Proc Indian natn Sci Acad **78** No. 2 June 2012 pp. 171-187

Research Paper

Reassessment of the Taxonomic Status of *Astragalus malacophyllus* Benth. ex Bunge (Fabaceae) Based on Morphology and nrDNA Internal Transcribed Spacer Sequence Data

KUMAR KAMAL ANAND[1*], SATYA NARAYAN JENA[2] and LAL BABU CHAUDHARY[1]
[1]Plant Diversity, Systematics & Herbarium Division, [2]Plant Molecular Biology and Genetic Engineering Division, CSIR-National Botanical Research Institute, Lucknow 226 001, India

(Received 03 November 2011; Revised 09 February 2012; Accepted 14 February 2012

Astragalus malacophyllus Benth. ex Bunge occurring in the N. W. Himalaya with restricted distribution is a well known medicinal plant 'Rudravanti' or 'Rudanti'. Sometimes, it is treated as a synonym of widely scattered and quite variable species *A. rhizanthus* Royle ex Benth. However, the critical morphological examination carried out on large number of herbarium specimens housed at different herbaria as well as plants in natural populations and molecular study based on nrDNA Internal Transcribed Spacer (ITS) separate them distinctly. A detailed account of both the species with photographs of habits and key to the species are presented here for their differentiation. In the present study, the ITS sequence of the candidate species has also been compared with its allied species *A. candolleanus* and *A. pindreensis*.

Key Words: Morphology; nrDNA ITS Sequences; Taxonomic Status; *Astragalus malacophyllus*

1. Introduction

The genus *Astragalus* L. with about 3000 species amongst 250 sections is chiefly distributed in the cold arid and semiarid mountains regions of Northern Hemisphere and South America [1-3]. In India, the genus contains *c.* 80 species belonging to 29 sections distributed in the Himalaya [4]. Among them, *A. malacophyllus* Benth. ex Bunge of the section *Caprini* DC. is considered highly medicinal. The species is well known as 'Rudanti' or 'Rudravanti' in Indian systems of medicine since ancient time for the treatment of blood impurities, diabetes, skin disease, cough and cold [4] and extensively used by local people especially in Gangotri-Gaumukh areas of N. W. Himalaya. However, the taxonomic status of the species is still uncertain as it has been treated differently by various workers since long. *A. malacophyllus* Benth. ex Bunge was described by Bunge in 1868 based on the specimens collected from the Himalaya. Later on, Parker [5] synonymised the species under *A. rhizanthus* Royle ex Benth. In the recent revision of the section *Caprini* DC., Podlech [6] has treated *A. rhizanthus* Royle ex Benth. in a broader concept with three infraspecific taxa (i. e. *A. rhizanthus* ssp. *rhizanthus* var. *rhizanthus*, *A. rhizanthus* ssp. *rhizanthus* var. *pindreensis* (Benth. ex Baker) Podl. and *A. rhizanthus* ssp. *candolleanus* (Royle ex Benth.) Podl.) and treated *A. malacophyllus* as a synonym under *A. rhizanthus* ssp. *rhizanthus* var. *rhizanthus* similar to Parker [5]. This treatment has been accepted in ILDIS (International Legume Database and Information System: http://www.ildis.org/) and Kumar & Sane [7]. However, Baker [8], Ali [9, 10] and Sanjappa [11] have treated *A. rhizanthus* and *A. malacophyllus* as two distinct species. While working on the taxonomy of the Indian *Astragalus*, one of us (LBC) also observed sufficient morphological characters between *A. malacophyllus* and *A. rhizanthus* [4] in spite of distinct variations in

*Author for Correspondence : E-mail: kamalanand53@rediffmail.com; Tel: + 91-0522-2205831-35

A. rhizanthus which have been dealt elaborately by Anand *et al.* [12]. Based on random markers (i.e., RAPD, ISSR and DAMD) analysis, Anand *et al*. [13] have also observed *A. malacophyllus* quite different from *A. rhizanthus* ssp. *rhizanthus* var. *rhizanthus* and their allied species like *A. candolleanus* and *A. pindreensis*. Here more specific and reliable marker nrDNA ITS has been used to ascertain the taxonomic status of *A. malacophyllus* along with detailed taxonomic study. Further, the ITS sequence of both the species have also been compared with their closely allied species like *A. candolleanus* and *A. pindreensis* to develop more robust and authentic data.

Genomics technology is rapidly improving for knowing the correct identity and reconstructing the phylogenies of closely related species. Non-coding regions of the nuclear genome typically present higher levels of molecular variation compared to chloroplast and mitochondrial DNA. Recently, sequencing of the ITS regions has provided a new source of nuclear DNA characters for inferring evolutionary relationships in many plant groups. The sequence comparison of the ITS region is commonly used in molecular systematics because it is easy to amplify even from small quantities of DNA and has a high degree of variation even between closely related species [14-15]. The molecular markers such as nrDNA ITS as well as chloroplast regions have been used by many workers to study the phylogenetic study in *Astragalus*, especially to verify the subgeneric and sectional morphological classification of the genus [16-23]. So far, only 212 species of *Astragalus* have been used in phylogenetic studies [21]. None of these phylogenetic studies carried out by various worker have investigated the species which have been included in the present study. Similarly, in other plant groups [24-25] also these techniques have been used for phylogenetic study.

