RESEARCH ARTICLE

Delimitation of Species of the *Astragalus rhizanthus* Complex (Fabaceae) Using Molecular Markers RAPD, ISSR and DAMD

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ABSTRACT: Forty accessions belonging to four closely related species of the *Astragalus rhizanthus* complex (i. e., *A. rhizanthus, A. candolleanus, A. malacophyllus* and *A. pindreensis*) were collected from different parts of the Indian Himalaya and analyzed for intra- and inter-specific relationship using Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR) and Directed Amplification of Minisatellite DNA (DAMD). Eighteen RAPD, 15 ISSR and 6 DAMD primers were used to study the genetic diversity between the species. A total of 449 bands for RAPD, 326 bands from ISSR and 179 bands from DAMD were obtained. Nearly 98.44% of RAPD, 99.38% of ISSR and 98.32% of DAMD bands were polymorphic. Pair-wise genetic similarity for a cumulative data was determined using Jaccard's similarity coefficient which varied from 0.23 to 0.82 and a Neighbor-joining (NJ) dendrogram was generated. The genetic similarity from the data matrix estimated by Jaccard's coefficient shows that all four species are distinct in accordance with our morphological findings as well as previous taxonomic treatment in which these species have been treated distinctly. The highest degree of genetic similarity was observed within *A. rhizanthus* followed by *A. malacophyllus*, *A. candolleanus* and *A. pindreensis*. This study has proved that these markers have the ability to distinguish the closely allied species as well as analyze the genetic diversity within and between the species.

KEY WORDS: *Astragalus*, differentiation, Fabaceae, RAPD, ISSR, DAMD.

INTRODUCTION

Astragalus L., the largest genus in angiosperms, comprises 2500-3000 species which are chiefly distributed in cold arid and semiarid mountain regions of the Northern Hemisphere and South America (Polhill, 1981; Podlech, 1986; Zarre and Podlech, 1997; Maassoumi, 1998; Lock and Schrire, 2005; Scherson et al., 2008). In India, it has about 80 species, distributed primarily in the temperate to alpine regions of the Himalaya with major center of diversity in the cold deserts of Lahul-Spiti (Himachal Pradesh) and Leh and Ladakh (Jaummu and Kashmir) (Sanjappa, 1992; Kumar and Sane, 2003; Chaudhary and Srivastava, 2007; Chaudhary et al., 2007a; Chaudhary et al., 2008).

During the course of a taxonomic study on Indian *Astragalus* one of us (LBC) observed that some of the members of section *Caprini* DC. like *A. rhizanthus* Royle ex Benth., *A. candolleanus* Royle ex Benth., *A. malacophyllus* Benth. ex Bunge and *A. pindreensis* (Benth. ex Baker) Ali have been variously treated by different workers (Table 1). These four species are very closely related to each other and often pose problems in their segregation and identification, hence they have been treated here as a taxonomic complex. Baker (1876) has treated all of them as distinct species, except *A. pindreensis*. He (1876) has described *A. pindreensis* as a variety under *A. candolleanus*. Although Parker (1921) also treated *A. pindreensis* as a variety under *A. candolleanus*, however, he observed it closed to *A. rhizanthus*. Unlike Baker (1876), Parker (1921) placed *A. malacophyllus* under *A. rhizanthus* while Ali (1961, 1977) treated all of them at species level followed by Sanjappa (1992). However, unlike Parker (1921), Ali (1977) found *A. pindeerensis* as a synthetic species between *A. candolleanus* and *A. malacophyllus*. In a recent study of the *Astragalus* section *Caprini* DC., Podlech (1988) observed *A. pindreensis* more closed to *A. rhizanthus* than *A. candolleanus* and treated it as a variety. Further, he (Podlech, 1988) treated *A. candolleanus* as a subspecies of *A. rhizanthus* unlike earlier treatments. Like Parker (1921), Podlech (1988) has also regarded *A. malacophyllus* as conspecific to *A. rhizanthus* whereas Baker (1876), Ali (1961, 1977) and Sanjappa (1992) have treated it as a distinct species.

In our morphotaxonomic study based on herbarium specimens and critical field observations, it was noticed that *A. malacophyllus* distinctly differ from *A. rhizanthus* by bushy habit, densely hairy nature, usually elongated stems, persistent, more or less stiff and subspiny leaf rachis, deciduous nature of terminal leaflet, axillary short raceme with only a few flowers and hairy style. Both species grow in quite different areas located distantly and can be separated by a number of morphological features

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Table 1. Taxonomic treatment of the species of *Astragalus rhizanthus* complex as described by different workers.

Baker (1876)	Parker (1921)	Ali (1961, 1977)	Podlech (1988)	Present study
A. rhizanthus	*A. rhizanthus* (= *A. malacophyllus*)	*A. rhizanthus*	*A. rhizanthus* ssp. *rhizanthus* var. *rhizanthus* (= *A. malacophyllus*)	*A. rhizanthus*
A. candolleanus var. *pindreensis*	*A. candolleanus* var. *pindreensis*	*A. pindreensis*	*A. rhizanthus* ssp. *rhizanthus* var. *pindreensis*	*A. pindreensis*
A. candolleanus	*A. candolleanus*	*A. candolleanus*	*A. rhizanthus* ssp. *candolleanus*	*A. candolleanus*
A. malacophyllus		*A. malacophyllus*		*A. malacophyllus*

as mentioned above. *A. malacophyllus* mainly grows from Gangotri (2500 m) to Gaumukh (4000 m) in Uttarakhand province while *A. rhizanthus* is sparsely distributed in the area of Lahul-Spiti in Himachal Pradesh province and occasionally in some other parts of the Himalayas from 3000-5500 m altitudes. *A. candolleanus* occasionally distributed in the different parts of the Himalayas from 2900-5000 m altitudes and comparatively closer to *A. malacophyllus* than *A. rhizanthus*. *A. pindreensis* is quite rare in distribution in the Himalayas between 3000-5000 m and was collected only from one locality in Badrinath (Uttarakhand) region. This species exhibits close resemblance with *A. rhizanthus* than *A. candolleanus* in overall gross morphology. In the present work both *A. candolleanus* and *A. pindreensis* have been treated as distinct species. These four species (Figs. 1A-D) can be separated by following taxonomic key.

