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Short Communication

Genetic variation of extremely threatened medicinal plant Nepalese Kutki (*Picrorhiza scrophulariiflora*)

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The *Picrorhiza scrophulariiflora* Pennell. (Scrophulariaceae), commonly known as Nepalease Kutki in English, is an extremely endangered, medicinally important, high altitude plant of the Eastern Himalayas of India [1]. The rhizomes which are the main propagule for multiplication, used in Indian, Bhutanese, Nepalese, Tibetan and Chinese traditional medicines to treat various ailments such as liver disorders, fever, asthma and jaundice [2]. However, the medicinal value of this plant is due to the presence of higher amount of picroside [3].

In general, medicinal plants are valued for their secondary metabolites and hence most attention has been paid to identify the elite lines containing higher amount of secondary metabolites. Plant improvement through breeding depends on the magnitude of the genetic diversity and extent to which this diversity is utilized. Despite the economic and agricultural value of this endangered plant, no attempts have been made to evaluate the potential value of genetic markers in aiding breeding and conservation. Random amplified polymorphic DNA (RAPD) technique is a method of choice for studying genetic diversity for crop species with little or no sequence information available [4].

In our previous studies, we found a wide variation of picroside content ranging from 6.35 % to 7.33% (dry weight basis) among the populations of different altitudes [5]. Therefore, present research was aimed to investigate the extent of genetic diversity among these natural populations for devising conservation strategy in *P. scrophulariiflora.*

Young leaf of sixteen landraces were sampled from their natural habitat found in eastern Himalayas comprising of East Sikkim, West Sikkim and Nepal region from the high altitude which rages from 3636 to 4500 msl. The DNA was isolated using cetyl trimethylammonium bromide (CTAB) method [6]. The DNA quality and quantity were evaluated spectrophotometrically at $OD_{260/280\ nm}$ and by visual assessment of band intensities on a 0.8% agarose gel in comparison to Lambda DNA marker (Himedia, India). Initially forty primers were screened (Operon Technologies Inc., CA, USA) using DNA as template from four different landraces. The DNA amplification was performed in a thermal cycler (Biorad, USA). The PCR reaction was carried out with a final volume of 25 µl reaction mixture containing 1X Taq DNA polymerase buffer (10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mm $MgCl_2$, 0.01% gelatin), 100 µM each of dNTPs (Bangalore Genei, India), 5 pmoles primer, 20 ng of genomic DNA and 0.3 U of *Taq* DNA polymerase (Bangalore Genei, India). PCR was performed with the following conditions: an initial cycle of 5 min at 94°C, followed by 35 cycles each of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C and an extension step at 72°C for 7 min. Around 20 µl PCR products

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resolved in 1.4% agarose gel stained with ethidium bromide (10 mg/ml) along with 1 kb ladder (Bangalore Genei) and visualized under ultra violet light in a gel documentation system (Biorad, USA). The Polymerase Chain Reaction (PCR)-RAPD analysis was repeated at least twice to identify primers that could produce strong and reproducible bands. Based on strong reproducible bands, 10 primers were selected for final analysis for all the genotypes. Accordingly, those 10 primers were used in the final analysis for all landraces. Band profiles were manually scored and compiled into a rectangular binary matrix (not shown). Positive amplifications were treated as separate characters and scored for presence (1) or absence (0) of bands. Only intensely stained unambiguous bands were scored for analysis.

Data analysis was done on the basis of polymorphism that was calculated as the proportion of polymorphic bands over the total number of bands. The degree of polymorphism was quantified by using Shannon's index [7] of phenotypic diversity:

$$H_0 = - \Sigma \pi_i \, 1n \, \pi_i$$

where π_i is the frequency of phenotype i and n was the number of populations. Therefore, H_0 was calculated by comparing among different populations.

Let

$H_{pop} = 1/n \, \Sigma \, H_0$ was the average diversity over n different populations and let $H_{sp} = -\Sigma \, \pi 1n \, \pi$ was the diversity calculated from phenotypic frequencies π in all the populations considered together. Then the proportion of diversity present within populations (H_{pop}/H_{sp}) was compared with that between populations $\{(H_{sp}–H_{pop})/H_{sp}\}$.

Estimates of similarity between genotypes were based on the probability that an amplified fragment from one accession will also be present in another [8].

D_{AB} = 2 X number of shared bands/(number of fragments$_A$ + number of fragments$_B$).

In the present investigation, out of 40 primers screened, five were found to be highly polymorphic, which generated a total 46 bands. Amplicon range from 564 to 2027 bp in size. The use of a few but highly informative primers lowers the cost, time and labor efforts in diversity analysis. Primers also differed in their capacity to detect the polymorphism. For an example, highest level of polymorphism was recorded with both OPE-19 (100%) and OPE-20 (100%), followed by OPE-6 (92.31%), OPE-15 (77.77%) and OPE-3 (60%) (Table 1). The number of bands by each primer varied between a minimum of 5 (OPE-3) to a maximum of 13 (OPE-6) with the average 9.2 bands per primer. Simple measure of intra population variability based on the number of polymorphic products scored in a single population over the total number of polymorphic products ranged from a minimum of 30.43 % of the Nepal population to maximum of 76.08 % of East Sikkim and Bhutan population (Table 1). Being mainly a vegetatively propagated plant, the variation at DNA level may be attributed either to mutations or somatic recombination occurring over time which have been selected and fixed in the population, nevertheless, genetic recombination with a very lower frequency may be contributed. Flowering and fruit setting has also been reported rarely in this species [1]. Since, the number of individual sampled for analysis was small due to extreme rareness of the species, the variation measured perhaps not fully represented the total available genetic diversity of *Picrorhiza*, nevertheless, the percentage of polymorphic bands (86.96%) (Table 1) obtained by RAPD primers in the species was observed very high [9]. The low percentage variation detected among the Nepal (30.43%) population might be attributed to the very low number of sample analyzed (Table 1). Thus, germplasm collection mission in future should give special attention for such regions to broaden the genetic basis of gene bank collection and to increase the chance of conserving important genotypes that can be used for improvement programs.