2. Materials and Methods

2.1 Morphological Study

The taxonomic study of the candidate species *A. rhizanthus* and *A. malacophyllus* was conducted on a large number of herbarium specimens housed at different herbaria (BM, BSD, BSHC, CDRI, CAL, DD, K, LWG) to know the range of variation and taxonomic limit of the species. The species were also investigated in the field from almost throughout their range of distribution. The voucher specimens of our own collections have been deposited at LWG for future record.

2.2 Molecular Study

The fresh leaf tissues of all species included here were collected from their natural habitats in silica gel [26] for DNA extraction. The details of the accession numbers of the sequences of the target species along with their close relatives and one out group species are shown in Table 1. Nuclear DNA of silica gel dried leaf tissues was extracted using DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) as per the instructions of the manufacturer. The ITS region including 5.8S rRNA of selected eight accessions were amplified using universal primers [27] following the protocols of Kazempour Osaloo *et al.* [21]. Gel purified double stranded DNAs were used in cycle sequencing reactions that were conducted using the Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Inc., USA). The same nrDNA ITS primers ITS4 and ITS5 used for cycle sequencing reactions. The reaction pellet were resuspended in 10 µl of HiDye formamide and analyzed in an ABI's 3730 $_{XL}$ DNA automated sequencer (Applied Biosystems Inc., USA). The sequences were aligned using MEGA 4.0 [27]. A genetic distance matrix was calculated by Kimura-2-parameter [29] and phylogenetic trees were constructed (Fig. 6) applying Neighbor-joining (NJ) method [30]. Support values of the internal branches of NJ tree were evaluated through bootstrap method (500 replicates). *A. curvipes* Trautv. has been selected as an out group as it also belongs to the section *Caprini* DC. along with two other closely associated species *A. candolleanus* and *A. pindreensis* to make the analysis more robust and authentic. The ITS sequence of the out group were obtained from the DDJB (DNA data bank of Japan). Another phylogenetic tree has also been derived with nucleotide *BLAST* of the genus against NCBI database along with the target species to show the status of investigated species (Fig. 7).

Fig. 1: *Astragalus rhizanthus* Royle ex Benth. – (a) Habitat, (b) A close view.

3. Results

3.1. Taxonomic Treatment

Key to *Astragalus rhizanthus* and *A. malacophyllus*

1a. Plants stemless or very occasionally with short stem (hardly reaches up to 10 cm long), grow singly or tufted with several short stems at the top of rootstock; leaf rachis neither persistent nor indurated, not spiny at tip; leaflets generally glabrous on upper surface, moderately pilose on lower surface, persistent; terminal leaflet never reduces into needle like structure and does not fall; flowers always aggregate at the tip of stem and easily visible; style of carpel generally glabrous throughout or slightly hairy below ..…..…… ***A. rhizanthus***

1b. Plants always with long stems (up to 90 cm long), usually caespitose, branched and forming busy habit; leaf rachis persistent and subindurated, subspiny at tip after the fall of terminal leaflet; leaflets densely tomentose on both surfaces, deciduous; terminal leaflet reduces in size and sometimes converted into needle like structure and falls before lateral leaflets; flowers on the peduncle in the axils of leaves and generally not visible as they concealed in the busy habit; style of carpel hairy throughout ….....… ***A. malacophyllus.***

Enumeration of the Species

Astragalus rhizanthus Royle ex Benth. in Royle, Illust. Bot. Himal. Mount. 200. 1835 ssp. rhizanthus var. rhizanthus; Baker in Hook.f., Fl. Brit. India 2:131. 1876; Ali in Biologia 7: 57. 1961 & in Nasir & Ali, Fl. W. Pakistan 100: 170. 1977; Podlech in Mitt. Bot. Staatss. Munchen 25: 177, f.19. 1988 *p. p.* (excluding syn. *A. malacophyllus* Benth. ex Bunge); Sanjappa, Legum. India 93. 1992; Kumar & Sane, Legum. South Asia: Checkl. 239. 2003 *p. p.* (excluding syn. *A. malacophyllus* Benth. ex Bunge). *Tragacantha rhizantha* (Royle ex Benth.) Kuntze, Rev. Gen. 2: 947. 1891. *Lectotype* (Ali, 1961): Leeo in Kunawur, Royle (LIV).

