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Original Research Article

Molecular Characterization of Endangered Species *Saussurea simpsoniana* and *Saussurea candicans* from Haramosh Valley, Gilgit, Pakistan

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Abstract

Medicinal plants are very valuable natural resource of Pakistan especially for the Northern mutinous areas of Gilgit Baltistan (GB), Pakistan. Saussurea species are traditionally used for curing various diseases like intense fever, cough and pneumonia. Present study attempted for the first time from GB to document genetic diversity of *Saussurea simpsoniana* and *Saussurea candicans* using morphological, biochemical and DNA based markers. All three parameters in both species exhibited highly significant differences. Total crude protein concentration was estimated in bark and leaves ranged from 13.8 % to 21.5 %. Maximum bark and leaf protein concentration was observed in *Saussurea candicans*. SDS-PAGE showed almost similar banding pattern except an extra band in leaf of *S. simpsoniana*. Total genomic DNA was isolated using modified CTAB protocol and 19 RAPD primers were used for the amplification. Genetic distances (GD) were estimated (0.0 to 100 %) using UPGMA procedure. Three primers showed complete homozygosity while 7 showed 100 % genetic distances among the two species. Average GD estimated (based on RAPD data) among two species was 63.16 %. Research indicated that enough genetic diversity is present in local material of genus Saussurea which can be utilized for the improvement / genetic studies in this medicinally important genus. It is also suggested that protocols developed / optimized during present research can be utilized at larger scale for better understanding of genome structure in these medicinally important species.

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Introduction

Approximately 6000 plant species have been reported in Pakistan. Among these approximately 3200 species have traditionally being used for medicinal purposes (Perveen and Hussain, 2007).Relatively recently established province of Gilgit-Baltistan (previously called Northern areas of Pakistan) is the most magnificent mountainous area of the world and is surrounded by three mighty mountain systems; Karakoram, Himalaya, and Hindukush. It is situated at 35.35 N 75.9 E latitude. Total area of GB is 72,971 km^2 (28,174 sq mi) with a population is 1,800,000 and a population density of 25/Km$^{2.}$ (64/Sq Mile). A large number of medicinally important plant families have been reported from various areas of Gilgit Baltistan. Among these, family

Asteraceae is an important family. Important genera of the family found in GB include Saussurea. Genus Saussurea is an important genus of medicinal plants comprising approximately 300 species. Members of the genus are perennial herbs, ranging in height from 5–10 cm up to 3 m. The leaves are produced in a dense basal rosette, and then spirally up the flowering stem. The flowers form in a dense head of small capitula, often surrounded by dense white to purple woolly hairs.

Two important species of the genus present in GB are *S. simpsoniana* and *S. candicans*. Even though medicinal value of *Saussurea* species is great, not much work on DNA based genetic studies have been carried out in the past. According to a relatively recent publication (Gailite et al., 2011) there are not any co-dominant DNA markers available for Saussurea species. As initial attempts, some scientists have initiated using PCR based markers for study of genetic diversity in *Saussurea* species.

Previous DNA based studies in genus Saussurea are summarized below. Gailite et al. (2011) studied genetic diversity in *S. esthonica* from Latvia and Esthonia using DNA based variation. They reported that most of the variation in their material was found within populations. They reported variation between populations 3 to 5%. In an earlier study (Gailite et al., 2010) estimated genetic diversity in two Latvian populations of *S. esthonica* using Amplified Fragment Length Polymorphism (AFLP) and inter-primer binding site (iPBS) methods. They reported that iPBS primers are more useful for the estimation of genetic diversity in *Saussurea* species as compared to AFLP primers.

Dashzeveg et al., (2016) estimated genetic diversity in *Saussurea involucrata* from western Mangolia using AFLP markers are reported 92 % variation exists among various populations. Jeong et al., (2012) studied genetic diversity in *Saussurea chabyoungsanica* from Korea using ISSR (inter simple sequence repeat) primers and found 45.6 % genetic diversity among various populations.