Taxonomic key to the species based on morphological characters

1. Plants stemless or occasionally with short stem; flowers aggregate at the tip of stem ……..……...… 2
1. Plants distinctly stemed; stem up to 90 cm long; flowers on the peduncle in the axil of leaf 3
2. Leaf rachis not persistent and not indurated; terminal and lateral leaflets not deciduous ……..… *A. rhizanthus*
2. Leaf rachis persistent and indurated; terminal and lateral leaflets deciduous ……..…. *A. pindreensis*
3. Plants densely hairy; leaflets hairy on both sides, terminal leaflet falls before lateral leaflets ……..…..… *A. malacophyllus*
3. Plants almost glabrous to moderately hairy; leaflets almost glabrous on upper surface and moderately hairy on lower surface, terminal leaflet does not fall before lateral leaflets ……..… *A. candolleanus*

A. malacophyllus Benth. ex Bunge well known as Rudanti or Rudravanti is used for tuberculosis, skin diseases, cough and blood purification in India particularly in Gangotri-Gaumukh areas since ancient time especially by local people and saints (Chaudhary et al., 2008). Since it is a quite useful as drug its correct taxonomic identification is highly essential for any kind of pharmacognostic studies of the species.

In view of the taxonomic problems mentioned above among the species of the *A. rhizanthus* complex and to reconfirm our morphological observations, it is required to investigate them also at molecular level to establish their taxonomic identity and relationship. The DNA based markers could serve as better taxonomic tools in such cases to overcome the limitations of morphological markers. The molecular markers are unlimited in numbers, devoid of any environmental and developmental effects as well as devoid of pleiotropic and epistatic effects and exhibit high level of polymorphism (Dangi et al., 2004).

Several molecular techniques such as RFLP (Restriction fragment length polymorphism), different chloroplast regions (e.g., *rpo*C1 and *rpo*C2) and analysis of nuclear genes (Nuclear ribosomal DNA ITS) have been employed in *Astragalus* for phylogenetic studies (Sanderson, 1991; Sanderson and Doyle, 1993; Liston, 1992; Liston and Wheeler, 1994; Wojciechowski et al., 1993; 1999; Kazempour Osaloo et al., 2003, 2005; Wojciechowski, 2005). In recent years PCR based methods such as RAPD, ISSR and DAMD have also been used quite extensively due to their ease, rapidity and reliability for analysis of molecular differentiation and for resolving taxonomic problems in plants. These methods have also been applied to study intra and inter-specific relationship among different species of *Astragalus* (Luo et al., 2000; Mehrina et al., 2005; Alexander et al., 2004; Na et al., 2004; Chaudhary et al., 2007b) and in many other plant groups (Tautz and Renz, 1984; Nagaoka and Ogihara, 1997; Fang and Roose, 1997; Sant et al., 1999; Dangi et al., 2004). The RAPD analysis utilizes single arbitrary sequence of ten bases as oligonucleotide primers to amplify discrete fragments of DNA in low-stringency of polymerase chain reactions (Welsh and McClelland, 1990; Williams et al., 1990; Wolfe and Liston, 1998; Harris, 1999). The ISSR markers have recently become widely used in population and systematic studies because they are highly variable and exhibits Mendelian fashion (Gupta et al., 1994; Tsumara et al., 1996). This marker analysis utilizes single primer 16-18 bp long composed of a repeated sequence and can produce larger numbers of fragments per primer, with the advantages of high reproducibility and relatively low cost (Powell et al., 1996). Similarly, DAMD reveals polymorphism due to minisatellites (Heath et al., 1993). These are the tandem repetition of a 10-60 bp DNA sequence known as the "Core" sequence. Therefore, the techniques like RAPD, ISSR and DAMD, which provide many polymorphic bands are efficient to unravel the intra and inter-specific relationships amongst different genotypes of *A. rhizanthus*.

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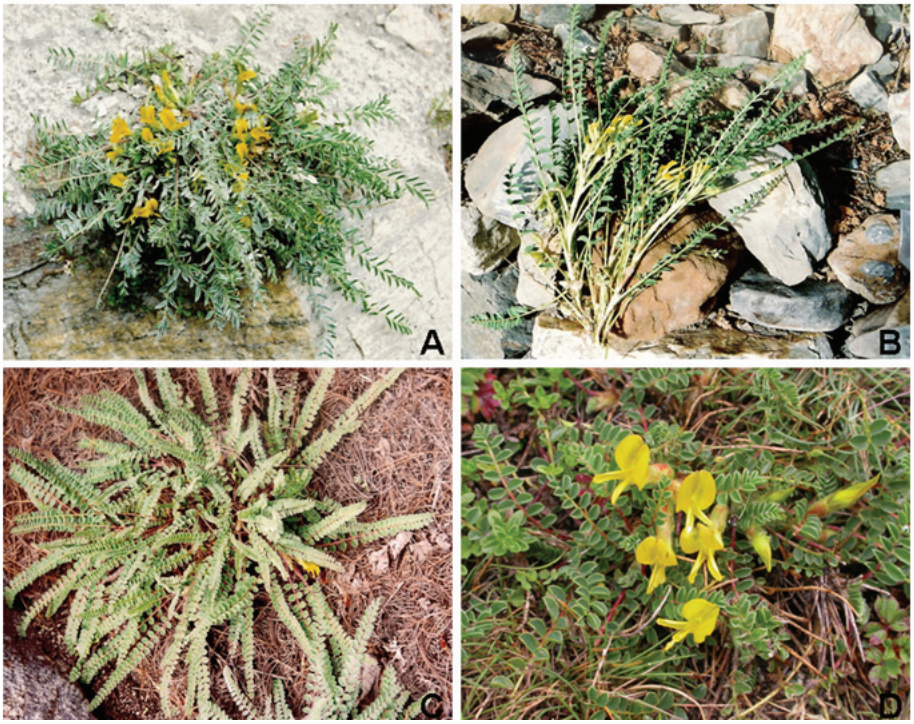