Perennial herbs, stemless or tufted with several minute to 3-10 cm long stems arising from the top of rootstock, internodes indistinct, completely covered with stipules and leafbase, white pilose, glabrous to glabrescent with age. *Stipules* 5-17 x 1-4 mm, persistent, adnate to the petiole at base, ovate–lanceolate, acuminate at tip, sparsely to densely pilose outside, glabrous inside, sometimes only ciliate along margins, prominently mult-veined. *Leaves* (3.5-)(8.5-22)(-31) cm long, very close and crowded together particularly towards the tip of stem, imparipinnate; petioles (3-) 4.5-7.5 cm long, white pilose with spreading or more or less forwardly oriented hairs; rachis (5.5-) 10-16 (-23) cm long, filiform, neither persistent nor indurated, hairy as petioles; leaflets (29-45, (3-) 10-15 (-19) x 3.5-6.5 mm, quite variable, subsessile, opposite, subopposite or alternate, narrowly to broadly obovate or ovate, lanceolate, oblanceolate, oblong, oblong-elliptic or sometimes almost orbicular, cuneate at base, entire along margins, acute, obtuse-apiculate, rounded to subtruncate–mucronate or occasionally emarginate at apex, glabrous or sometimes moderately pilose above, moderately to densely white, spreading, pilose below, terminal leaflet variable, usually smaller in size than other leaflets but never becoming needle and not deciduous like. *Inflorescence* aggregate at tip of stems, condensed-raceme, sessile to shortly peduncled, 4-8-flowered; peduncles up to 10 mm long, spreading or adpressed white pilose. *Bracts* 4-8 (-15) mm long, equal to or longer than pedicels, linear to linear-lanceolate, ciliate along margins, pilose outside, glabrous inside; bracteoles sometimes present on pedicel, 2, *c.* minute to 2 mm long. *Flowers c.* 18-28 mm long (excluding pedicels), yellow; pedicels 3-6 mm long, white pilose with spreading hairs. *Calyx* (-10) 12-18 mm long, persistent, tubular, splitting at maturity, pilose with white, spreading hairs outside, tube 7-10 mm long, lobes (-2) 5-8 mm long, usually shorter but sometimes ± equal to tube, subequal, shorter on dorsal side, linear. *Corolla* glabrous; standard 18-23 mm long, lamina 9-12 x 6-10 mm, broadly obovate or oblong-elliptic, emarginate at tip, claw 8-11 x 1.5 mm; wing petals 17-23 mm long, shorter than or ± equal to standard, lamina 8 x 4 mm, oblong, rounded at tip, upper auricle *c.* 1 mm long, lateral pockets present, claw 9 mm long; keel petals 13.5-19 mm long, shorter than wing petals, lamina 5-5.5 x 3.5 mm, curved in upper part

Fig. 2: ***Astragalus rhizanthus*** **Royle ex Benth. – Habit**

on ventral margins, upper auricle minute, lateral pockets present, claw 8.5-9 mm long. *Stamens* diadelphous, (9+1); vexillary filament *c.* 12 mm long, free, filiform; staminal sheath *c.* 11 mm long, slit on dorsal side, obtuse at tip, with 1-2 mm long upper free filaments; anthers dorsifixed. *Ovary c.* 4 mm long, linear, minutely stipilate (stipe 1.5 -2 mm long), densely sericeous, *c.* 18-ovuled; style *c.* 8 mm long, incurved in upper portion, glabrous; stigma capitate, glabrous. *Pods* 12-20 x 6-7 mm long, subsessile, oblong, straight, turgid, abruptly tapering into a *c.* 3 mm long beak at tip, moderately to densely white pilose with spreading hairs, dehiscent along dorsal suture, bilocular,. *Seeds* 2.5-3.2 x 2 mm, reniform, turgid, brown or blackish-brown, smooth, glabrous.

Distribution: India (Jammu & Kashmir, Himachal Pradesh), Nepal, Pakistan, Afghanistan, China.

Phenology: July–Aug. (-Sept.)

Habitat: Widely scattered on stony or grassy hill slopes, along river sides and agriculture fields in sandy and hard soils, at altitudes ranging from 3000-5400 m.

