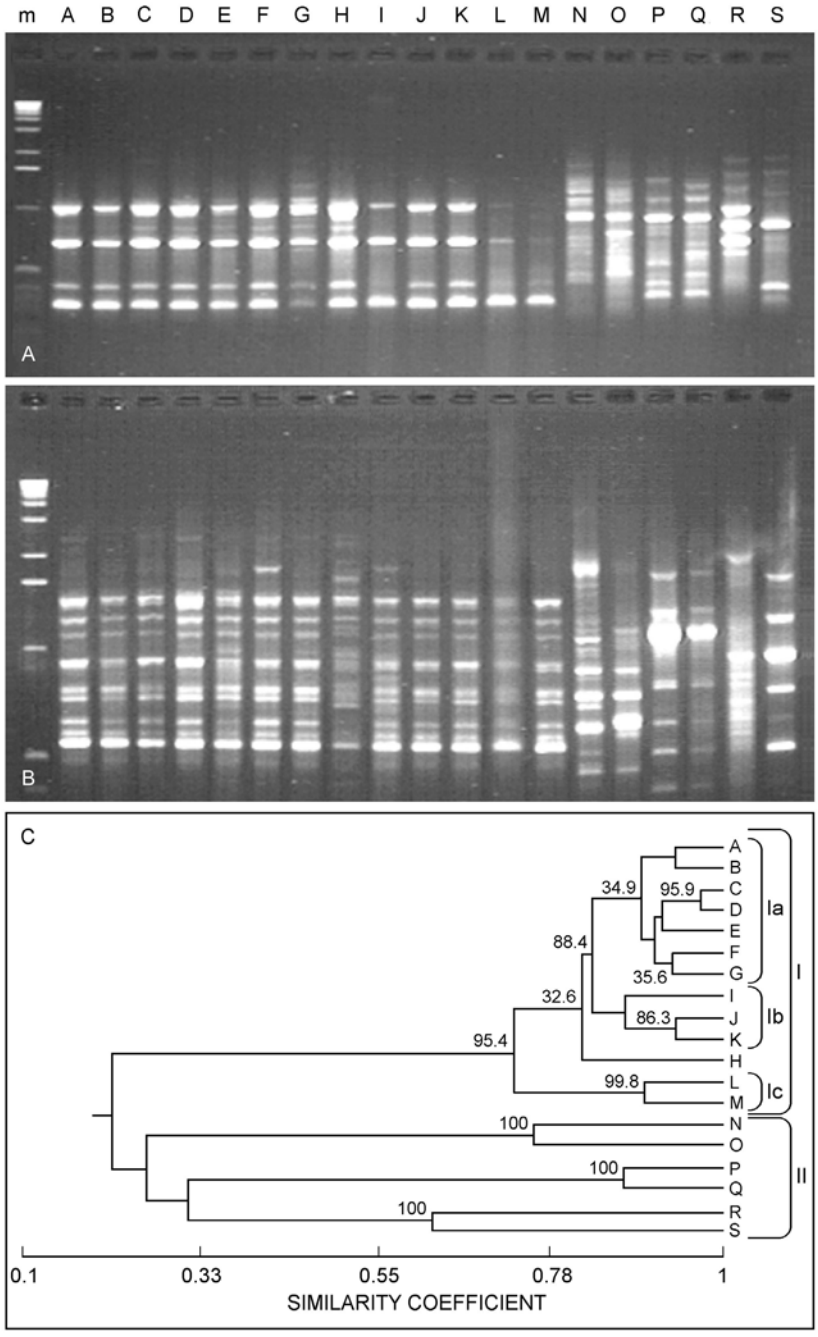

Fig. 1. ISSR fingerprint generated on employing primer UBC 809 (*A*) and UBC 818 (*B*), respectively. *Lanes A* to *M* represent genotypes of *Swertia chirayita; lanes N* and *O* represent the genotypes of *S. cordata*, *P* and *Q* those of *S. paniculata,* and *R* and *S* represent *S. purpurascens* genotypes. *Lane m* represents the marker lane. *C* - Dendrogram representing the UPGMA based clustering obtained among a population of *Swertia chirayita* of Mandal region. The similarity matrix was obtained using Jaccard's coefficient from 315 ISSR marker bands. The values at the nodes denote the percentage bootstrap values.