These two species (*Saussurea simpsoniana* and *Saussurea candicans*) are used for treatment of fever, snake bite, cough and pneumonia by local communities in mountainous areas of Gilgit Baltistan. Present work is therefore first documented attempt to utilize DNA based technology (along with morphological and biochemical characterization) for estimation of genetic diversity in two medicinally important species *S. simpsoniana* and *S. candicans*.

Materials and methods

Plant material for the study was collected from Haramosh area, district Gilgit. *S. simpsoniana* was collected from village Jutial, Haramosh area, District Gilgit approximately 5000 meter (approximately 16000 feet) above sea level. This specie (*S. simpsoniana*) is not present at lower altitudes. *S. candicans* was collected from low attitude area i.e. village Sassi, Haramosh area, district Gilgit (approximately 2500 meter above sea level, equivalent to approximately 8000 feet above sea level). *S. candicans* is not present on higher altitudes. Five plants per species were collected. Samples of *S. simpsoniana* were collected during October, 2015. Collection of *S. candicans* was carried out during April, 2016. (Representative pictures of *S. simpsoniana* and *S. candicans* are presented in Fig 1.

Small scale DNA isolation procedure (miniprep) of Doyle and Doyle (1990) was used to isolate total genomic DNA from the leaf samples. The quality and quantity of the DNA was checked on 1% agarose/TBE gel. Nineteen Randomly Amplified Polymorphic DNA primers (obtained from Gene Link, Inc, USA) were used to amplify genomic DNA isolated from *Saussurea simpsoniana* and *Saussurea candicans*. Basic Statistical analysis including mean, median, minimum, maximum, standard deviation, standard error and coefficient of variation, graphical representation and analyses of variance were carried out using computer program PAST (Paleontological Statistics, Hammer, 2016) version 3.11. Genetic diversities among all the possible comparisons were estimated using following formula:

$$GD = 1-dxy/dx+dy-dxy$$

Fig. 1: (L-R) *S. simpsoniana* and *S. candicans* from Haramosh.

Results and discussion

Statistical significance among morphological characters (plant height, flower/head length, flower/head width and flower/head diameter) between *S. simpsoniana* and *S. candicans* were calculated using computer program Past (Hammer, 2016). t-values and probabilities (*p*-values) for 4 morphological characters among 2 species (*S. simpsoniana* and *S. candicans*) are presented in Table 1.

Table 1. Statistical significance for morphological characters of *S. simpsoniana* and *S. candicans.*

Mean	Plant height	Flower/ Head length	Flower/ Head width	Flower/ Head diameter
S. simpsoniana	16.5	6.0	5.0	13.5
S. candicans	46	2.4	2.0	6.2
t value	5.2 **	16.6**	13.7**	16.3**
p value	0.004	4.6-E11	6.6.E-10	5.8E-11

Bark and leaf crude protein concentration was estimated using spectrophotometric procedure described by Grimsley and Pace (2003) and average values are presented in Table 2. Average crude bark and leaf protein concentration in two *Saussurea* species ranged from 13.8% to 21.5%. Average bark and leaf protein concentration in *Saussurea simpsoniana* and *S. candicans* were 13.8, 16.3, 19.4 and 21.5%, respectively. Maximum bark protein concentration (19.4%) was observed in *S. candicans*. For leaf protein, maximum concentration (21.5%) was observed in *S. candicans*. Overall protein concentration (for leaf and bark) in *S. candicans* was higher as compared to *S. simpsoniana*. Statistically protein concentration in leaves and bark of *S. simpsoniana* and *S. candicans* was highly significantly different (t-value = 10.69**; *p*-value = 0.001).

Table 2. Estimates of crude protein content in leaves and bark of *S. simpsoniana* (SS) and *S. candicans* (SC).

	SS bark	SS leaf	SC bark	SC leaf
Sample 1	13.1	16.1	18.4	22.3
Sample 2	13.8	16.1	19.2	21.5
Sample 3	14.6	16.9	20.7	20.7
Average	13.8	16.3	19.4	21.5
t-value = 10.69; *p*-value = 0.001.				