Specimens examined: INDIA, JAMMU & KASHMIR: Karakoram: Sokha Glacier, right bank, 214-15,000 ft, 24.8.1939, *R. Scott Russell* 1705 (BM); Kero Lugma Glacier, left bank, 10000 – 11000 ft., 26.7.1939, *R. Scott Russell* 1352 (BM); Haramosh range, Mani Basin, 12.8.1957, *R. C. Culbert* 11 (BM); Without locality, 11500 ft., 14.8.1876, *C. B. Clarke* 30470 (BM); Sinel valley below Baltal, 9 – 10,000 ft., 27.6.1992. *J. F. Duthie* 11572 (BM); Sassir Pass, 14,700 ft., July 1925, *Meinertzhagem s. n.* (BM); Ladak, Leh, Khardung La 15500', 18.6.1941, *Ludlow & Sherriff* 8424 (BM); Ladakh, Rungdum Gompa, 13,000 ft., July, 1905, *A. Meebold* 1367 (CAL); Ladakh, Drass, 3100 m, 4.8.1980, *U. C. Bhattacharyya* 71363 (BSD); Dras, 10000 ft., 7.6.1977, *J. D. A. Stainton* 7793 (BM); Kashmir, *N. C. Nair* 37015 (BSD); Gulmarg, *Prescott - Decie s. n.* (BM); Dras, 11000 ft., 30-31.8.1922. *R. R. Stewart* - separated from no. 7402 (BM); East of Zojila River, 3250 m, 16.7.1976, *B. M. Wadhwa* 58621 (BSD: photo LWG); sankoo, 3100 m, 21.8.1976, B. M. Wadhwa 59845 – holo and isotype of *A. ladakhense* (BSD). Kashmir, Pachtsani, 3600 m, 27.7.1966, *N. C. Nair* 37015 (BSD); Ladakh, Lakong, 4500 m, 23.7.1973, *U. C. Bhattacharyya* 52183 (BSD); Ladakh, Matyan, 3330 m, 17.7.1976, *B. M. Wadhwa* 58688 (BSD); Ladakh, between Leh & Khardunga La, 5000 m, 8.8.2003, *L. B. Chaudhary & Z. H. Khan* 225052 (LWG); Pahlgam, 7000 ft.,1909, *Douie* 1 (CAL); Zojila, 13,000 ft., 31.07.1891, *G. A. Gammie* (CAL); Kashmir, 1864, *Falconer* 423 (CAL); Gilgit, 1885, *G. M. Giles* (CAL); Kungwatan, 3000 m, 9. 6.1959, *T. A. Rao* 9286 (BSD); Bamaj nullah near Sapphire mines, Kishtwar, 11000 ft, 11.7.1943, *Ludlow & Sheriff* 9179 (BM); Bringli valley, 9000 ft., 15.5.1939, *F. Ludlow* 16 A (BM); Kishenganga valley and the road to Naga Parbat, Rattu to Rupal Nullah, 8500 ft., Aug. 22, 1939, *R. R. & I. D. Stewart* 18853 (BM). HIMACHAL PRADESH: Lahul – Spiti: Khoksar to Sissu, 10,500 ft., 29.07.1935, *Mohindernath* (CAL); Pagni, *Stoliczka* (CAL); Pangi, Bhut Nullah, Tau, 11,000 ft., 19.6.1981, *C. D. Sayers* 3731(BM); Gondla, 3000 m, 15.9.2001, *L. B. Chaudhary* 220669 (LWG); Khoksar, 3200 m, 16.9.2001, *L. B. Chaudhary* 220678 (LWG); Khoksar, 3150 m, 2.8.2002, *L. B. Chaudhary & Z. H. Khan* 206737 (LWG); Khoksar, 3300 m, 3.8.2003, *L. B. Chaudhary & Z. H. Khan* 225004 (LWG); 4 km away from Khoksar, 3200 m, 3.8.2003, *L. B. Chaudhary & Z. H. Khan* 225007 (LWG); Khoksar, 3200 m, 23.8.1982, *B. S. Aswal* 11059 (CDRI); Khoksar, 3200 m, 2.7.1943, *U. C. Bhattacharyya* 51729 (BSD); Khoksar-Sissu, 3400 m 26.5.1957, *M. A. Rau* 5877 (BSD); Khoksar, 3200m, 18.7.1972, *U. C. Bhattacharyya*, 48679 (BSD); Khoksar, 3200 m, 4.8.1970, *U. C. Bhattacharyya* 40524 (BSD); Khoksar-Sissu, 3400 m, 26.6.1958, *M. A. Rau* 5877 (BSD); near Chhatru, 3 Km before from Khoksar side, 3300 m, 3.8.2002, *L. B. Chaudhary & Z. H. Khan* 206746 (LWG); Sissu, 3200 m, 17.7.1977, *B. S. Aswal* 6790 (CDRI); Sissu-Gondla, 3300 m, 25.7.1979, *B. S. Aswal* 10475 (CDRI); between Gondla & Tandi, 3100 m, 3.8.2003, *L. B. Chaudhary & Z. H. Khan* 225014 (LWG); Darcha river bed, 3600 m, 15.8.1982, *B. S. Aswal* 10948 (CDRI); Jhala, 12,000 ft, 15.6.1941, *N. L. Bor* 9722 (DD); Chotadhara, 3900 m, 21.7.1972, *U. C. Bhattacharyya* 48775 (BSD);

Fig. 3: *Astragalus malacophyllus* Benth ex Bunge – (a) Habitat, (b) A close view

Fig. 4: ***Astragalus malacophyllus*** **Benth ex Bunge – Habit**

Chotadhara, 3700 m, 3.8.2002, *L. B. Chaudhary & Z. H. Khan* 206750 (LWG); Chotadhara, 3900 m, 21.7.1972, *U. C. Bhattacharyya* 48775 (BSD); Pangi, *Mrs. Douie* 16 (CAL). 11 Km. before Kunzom towards Khoksar side, 4000 m, 6.8.2002, *L. B. Chaudhary & Z. H. Khan* 206788 (LWG); near Patsio, 3800 m, 4.8.2003, *L. B. Chaudhary & Z. H. Khan* 225021 (LWG); Rakcham, on left bank of river near glacier (Baspa valley), *c.*11500, 20.5.1972, *K. P. Janardhanan* 47343 (BSD); Chini, 3200 m, 4.6.1962, *N.C. Nair* 22410 (BSD); Lahul, 3200 m, 2.8.1971 *U. C. Bhattacharyya* 45004 (BSD); Trilokinath, 20.8.1985. *B. S. Aswal* 15356 (CDRI); Sangla near Thinb (Baspa valley), *c.* 2600 m, 16.5.1972, *K. P. Janardhanana* 46757 (BSD); Nagasti hill slope above, Intelligence bureau barracks (Baspa valley), *c.* 3750 m, 25.5.1972, *K. P. Janardhanan* 47449 (BSD); Chitkul near yak form (Baspa valley)