Fig. 1. Habit of *Astragalus* species. A: *A. rhizanthus*. B: *A. candolleanus*. C: *A. malacophyllus*. D: *A. pindreensis*.

The main objectives of the work are to study the inter and intra-specific relationship and delimitation of the species of *A. rhizanthus* complex using RAPD, ISSR and DAMD markers. The scrutiny of the literature reveals that these species have not been tested so far at molecular level to confirm their taxonomic identity and relationship.

MATERIALS AND METHODS

Sample collection

The leaf samples of candidate species (viz. *A. rhizanthus, A. malacophyllus, A. candolleanus* and *A. pindreensis*) were collected from their place of occurrence in silica gel (Chase and Hills, 1991). The accessions were randomly sampled from throughout the known populations of cold desert of Lahul-Spiti in Himachal Pradesh and Gangotri-Gaumukh and Badrinath in Uttarakhand provinces. *A. rhizanthus* was sparsely scattered at different places of Lahul-Spiti region whereas *A. candolleanus* was chiefly confined to Losar of this region. On the other hand, *A. malacophyllus* was found common from Gangotri to Gaumukh, a region quite distant and different from Lahul-Spiti. *A. pindreensis* was found rare and observed only in Badrinath area and therefore only one accession of this species has been included in the present investigation. A total of 40 accessions of all four species have been used here for RAPD, ISSR and DAMD analyses (Table 2). The additional accessions (not mentioned here to minimize the space) were also studied in the field as well as in different herbaria (BM, BSD, CDRI, CAL, DD, K, LWG) to know the range of morphological variations and taxonomic limits of the target species. The voucher specimens of all collected materials have been deposited in the herbarium of National Botanical Research Institute, Lucknow, India (LWG) for future record.

DNA isolation

Silica gel dried leaf tissues (100 mg) were frozen in liquid nitrogen and grounded in mortar and pestle. The genomic DNA was extracted from the powdered leaf material using Dneasy Plant Mini Kit (Qiagen, Valencia, CA, USA) as per the instructions of the manufacturer with minor modifications. Extracted DNA was quantified using ND-1000 spectrophotometer (Nanodrop, USA) and their quality was evaluated by gel electrophoresis on 0.8% agarose stained with ethidium bromide (Sambrook et al., 1989).

RAPD-PCR

The RAPD primers were procured from Operon Tech. Inc. Alameda, CA, USA. Total 80 decamer primers (B, C, N and U kits) were screened for PCR amplification. Out of which 18 primers that generated polymorphic profiles

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Table 2. List of species belonging to *Astragalus rhizanthus* complex with their collection details. The first column corresponds to the code assigned to the accession number.