For instance, in *Trigonella foenum-graecum* 14 ISSR primers revealed 72 % polymorphism in 17 accessions and 93.64 % polymorphism was detected in 9 accessions of *T. caerulea* (Dangi *et al.* 2004). Similarly Wu *et al.* (2004) observed 64 % polymorphism among the 14 populations of *Oryza granulata* and 26 and 22 % polymorphism within two different populations by employing ISSR marker. Essentially, the choice of markers does influence the levels of polymorphisms observed and this can likely be the other reason for high polymorphisms deduced in our study.

The similarity values based on Jaccard's coefficient ranged from 0.97 to 0.68 within the population of *S. chirayita*. High similarity coefficient values of 0.970, 0.939, 0.937 and 0.933 were scored between genotypes C and D; A and B; J and K; and F and G, respectively. Lowest similarity value of 0.679 was observed between G and M genotypes of *S. chirayita*. On inclusion of the outlier species, the similarity coefficients decreased considerably and the genotypes coded H (*S. chirayita*) and R (*S. paniculata* genotype) were found to share the lowest similarity value of 0.16.

The dendrogram based on UPGMA method consisted of two major clusters (Fig 1*C*). Cluster I comprised of the *S. chirayita* genotypes, and cluster II included the allied species of the *Swertia* genus, which were used in the study as outliers. Interestingly, three sub-groups (Ia, Ib and Ic) could be deciphered within cluster I. Subgroup Ia included all the individuals that were in the flowering stage during the collection period, with the exception of genotype H. Individuals at the vegetative phase formed a separate sub-cluster Ib. The genotypes representing one-year-older population (L and M) grouped separately as sub-cluster Ic. These groupings obtained within cluster I are relevant as they correlate with the phenological differences of the genotypes. *S. chirayita* is a biannual herb, and plants at vegetative as well as reproductive phase were found to coexist in natural populations simultaneously. These phenological differences were conspicuously brought out in our study. Future studies with larger sample size would help us validate this observation.

In cluster II, the three species of *Swertia* genus formed three separate clusters. The two *S. cordata* individuals (coded N and O) grouped together at 0.75 similarity coefficients, those of *S. purpurascens* (coded P and Q) grouped at 0.86 and the two genotypes of *S. paniculata* (coded R and S) formed a cluster at 0.62 value of similarity coefficient. Considering that the genotypes of each species were collected from same localities the diversity noted among the species is very high.

Bootstrap analysis was performed to estimate the statistical support to the internal nodes of the UPGMA tree. The clusters which grouped genotypes of *S. cordata*, *S. paniculata* and *S. purpurascens* were supported by 100 % bootstrap values. The major cluster (I), which grouped all the genotypes of *S. chirayita*, was also supported by 100 % bootstrap P-value. Within this cluster a few nodes had low bootstrap support (32.6, 34.9, and 35.9 %). However, for most of the other nodes this value ranged between 69.2 to 95.9%. These values suggest overall robustness of the UPGMA tree.

The principal component analysis performed on the correlation matrix of the original data set revealed four distinct clusters for the different *Swertia* species. The *S. chirayita* population formed a tight cluster along the PC-1 axis and the other three species of *Swertia* separated along the second principal axis, thus corroborating the clustering pattern obtained by the UPGMA cluster analysis.

The present study helps us to validate that high levels of genetic diversity are still maintained within the dwindling populations of *S. chirayita*. Therefore, it is imperative to preserve this valuable germplasm and protect its habitat from further destruction.

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