24.5.1972, *K. P. Janardhanan* 47415 (BSD); Chitkul, Baspa valley, 12500', 17.7.1939, *G. Sherriff* 7435 (BM); Spiti, Pin Valley National Park, Tariya – Pin Parvati Pass, 4300 m, 24.7.2003, *K. Chandra Sekhar* 103672 (BSD); Spiti, Pin Valley National Park, Chhohem, 4100 m, 13. 7. 2003, *K. Chandra Sekhar* 103676 (BSD); Spiti, Pin Valley National Park, Chhohem, 4400 m, 14.7.2003, *K. Chandra Sekhar* 103692 (BSD). Before Khoksar, 3814 m, 28.7.2006, *L. B. Chaudhary, T. S. Rana & K. K. Anand*, 229407 (LWG); Before Khoksar, 3814 m, 28.7.2006, *L. B. Chaudhary, T. S. Rana & K. K. Anand*, 229408 (LWG); Before Khoksar, 3814 m, 28.7.2006, *L. B. Chaudhary, T. S. Rana & K. K. Anand*, 229409 (LWG); Khoksar proper in potato field, 3136 m, 28.7.2006, *L. B. Chaudhary, T. S. Rana & K. K. Anand*, 229416 (LWG); Khoksar proper towards river side, 3136 m, 28.7.2006, *L. B. Chaudhary, T. S. Rana & K. K. Anand*, 229417 (LWG); Khoksar, 3134 m, 28.7.2006, *L. B. Chaudhary, T. S. Rana & K. K. Anand*, 229421 (LWG); Khoksar, 3134 m, 28.7.2006, *L. B. Chaudhary, T. S. Rana & K. K. Anand*, 229422 (LWG); Gondla, 2905 m, 30.7.2006, *L. B. Chaudhary, T. S. Rana & K. K. Anand*, 229463 (LWG); Gondla, 2905 m, 30.7.2006, *L. B. Chaudhary, T. S. Rana & K. K. Anand*, 229464 (LWG); Gondla, 2905 m, 30.7.2006, *L. B. Chaudhary, T. S. Rana & K. K. Anand*, 229466 (LWG); Gondla, 2905 m, 30.7.2006, *L. B. Chaudhary, T. S. Rana & K. K. Anand*, 229468 (LWG); Near Trilokinath temple, 3845 m, 30.7.2006, *L. B. Chaudhary, T. S. Rana & K. K. Anand*, 229476 (LWG); Between Gondla and Sissu, 2996 m, 30.7.2006, *L. B. Chaudhary, T. S. Rana & K. K. Anand*, 229480 (LWG); Chota Dara, 3475 m, 31.7.2006, *L. B. Chaudhary, T. S. Rana & K. K. Anand*, 229485 (LWG); Between Chatru and Chota Daraa, 3027 m, 31.7.2006, *L. B. Chaudhary, T. S. Rana & K. K. Anand*, 229493 (LWG); Near Chatru, 3034 m, 31.7.2006, *L. B. Chaudhary, T. S. Rana & K. K. Anand*, 229498 (LWG); Gramphu, 2996 m, 31.7.2006, *L. B. Chaudhary, T. S. Rana & K. K. Anand*, 229500 (LWG).

Note: A quite variable species especially in the length of stem, leaf and bracts, shape, size and numbers of leaflets, flower size, length of calyx lobes, density of hairs on different parts, etc. All these variations have been found within the same population or sometimes even on the same plant. Usually, the calyx lobes are shorter than tube, however, they may be more or less equal too.

Astragalus malacophyllus Benth. ex Bunge in Mem. Acad. Sci. St.-Pet. ser. 7, 11: 36. 1868 & 12: 61. 1869; Baker in Hook.f., Fl. Brit. India 2: 133. 1876; Ali in Biologia 7: 54.1961 & in Nasir & Ali, Fl. W. Pakistan 100: 168, f. 17 N. 1977; Sanjappa, Legum. India 91. 1992. *Tragacantha malacophylla* (Benth. ex Bunge) Kuntze, Rev. Gen. 2: 946. 1891. *A. rhizanthus* Royle ex Benth. ssp. *rhizanthus* var. *rhizanthus* sensu Podlech in Mitt. Bot. Staatss. Munchen 25: 177. 1988 p. p. (only *A. malacophyllus*) *Lectotype* (Podlech, 1988): In Himalaya Bor. Occ., 8 – 10000 ft., Thomson (P; iso: K!).