Abbreviation of species	Species	Locality	Longitude / Latitude	Altitude	Collection number
AR1	*A. rhizanthus*	Before Koksar, Lahul (H.P.)	N 38° 24.427' E 77° 14.806'	3814 m	229407
AR2	*A. rhizanthus*	Before Koksar, Lahul (H.P.)	N 38° 24.427' E 77° 14.806'	3814 m	229408
AR3	*A. rhizanthus*	Before Koksar, Lahul (H.P.)	N 38° 24.427' E 77° 14.806'	3814 m	229409
AR4	*A. rhizanthus*	Koksar proper in potato field, Lahul (H.P.)	N 32° 24.755' E 77° 14.180'	3136 m	229416
AR5	*A. rhizanthus*	Koksar proper towards river side, Lahul (H.P.)	N 32° 24.755' E 77° 14.180'	3136 m	229417
AR6	*A. rhizanthus*	Koksar, Lahul (H.P.)	N 38° 24.809' E 77° 14.225'	3134 m	229421
AR7	*A. rhizanthus*	Koksar, Lahul (H.P.)	N 38° 24.809' E 77° 14.225'	3134 m	229422
AR8	*A. rhizanthus*	Gondla, Lahul (H.P.)	N 32° 36.286' E 76° 55.996'	2905 m	229463
AR9	*A. rhizanthus*	Gondla, Lahul (H.P.)	N 32° 36.286' E 76° 55.996'	2905 m	229464
AR10	*A. rhizanthus*	Gondla, Lahul (H.P.)	N 32° 36.286' E 76° 55.996'	2905 m	229466
AR11	*A. rhizanthus*	Gondla, Lahul (H.P.)	N 32° 36.286' E 76° 55.996'	2905 m	229468
AR12	*A. rhizanthus*	Near Triloki nath temple, Lahul (H.P.)	N 32° 47.265' E 77° 17.099'	3845 m	229476
AR13	*A. rhizanthus*	Between Gondla and Sissu , Lahul (H.P.)	N 30° 30.842' E 76° 58.986'	2996 m	229480
AR14	*A. rhizanthus*	Chota Dara, Lahul (H.P.)	N 32° 18.035' E 77° 27.704'	3475 m	229485
AR15	*A. rhizanthus*	Between Chatru and Chota Dara, Lahul (H.P.)	N 32° 20.079' E 77° 20.413'	3027 m	229493
AR16	*A. rhizanthus*	Near Chatru, Lahul (H.P.)	N 32° 21.654' E 77° 18.247'	3034 m	229498
AR17	*A. rhizanthus*	Gramphu , Lahul-Spiti (H.P.)	N 32° 30.842' E 77° 58.987'	2996 m	229500
AC18	*A. candolleanus*	Gondla, Lahul (H. P.)	N 32° 36.286' E 76° 55.996'	2905 m	229465
AC19	*A. candolleanus*	Before 1 km of Losar from Kunjam pass, spiti valley (H. P.)	N 32° 26.590' E 76° 44.573'	4106 m	229506
AC20	*A. candolleanus*	Before 1 km of Losar from Kunjam pass, spiti valley (H. P.)	N 32° 26.590' E 76° 44.573'	4106 m	229508
AC21	*A. candolleanus*	Losar , spiti valley (H. P.)	N 32° 25.848' E 77° 55.0460'	4079 m	229517
AC22	*A. candolleanus*	1 km Before of Losar from Kunjam pass, spiti valley (H. P.)	N 32° 26.571' E 76° 44.571'	4088 m	229521
AC23	*A. candolleanus*	1 km Before of Losar from Kunjam pass, spiti valley (H. P.)	N 32° 26.571' E 76° 44.571'	4088 m	229522
AC24	*A. candolleanus*	1 km Before of Losar from Kunjam pass, spiti valley (H. P.)	N 32° 26.571' E 76° 44.571'	4088 m	229523
AC25	*A. candolleanus*	1 km Before of Losar from Kunjam pass, spiti valley (H. P.)	N 32° 26.571' E 76° 44.571'	4088 m	229524
AM26	*A. malacophyllus*	Between Chirbasa to Bhojwasa (Uttarakhand)	N 30° 58.389' E 79° 02.063'	3680 m	229545
AM27	*A. malacophyllus*	Gangotri to Chirbasa, Uttarakhand	N 30° 58.827' E 79° 01.855'	3343 m	229547
AM28	*A. malacophyllus*	Chirbasa (Uttarakhand)	N 30° 58.949' E 79° 01.355'	3313 m	229551
AM29	*A. malacophyllus*	Gangotri to Chirbasa (Uttarakhand)	N 30° 59.288' E 79° 00.081'	3277 m	229552
AM30	*A. malacophyllus*	Gangotri to Chirbasa, near Chirbasa (Uttarakhand)	N 30° 59.795' E 79° 57.564'	3171 m	229554
AM31	*A. malacophyllus*	Gangotri to Chirbasa (Uttarakhand)	N 30° 59.571' E 79° 59.613'	3241 m	229555
AM32	*A. malacophyllus*	Gangotri (Uttarakhand)	N 31° 02.288' E 79° 45.580'	2509 m	229557
AM33	*A. malacophyllus*	Gangotri (Uttarakhand)	N 31° 02.288' E 79° 45.580'	2509 m	229558
AM34	*A. malacophyllus*	Bhojwasa (Uttarakhand)	N 30° 57.0508' E 79° 03.032'	3871 m	229560
AM35	*A. malacophyllus*	Bhojwasa (Uttarakhand)	N 30° 57.0508' E 79° 03.032'	3871 m	229561
AM36	*A. malacophyllus*	Bhojwasa (Uttarakhand)	N 30° 57.0508' E 79° 03.032'	3871 m	229562
AM37	*A. malacophyllus*	Bhojwasa (Uttarakhand)	N 30° 57.0508' E 79° 03.032'	3871 m	229563
AM38	*A. malacophyllus*	Bhojwasa (Uttarakhand)	N 30° 57.0508' E 79° 03.032'	3871 m	229564
AM39	*A. malacophyllus*	Bhojwasa (Uttarakhand)	N 30° 57.0508' E 79° 03.032'	3871 m	229565
AP40	*A. pindreensis*	Badrinath, Near Vasundhara Fall (Uttarakhand)	N 30° 33.0508' E 79° 36.024'	3500 m	225088
OG	*Oxytropis tatarica*	Ladakh, Tanglang La (J. & K.)	N 33° 30.275' E 77° 46.126'	5200 m	225038

were selected for scoring data for all the accessions in the present study (Table 3). The final RAPD reactions were carried out in 25 μL volumes which contain 25 ng of template DNA, 10 pmoles primer, 200 μM of each dNTP, 2.5 mM Mg^{2+} ion concentration in suitable 1X assay buffer supplied along with the enzyme and 0.5 Units of the thermostable *Taq* DNA polymerase (Bangalore Genei, India). The amplification of DNA was performed on a PTC-200^{TM} thermocycler (MJ Research, Inc. USA), which was programmed to include pre-denaturation at 94°C for 1 min, followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 35°C for 1 min and extension at 72°C for 1 min. The final cycle allowed an additional 5 min period of extension at 72°C.

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Table 3. Information conveyed by eighteen, fifteen and six primer combination employed to detect polymorphism based on RAPD, ISSR and DAMD among forty genotypes of *Astragalus*.