The band frequencies detected with the 5 primers were used in estimating diversity (H_0) within the population types. Although, both the West Sikkim as well as East Sikkim and Bhutan exhibited high intra population variability, the intra population variability of Nepal was low due to the smallest population size which ultimately affects H_0. Shanon's index of phenotypic diversity was then used to partition the diversity into intra and inter population components (data not shown). On an average the proportion of diversity present within population (Hpop/Hsp) was 53%, while between the population {(Hsp-Hpop)/Hsp} was 47%. The primer differed in their capacity to detect intra and inter population variability (data not shown). Thus, when the diversity recorded for each locus was averaged across the loci within a primer, it becomes clear that the primer OPE-06 (61%) detected the highest intra population variation, which means that this primer might be better suited to detect the intra population variation of *Picrorhiza* genotypes. On the other hand, primer OPE-20 seem to be better suited to identify populations that

Table 1. Number of amplification products generated with 5 polymorphic RAPD primers

Primers	Sequence	No. of polymorphic bands*	Percentage of polymorphism	No. of polymorphic PCR products		
				West Sikkim	East Sikkim and Bhutan	Nepal
OPE-03	CCAGATGCAC	4	60	4	4	3
OPE-06	AAGACCCCTC	12	92.31	8	12	3
OPE-15	ACGCACAACC	7	77.77	5	5	3
OPE-19	ACGGCGTATG	11	100	5	7	2
OPE-20	AACGGTGACC	8	100	4	7	3
Total		42	-	26	35	14
Average % polymorphism			86.96	56.52	76.08	30.43

*Data in parenthesis represent % of product detecting polymorphism

have lost amplifiable alleles, as they revealed a relatively higher proportion of inter population (56 %) variations. Analysis of genetic similarity value revealed that no two genotypes are identical and similarity index for each pair of population varied from 0.965 to 0.185. The evolution of landraces in distinct climatic zones demonstrates significant levels of variations in response to the selection pressure in their native environment. It is, therefore, not surprising to find significant levels of polymorphism among the 17 genotypes as revealed by RAPD analysis here.

A dendrogram constructed on the basis of shared fragment revealed broadly in three clusters (Fig. 1). Cluster-I or West Sikkim was linked to Cluster-II or East Sikkim and Bhutan at low similarity coefficient of 53%. The dendrogram also revealed that the clustering matched exactly with the existing knowledge of the place of collection. For example, cluster-III and IV, which are geographically distinct from the origin of others, may be explained for the formation of separate clustering. The complete identity of the genotypes in the dendrogram perhaps may be due to the fact that the samples were wild in nature and hence no chances of mixing by human intervention. The importance of high level of genetic variation has been emphasized as a safeguard against co-evolving biotic factors such as pests and diseases. The distribution of genetic diversity in the natural population of *P. scrophulariiflora* with high level of variation agrees with the behavior of an out crossing species [10].

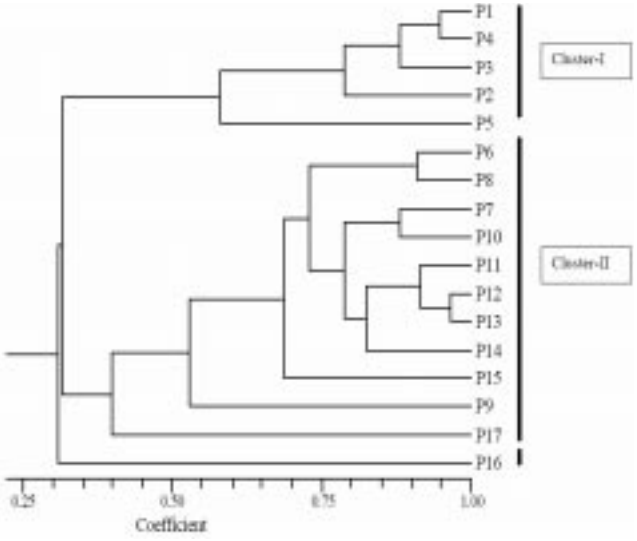

Fig. 1. Dendrogram of different genotypes of *P. scrophulariiflora* based on average linkage cluster analysis

It is noteworthy to mention that, till today no genetic diversity study in this species has been studied through any molecular markers. The genetic structure of *P. scrophulariiflora* populations indicates that *in situ* conservation strategies for this species should be based on establishment of several small-scale protected areas chosen so as to represent the species genetic variability.

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