Perennial herbs with well developed stems and busy habit, caespitose, prostrate; *stems* up to 90 cm long with condensed to distinct internodes, branched, white tomentose. *Stipules* 6-18 x 5-3 mm, persistent, shorter to longer than internodes, adnate to the petiole at base, lanceolate to narrowly lanceolate with acute to very long narrow tip, prominently multi-veined, white pilose with spreading hairs outside, glabrous inside. *Leaves* 5.5-19 cm long, imparipinnate; petioles 2-3.5 cm long; rachis 8-15 cm long, persistent, subindurated, subspiny at tip after the fall of terminal leaflet, white pilose with spreading hairs; leaflets 37-43, close, 3-11 x 2-5 mm, subsessile, obovate, oblong, oblong-elliptic or almost orbicular, cuneate at the base, entire along margins, acute, subobtuse, obtuse or subtruncate at apex, tomentose on both surfaces; terminal leaflet smaller in size than lateral ones and sometimes reduced into needle like structure. *Inflorescence* axillary, shortly peduncled 3-5-flowered raceme; peduncles *ca.* 2-2.5 cm long, mixed white and black (predominantly white) pilose with spreading hairs. *Bracts* 6-10 mm long, linear, longer than pedicels, sparsely pilose outside, glabrous inside, white ciliate along margins. *Flowers* 17-23 mm long, yellow; pedicels 4-5 mm long, mixed black & white tomentose. *Calyx* 12-14 mm long, persistent, tubular, mixed brown and white tomentose outside, glabrous inside, tube 7-10 mm long, longer than lobes, lobes 3-5 mm long, subequal, shorter on dorsal side, linear.

Table 1: Collection details of the plant materials with NCBI/DDJB accession number and length of ITS region

Species	Locality	Longitude/ latitude	Altitude	Collection number	NCBI/DDJB Accn. No.	Total ITS length (bp)
*A. candolleanus*1	Kunjam pass, Spiti (Himachal Pradesh)	N 32° 26.590' E 76° 44.573'	4106 m	229506	GU238423	622
A. candolleanus 2	Gondla, Lahul (Himachal Pradesh)	N 32° 36.286' E 76° 55.996'	2905 m	229465	GU238424	622
A. malacophyllus 1	Gangotri to Chirbasa (Uttarakhand)	N 30° 58.827' E 79° 01.855'	3343 m	229547	GU238430	626
A. malacophyllus 2	Between Chirbasa to Bhojwasa (Uttarakhand)	N 30° 58.389' E 79° 02.063'	3680 m	229545	GU238425	636
A. pindreensis 1	Vasundhara Fall, Badrinath (Uttarakhand)	N 30° 33.050' E 79° 36.024'	3500 m	225088	GU238428	629
A. rhizanthus 1	Gondla, Lahul (Himachal Pradesh)	N 32° 36.286' E 76° 55.996'	2905 m	229463	GU238426	644
A. rhizanthus 2	Near Triloki Nath temple, Lahul (Himachal Pradesh)	N 32° 47.265' E 77° 17.099'	3845 m	229476	GU238429	639
A. rhizanthus 3	Chota Dara, Lahul (Himachal Pradesh)	N 32° 18.035' E 77° 27.704'	3475 m	229485	GU238427	625
A. curvipes	Maass. Iran	14° E 13° N 94° E 53° N	11,000 ft.	47553	AB051955	603

Corolla glabrous; standard 17-22 mm long, deflexed, lamina 14 x 8 mm, obovate, emarginate at apex, claw *ca*. 8 mm long; wing petals *ca.* 21 mm long, more or less equal to standard, lamina 9 x 2.5 mm, oblong, obtuse at apex, upper auricle ca. 1 mm long, lateral pockets present, claw 12 mm long; keel petals *ca.* 16 mm long, further shorter than wing petals, lamina 5 x 3.5 mm, almost oblong, slightly incurved towards apex on lower margins, subacute at apex, upper auricle minute, lateral pockets present, claw 11 mm long. *Vexillary filament ca.* 15 mm long; staminal sheath ca. 12 mm long, obtuse at apex, with 2-3 mm long upper free filaments; anthers dorsifixed. *Ovary ca.* 6 mm long, hairy, stipitate (stipe *ca.* 2.5 mm long); style slightly incurved towards apex, hairy almost throughout; stigma capitate, glabrous. *Pods* 12-17 x 6-7 mm, subsessile, oblong, straight, turgid, abruptly pointed at apex with 2-3 mm long beak, hairy, dehiscent along dorsal suture, partially bilobed, stipe *c*. 1 mm long. *Seeds* 3-4 x 2-3 mm, oblong to orbicular-reniform, brown, turgid, glabrous.

Distribtuion – India (Jammu & Kashmir, Himachal Pradesh, Uttarakhand), Pakistan.

Phenology: (June-) July-Sept.

Habitat: On open or shady mountain slopes in dry as well as in moist sandy soils among boulders and under *Pinus* tree, at the altitudes ranging from 2600-4000 m.

Specimens examined: INDIA, JAMMU & KASHMIR: Kashmir, Nagmarg, 9,000 ft., 26.06.1913, *F. E. Koebel* 16 (CAL). HIMACHAL PRADESH: Baspa valley, Ranikhanda, 3800 m, 25.5.1972, *K. P. Janardhanan* 47473 (BSD); Pangi, Chakot forest, 9000-10,000 ft., 16.08.1897, *J. H. Lace* 1634 (CAL); Pangi, *Mrs Dovie* 25 (CAL); Lahul, Bhaga valley, below Kelong, 11,000 ft., 4.08.1901, *T. H. Holland* (CAL); Chamba, August, 1880, *R. Ellis* 382 A (CAL). UTTARAKHAND: Uttarkashi: Gangotri, 11- 12,000 ft. Oct., 1881, *J. F. Duthie* 1491 (CAL, BM); Bhojwasa to Gangotri, 31.7.1987, *B. P.*