Primer	Sequence (5' – 3')	No. of loci amplified	Polymorphic Bands	% Polymorphism
RAPD				
OP-B06	TGCTCTGCCC	27	26	96.29
OP-B07	GGTGACGCAG	25	24	96.00
OP-B08	GTCCACACAG	24	24	100
OP-B09	TGGGGGACTC	25	25	100
OP-C05	GATGACCGCC	21	21	100
OP-C15	GACGGATCAG	20	20	100
OP-N06	GAGACGCACA	26	26	100
OP-N08	ACCTCAGCTC	27	27	100
OP-N10	ACAACTGGGG	18	17	99.44
OP-N17	CATTGGGGAG	32	32	100
OP-N18	GGTGAGGTCA	24	24	100
OP-N19	GTCCGTACTG	27	27	100
OP-N20	GGTGCTCCGT	26	26	100
OP-U01	ACGGACGTCA	31	30	96.77
OP-U07	CCTGCTCATC	26	26	100
OP-U08	GGCGAAGGTT	26	25	96.15
OP-U10	ACCTCGGCAC	22	22	100
OP-U12	TCACCAGCCA	22	21	95.45
ISSR				
808	(AG)8C	22	22	100
809	(AG)8G	24	24	100
810	(GA)8T	26	26	100
811	(GA)8C	23	23	100
812	(GA)8A	26	26	100
816	(CA)8T	22	22	100
817	(CA)8A	25	25	100
818	(CA)8G	19	18	94.73
823	(TC)8C	17	17	100
824	(TC)8G	18	18	100
825	(AC)8T	19	19	100
830	(TG)8G	23	23	100
840	(GA)8YT	26	26	100
848	(CA)8RG	13	13	100
855	(AC)8YT	23	22	95.65
DAMD				
M13	GAGGGTGGCGGTTCCT	32	32	100
33.6	GGAGGTTTTTCA	27	24	88.88
HVR	CCTCCTCCCTCCT	29	29	100
HVA	AGGATGGAAAGGAGGC	32	32	100
HVY	GCCTTTCCCGAG	28	28	100
HVV	GGTGTAGAGAGGGGT	31	31	100

ISSR-PCR

In ISSR-PCR, a set of 100 anchored microsatellite primers procured from University of British Columbia, Canada were screened, out of which 15 primers that generated reproducible polymorphic profiles were selected (Table 3). PCR amplification of 50 ng DNA was performed in 10 mM Tris–HCl pH 7.5, 50 mM KCl, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM primer, and 0.9 U *Taq* DNA polymerase (Bangalore Genei, India) in a 25 µL reaction using PTC 200^{TM} thermocycler (MJ Research, Inc., USA). After initial denaturation at 94°C for 4 minutes, each cycle consisted of 1 minute denaturation at 94°C, 1 minute of annealing at 52°C, 2 minutes extension at 72°C along with 7 minutes extension at 72°C at the end of 35 cycles.

DAMD-PCR

Similarly, DAMD-PCR was carried out according to Zhou and Gustafson (1995) which involves the use of minisatellite core sequence as a primer singly in the amplification reactions. Six minisatellite core sequence primers (Custom synthesized from Bangalore Genei, India) were analyzed (Table 3). The reaction mixture (25 µL) contained 10 mM Tris–HCl (pH8.3), 50 mM KCl, 2 mM Mg^{2+} ion concentration, 200 µM each dNTP, 20 pmoles primer, 1 unit *Taq* polymerase (Bangalore Genei, India) and approximately 60 ng genomic DNA. Optimal DNA amplification was obtained through 40 cycles at different temperature and time duration (92°C for 1 min, 55°C for 2 min and 72°C for 2 min). The amplification of DNA was performed on a PTC-200^{TM} thermocycler (MJ Research, Inc. USA).

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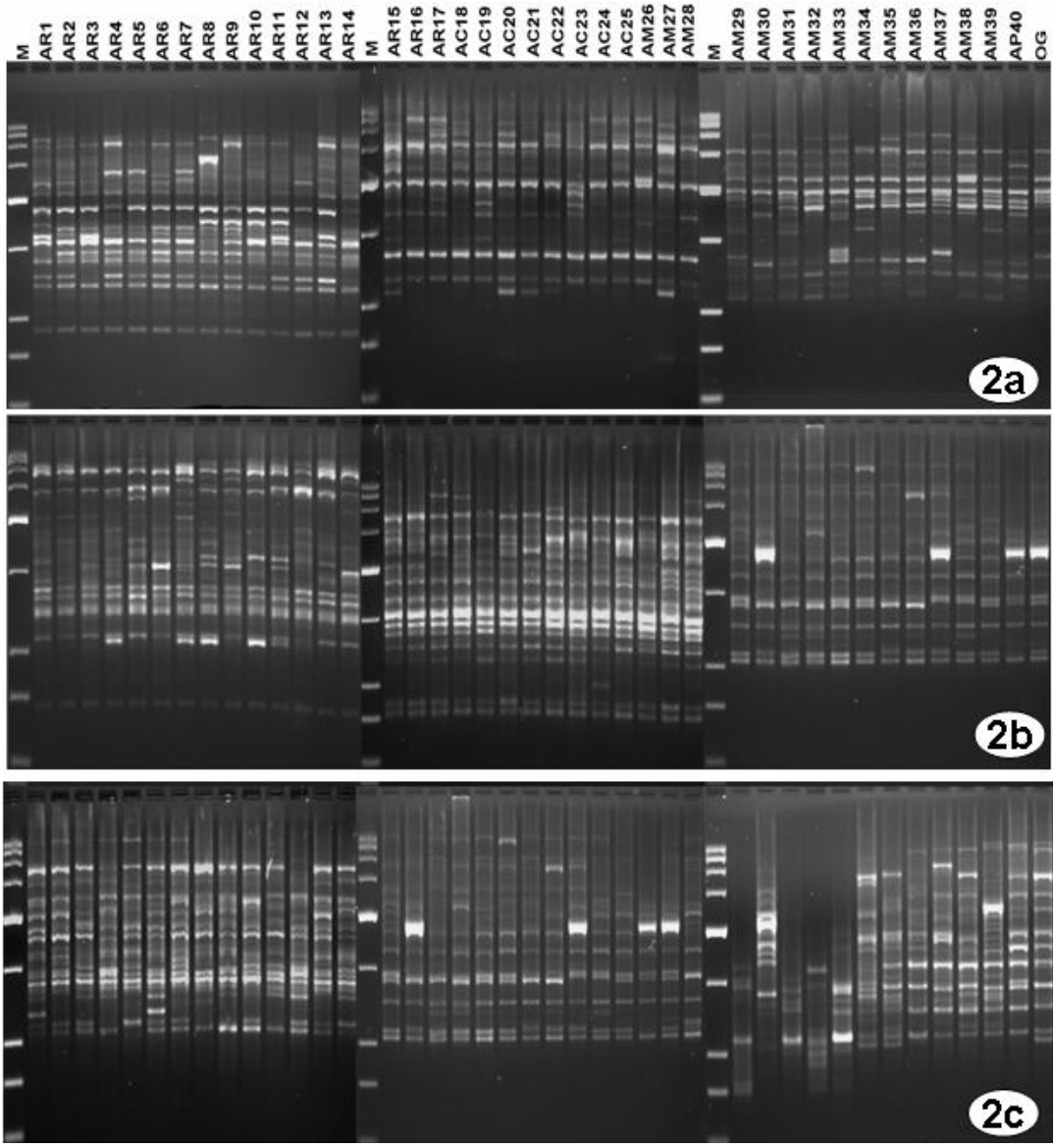