Total protein extracted from bark and leaf of *S. simpsoniana* and *S. candicans* separated using SDS-PAGE showed that protein banding pattern in the two species is almost same except an extra band in *S. simpsoniana* leaf Fig. 2).

Present finding strengthened a previous report by Dias et al. (2008) who demonstrated that altitudinal variation exist in genus *Trifolium*. It is suggested that this species / tissue specific band may be studied in detail for better understanding of proteomics in genus *Saussurea*.

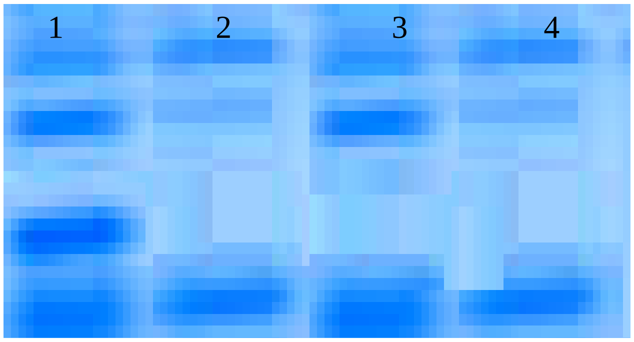

Fig. 2: SDS-PAGE analysis of *Saussurea* samples: 1= *S. simpsoniana* leaf, 2= *S. simpsoniana* bark, 3= *S. candicans* leaf, 4= *S. candicans* bark.

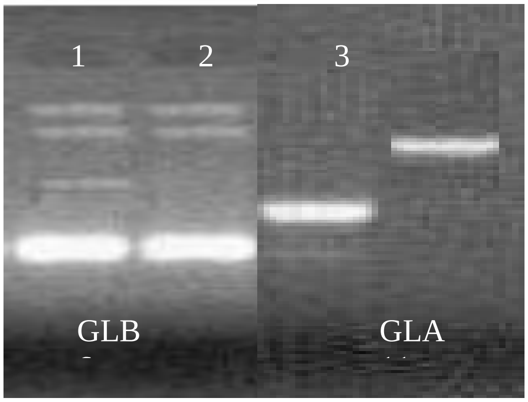

Fig. 3: Few examples of PCR amplification of *Saussurea simpsoniana* (lane 1) and *Saussurea candicans* (lane 2) using RAPD

Nineteen Randomly Amplified Polymorphic DNA (RAPD) primers were used for the amplification of total genomic DNA isolated from the two species and some examples of amplification profiles are presented in Fig. 3. DNA fragments amplified ranged from approximately 300 to 500 bp. Total of 85 DNA fragments were amplified during present research giving an averaged 4.5 DNA fragments amplified per primer.

Table 3. Genetic distances among *Saussurea* species using RAPD primers.

S. No.	RAPD	GD%	S. No.	RAPD	GD%
1	GLA-02	100	11	GLG-01	0
2	GLA-11	100	12	GLG-4	34
3	GLA-14	75	13	GLG-09	100
4	GLB-6	67	14	GLH-3	0
5	GLC-03	0	15	GLI -7	67
6	GLD-2	25	16	GLI-12	100
7	GLD-3	82	17	GLJ-01	100
8	GLD-5	50	18	GLJ-10	50
9	GLD-8	50	19	GLK-3	100
10	GLE-5	100		**Average**	**63.16**

DNA fragments were scored as present (1) or absent (0). Genetic distances (presented in Table 3) were estimated using unweighted pair group of arithmetic mean UPGMA procedure (Nei and Li, 1979). Genetic distances estimated among *S. simpsoniana* and *S. candicans* ranged from 0 to 100 % using 18 RAPD primers. Three out of 19 RAPD primers *viz.*, (15.8%) showed complete homozygosity among two species of *Saussurea* for the loci amplified. While 7 out of 19 RAPD primers (36.8%) showed 100% genetic distances among the two species. Average genetic distance estimated (based on RAPD data) among two species of genus *Saussurea* was 63.16%.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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