Uniyal & B. Balodi 80542 (BSD); Gangotri, 28.6.1986, *K. M. R. Raghuvanshi* 2 (LWG); Gangotri to Chirbasa, 1.6.1992, *B. Dutt* & *Brij Lal* 212240 (LWG); Chirbasa, 1.9.2000, *V. Kumar* 219365 (LWG); Gaumukh, 3000 m, 3.6.1980, *B. S. Aswal* 6489 (CDRI); Gangotri, 3100 m, 30.8.1983, *U. C. Bhattacharyya* 74668 (BSD); Chirbasa, 3500 m, 5.9.1983, *U. C. Bhattacharya* 74945 (BSD); Bhojwasa – Gaumukh, 3750 m, *U. C. Bhattacharya* 74774 (BSD); Chirbasa – Gangotri, 3000 m, *M. A. Rau* 51649 (BSD); Gangotri, 3300 m, 5.10.1967, *B. D. Naithani* 37498 (BSD); Gaumukh, 3922 m, 22.9.1964, *B. D. Naithani* 37407 (BSD); Between Chirbasa to Bhojwasa, 3680 m, 4.09.2006, *L. B. Chaudhary, K. K. Anand & R. K. Srivastava*, 229545 (LWG); Gangotri to Chirbasa, 3343 m, 4.09.2006, *L. B. Chaudhary, K. K. Anand & R. K. Srivastava*, 229547 (LWG); Chirbasa, 3313 m, 4.09.2006, *L. B. Chaudhary, K. K. Anand & R. K. Srivastava*, 229551 (LWG); Gangotri to Chirbasa, 3277 m, 4.09.2006, *L. B. Chaudhary, K. K. Anand & R. K. Srivastava*, 229552 (LWG); Gangotri to Chirbasa, 3171 m, 4.09.2006, *L. B. Chaudhary, K. K. Anand & R. K. Srivastava*, 229554 (LWG); Gangotri to Chirbasa, 3241 m, , 4.09.2006, *L. B. Chaudhary, K. K. Anand & R. K. Srivastava*, 229555 (LWG); Gangotri, 2509 m, 3.09.2006, *L. B. Chaudhary, K. K. Anand & R. K. Srivastava*, 229557 (LWG); Gangotri, 2509 m, 3.09.2006, *L. B. Chaudhary, K. K. Anand & R. K. Srivastava*, 229558 (LWG); Bhojwasa , 3871 m, 5.09.2006, *L. B. Chaudhary, K. K. Anand & R. K. Srivastava*, 229560 (LWG); Bhojwasa, 3871 m, 5.09.2006, *L. B. Chaudhary, K. K. Anand & R. K. Srivastava*, 229561 (LWG); Bhojwasa, 3871 m, 5.09.2006, *L. B. Chaudhary, K. K. Anand & R. K. Srivastava*, 229562 (LWG); Bhojwasa, 3871 m, 5.09.2006, *L. B. Chaudhary, K. K. Anand & R. K. Srivastava*, 229563 (LWG); Bhojwasa, 3871 m, 5.09.2006, *L. B. Chaudhary, K. K. Anand & R. K. Srivastava*, 229564 (LWG); Bhojwasa, 3871 m, 5.09.2006, *L. B. Chaudhary, K. K. Anand & R. K. Srivastava*, 229565 (LWG); Tihri – Garhwal; Nila valley, 10,000 ft., 19. 06. 1883, *J. F. Duthie* 991 (CAL-student herb); Ganges valley, near Jungla, 8000-9000 ft., 16. 7. 1883, *J. F. Duthie* 991a (BM).

3.2. Molecular Analysis

Alignment of all ITS sequences resulted in a matrix of 656 characters, out of which 20 positions from ITS1 (encoded at 1-20) and 8 positions from ITS2 (encoded at 648-656) were deleted prior to making phylogenetic analysis to devoid of alignment ambiguities (Fig. 5). After introducing gaps and eliminating ambiguous characters, a 628 bp matrix of ITS region were generated with 581 conserved characters, 47 variable characters, 21 parsimony informative characters and 23 singleton characters using *MEGA*4 software. The aligned sequences contained the conserved region of 164 bp of 5.8S rRNA encoded at 253 to 416 positions**.** There were 6, 10, 13 and 18 potentially phylogenetic species-specific informative sites were found in *A. pindreensis*, *A. candolleanus*, *A. rhizanthus* and *A. malacophyllus* respectively and length of ITS region along with conserved region, GC content (%), variable nucleotide sites and potentially phylogenetic informative sites have been discussed in Table 2.