Fig. 2. Gel profiles obtained typically with 2a: RAPD primer (OP-C05). 2b: ISSR primer (810). 2c: DAMD Primer (M13). All profiles were resolved in 1.5% agarose gels in TBE. The lanes marked as Marker (M) contain the Low Range Ruler (Bangalore Genei, Bangalore) as DNA fragment size marker. The other lanes are marked with species abbreviation in Table 2.

Agarose gel electrophoresis

Amplified PCR products were separated on 1.5% (w/v) agarose gel in 0.5 Tris-Borate EDTA (TBE) buffer at 100 V for 3 h, stained with ethidium bromide and then visualized and photographed on a UV Transilluminator using a Gel Documentation System (UV Tech, UK). Amplified products were estimated on the gel by comparison with molecular weight marker low-range DNA ruler. The reproducibility of the amplification products was checked twice for each polymorphic primer. The representative gels for each marker system have been provided in Figure 2.

Data analysis

Unambiguous and reproducible bands were scored. The marker bands were defined by their molecular weights based on size standard. Only intense bands were scored assuming that co-migrating bands were allelic and bands of different molecular weights were non-allelic. Amplified products were scored as "1" to indicate presence and "0" to indicate absence of a band to form a binary matrix for all the species included in the study. To compare RAPD, ISSR and DAMD assays, data were analyzed to calculate various parameters such as the number of amplified loci, the number of polymorphic

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bands, percentage polymorphism. A pair wise matrix of distances between genotypes was determined for the band data from each method using Jaccard's (Jaccard, 1908) similarity coefficient in the Free Tree program (Pavlicek et al., 1999). From the pair wise distance data, the Neighbor-joining (NJ) trees were computed after allowing a 1000 replicate bootstrap test using the same program. The trees were viewed, annotated and printed using Tree View (ver. 1.6.5; page 2001).

RESULTS

The three independent methods such as RAPD, ISSR and DAMD were carried out to have a comprehensive idea of variability amongst the genotypes of four candidate species of *Astragalus*. These markers are mostly dominant and detect variations in both coding and non-coding regions of the genome. Therefore, individual as well as a combined data analyses of these marker systems were considered that revealed a comprehensive pattern of genetic divergence amongst the genotypes.

Out of 80 decamer primers screened, only 18 RAPD primers were selected to detect polymorphism based on their discrete and reproducible banding pattern. Total 449 bands were obtained out of which 442 bands were polymorphic. The number of bands per primer ranged from 18 (OP-N10) to 32 (OP-N17) with an average of 25 bands per primer. The size of fragments ranged from 200 bp-3000 bp. The percentage of polymorphism detected with all primers was 98.44 % (Table 3). In case of ISSR, a set of 100 primers were used for initial screening of all accessions of which 35 primers gave amplification out of which only 15 ISSR primers that generated reproducible banding pattern were selected to study inter and intra-specific variation among candidate species. A total 326 bands were obtained out of which 324 bands were found polymorphic which amounted 99.38 % polymorphism. Number of bands varied from 13 (848) to 26 (810, 812, 840) with an average of 22 bands per primer and the size of the fragment ranged from 250 bp–3kb. Similarly, six DAMD primers resulted 176 polymorphic bands out of total 179 bands that showed 98.32 % polymorphism. Size of bands ranges from 200 bp to 3 kb and the number of bands per primer varied from 27 (33.6) to 32 (M13, HVA) with an average of 30 bands per primer (Table 3). The cumulative band data generated through the above three Single Primer Amplification Reaction (SPAR) methods has resulted 99.01 % polymorphism across all genotypes.

Genetic similarity was calculated from Jaccard's similarity index value for all the 40 genotypes of *A. rhizanthus* complex considering RAPD, ISSR and DAMD approaches individually as well as combined. The similarity coefficient values ranged from 0.20 to 0.84 in RAPD, 0.25 to 0.82 in ISSR, 0.13 to 0.82 in DAMD and 0.23 to 0.82 in combined analyses. These values were used to construct dendrograms using NJ methods. The overall grouping of the genotypes of the species is similar in all marker systems and cumulative analysis except some variations in the orientation of genotypes within species (Figs. 3-6). In a cumulative analysis (Fig. 6) two main branches (1 & 2) have evolved with 100 % bootstrap values. The first branch (1) contains the genotypes of *A. malacophyllus* (supported by 98% bootstraps) and second branch (2) is further divided into two subclusters of *A. rhizanthus* (2a) and *A. candolleanus* (2b) with 97 % bootstraps values. However, *A. pindreensis* has been separated out distinctly with 100% bootstraps. *Oxytropis tatarica* Camb. ex Bunge was used as an out group in the study which has been clearly separated from the species of *A. rhizanthus* complex. *Oxytropis* is morphologically very allied to *Astragalus* and belongs to same subtribe *Astragalinae* in the tribe *Galegeae*, hence, it has been used as an out group.

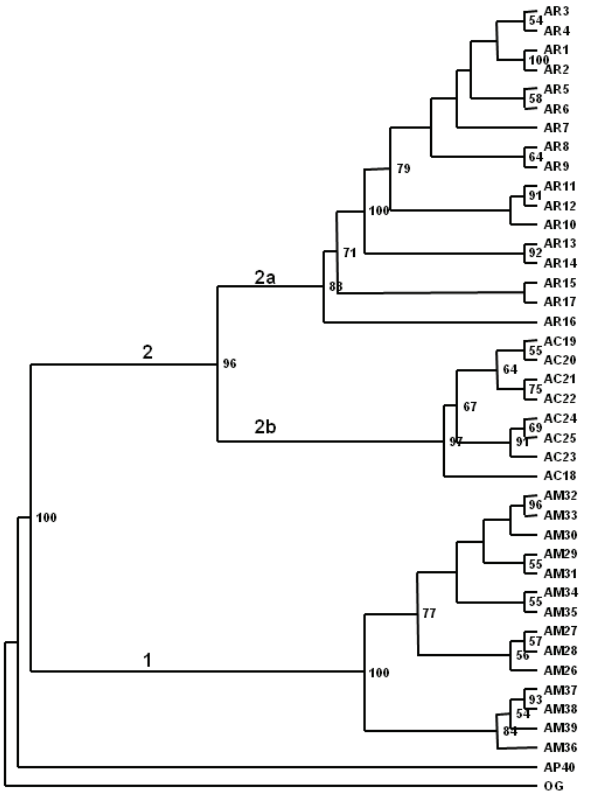


Fig. 3. NJ Tree generated on the basis of RAPD after 1000 bootstrap test. The accession names are abbreviated as in Table 2, and are indicated to the right side of each branch. The number at each node indicates the bootstrap percent having above than 50% values.

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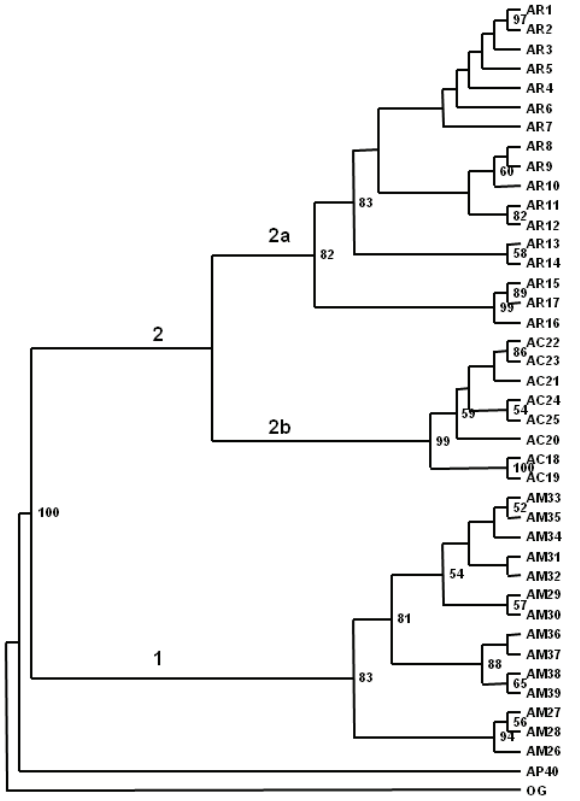


Fig. 4. NJ Tree generated on the basis of ISSR after 1000 bootstrap test. The accession names are abbreviated as in Table 2, and are indicated to the right side of each branch. The number at each node indicates the bootstrap percent having above than 50% values.

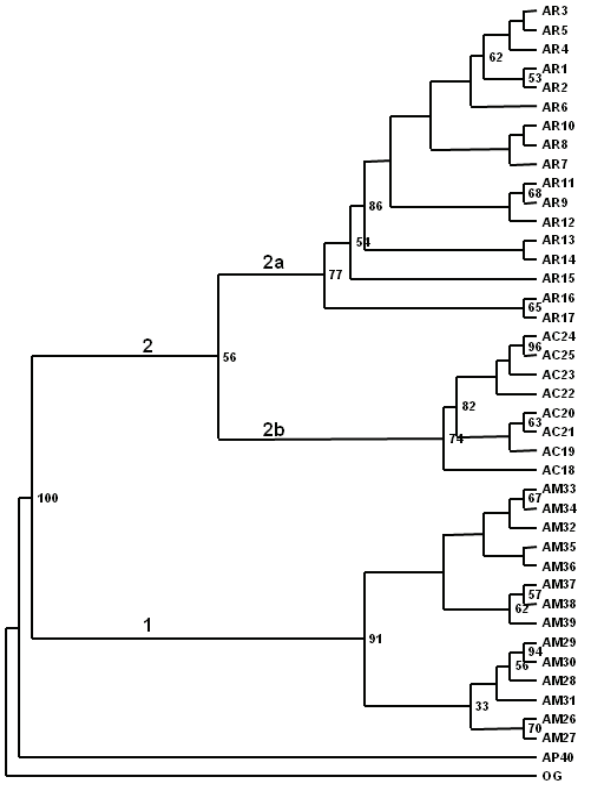


Fig. 5. NJ Tree generated on the basis of DAMD after 1000 bootstrap test. The accession names are abbreviated as in Table 2, and are indicated to the right side of each branch. The number at each node indicates the bootstrap percent having above than 50% values.

DISCUSSION

The RAPD, ISSR and DAMD data revealed high percentage of polymorphism between four species of *Astragalus* included in the present study. The greatest (0.82) and the smallest (0.23) distance observed in the study, exhibit 23% and 82% similarity between the species respectively. The data analysis revealed that 98.44 % bands in RAPD, 99.38% bands in ISSR and 98.32% bands in DAMD were polymorphic, while in a cumulative analysis 98.74% bands were observed polymorphic. Variation in the polymorphism in marker systems detected by different primers can be attributed to the fact that theirspecificity and efficiency are governed by nucleotide sequence, genomic DNA sequence and genome size. Effective PCR requires a perfect match between bases at the 3' end of the primer and DNA (Somner and Tautz, 1989).

In the intra-specific analysis, similarity found among accessions of each species ranged from 0.23-0.82. In further details, similarities coefficient value varied from 0.46-0.82 among *A. rhizanthus* accessions, 0.45-0.78 in *A. malacaophyllus*, 0.42-0.63 in *A. candolleanus* and 0.23-0.34 in *A. pindreensis*. The genetic similarity from the data matrix estimated by Jaccard's coefficient among four species of Indian *Astragalus* exhibited that all species are genetically distinct and the highest degree of genetic similarity was noticed in *A. rhizanthus* followed by *A. malacophyllus*, *A. candolleanus* and *A. pindreensis*. The present investigation has proved that these marker systems (viz. RAPD, ISSR and DAMD) have ability to distinguish the species in the genus *Astragalus*.

The groupings of the genotypes of the candidate species developed through NJ tree reflect the similarities of their genomic regions in all RAPD, ISSR and DAMD as well as in combined analyses. In comparison to individual marker, the cumulative profile obtained with more number of primers and more coverage of genome is reasonably quite attributable. The groupings of target species into four different clusters explain the distinctionamong the closely related species by PCR methods. The clustering of the candidate species into four groups clearly support our own as well as previous taxonomic treatments of the species done by Ali (1961,

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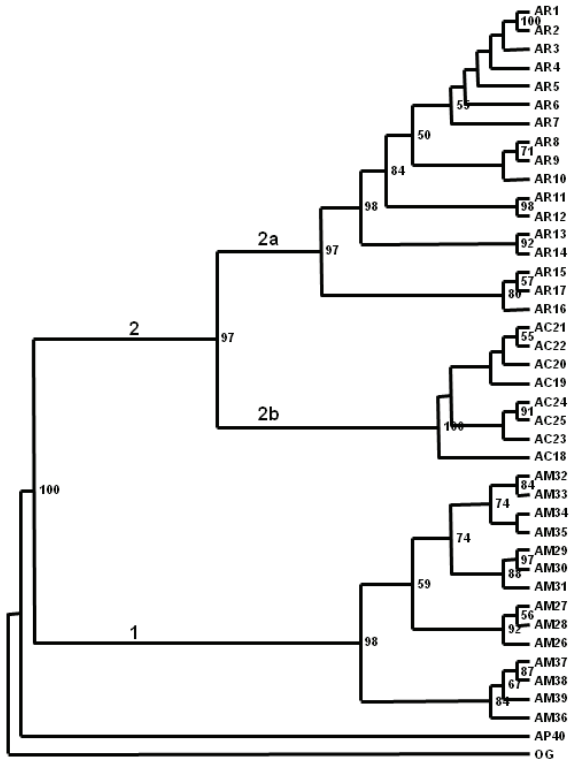


Fig. 6. NJ Tree generated on the basis of cumulative (RAPD, ISSR and DAMD) after 1000 bootstrap test. The accession names are abbreviated as in Table 2, and are indicated to the right side of each branch. The number at each node indicates the bootstrap percent having above than 50% values.

1977). However, Podlech (1988) in his taxonomic study has synonymised *A. malacophyllus* under *A. rhizanthus* while in the present study in both morphological and molecular marker systems these two species have come separately. The present findings suggest that these molecular techniques may be utilized for assessing diversity and species delimitation among the closely related species of *Astragalus*.

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利用分子標記 RAPD、ISSR 和 DAMD 來界定 *Astragalus rhizanthus* 複合群的物種

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摘要：從印度喜馬拉雅不同地區收集了四十個隸屬於 *Astragalus rhizanthus* 複合群中的四個相關物種 (*A. rhizanthus*、*A. candolleanus*、*A. malacophyllus* 和 *A. pindreensis*)；利用逢機擴增多型性 DNA (Random Amplified Polymorphic DNA, RAPD)、簡單序列重複間序列(Inter Simple Sequence Repeats, ISSR)和直接擴增小衛星 DNA (Directed Amplification of Minisatellite DNA, DAMD)來分析其種內及種間的關係。十八條 RAPD、十五條 ISSR 和六條 DAMD 引子用來研究物種間的遺傳歧異度，總共獲得 449 條 RAPD 條帶、326 條 ISSR 條帶以及 179 條 DAMD 條帶，其中將近 98.44%的 RAPD、99.38%的 ISSR 與 98.32%的 DAMD 條帶為多型性的。並利用 Jaccard 的相似度公式 (Jaccard's similarity coefficient) 計算出這些累計數據的相似性係數為 0.23-0.82，並得到一個鄰近連接 (Neighbor-joining) 樹狀圖。由 Jaccard 相似係數估算出的遺傳相似性結果與形態特徵及先前的分類處理相符合。其中 *A. rhizanthus* 的種內遺傳相似性最高，接著為 *A. malacophyllus*、*A. candolleanus* 最後為 *A. pindreensis*。本研究證實了這些分子標記能夠區分出關係密切的相近物種，並能夠分析其種內及種間的遺傳歧異度。

關鍵詞：黃耆屬、區分、豆科、逢機擴增多型性 DNA、簡單序列重複間序列、直接擴增小衛星 DNA。

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