Total six deletions have been observed in entire ITS sequence within target species. In ITS1, three single base pair deletions have been observed at 129, 130 and 193 positions in all species except *A. pindreensis* where one species specific insertion was observed at 193 position, while one another species specific deletion of one base pair has also been observed at position number 234 in *A. rhizanthus*. Similarly, three deletion of one base pair at 433, 476 and 523 positions have been noticed in all species in ITS2, whereas in *A. rhizanthus* species specific insertions have been found at the same positions. However, one base pair species specific deletion was noticed in *A. pindreensis* at 492 position. Multiple substitutions of varying lengths were found in all genotypes of *A. rhizanthus* (1 bp at 192 and 2bp at 194-195 in ITS1 and 1bp at 485 and 2bp at 482-483 in ITS2) and *A. malacophyllus* (1 bp at 189 in ITS1 and 1bp at 473, 481, 491 and 2 bp at 428-429, 458-459, 477-478 in ITS2) and *A. pindreensis* (1 bp at 231, 242 and 244 in ITS1), however, no substitution has been observed in case of *A. candolleanus* (Fig. 5).

In the NJ based phylogenetic tree, *A. malacophyllus* has distinctly segregated out in a separate clad with 99% bootstrap value from three species like *A. rhizanthus, A. candolleanus* and *A. pindreensis* with an average of pairwise distance 0.055, 0.013 and 0.08 respectively in kimura-2 parameter (Fig. 6). A total of seven (one in ITS1 and six in ITS2) species specific substitutions were observed which supports the distinct identity of the species. The species also differs in the percentage of overall GC content (52.77%) in comparison to other species. Similarly, *A. pindreensis* also forms a distinct

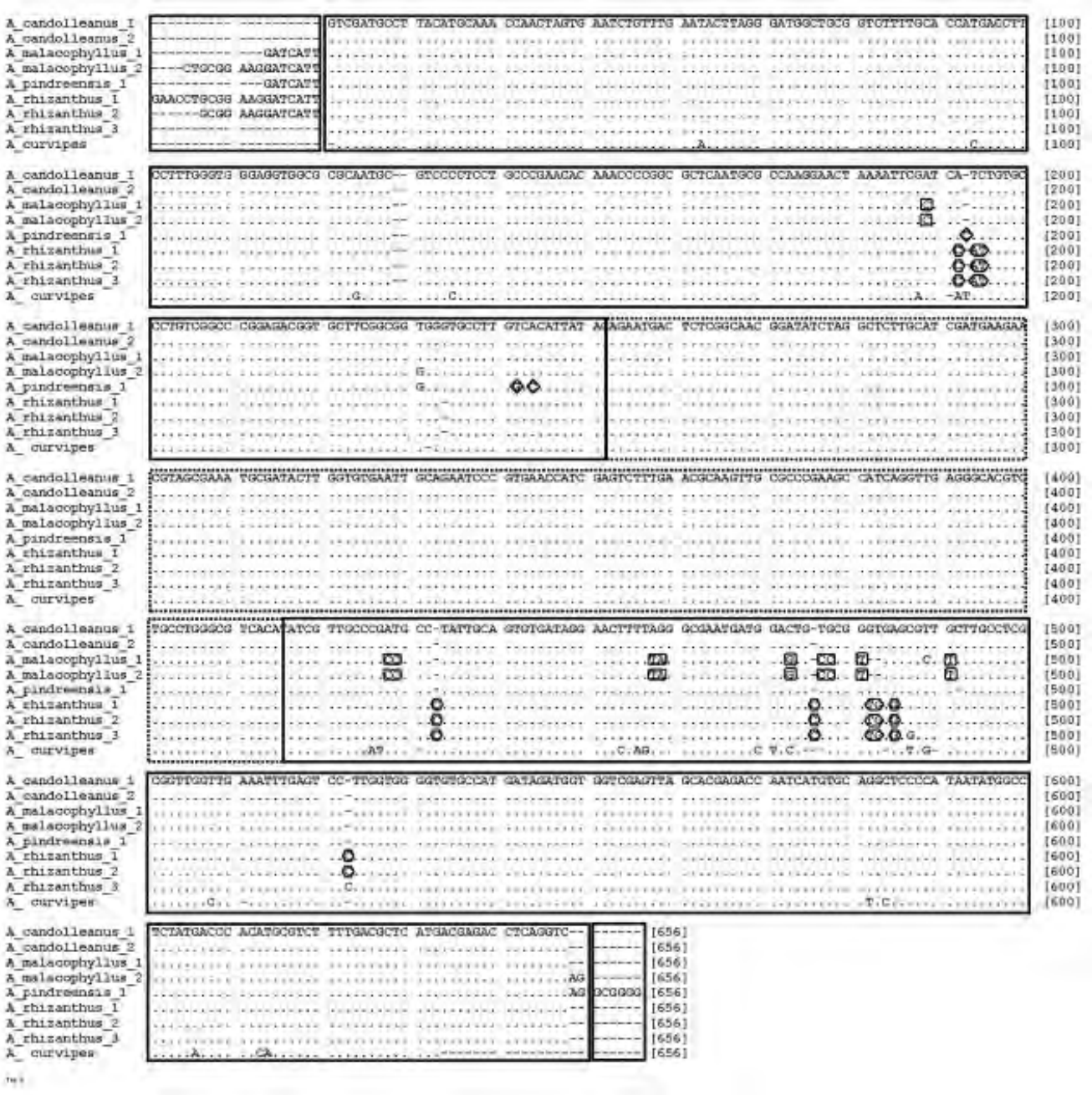

Fig. 5: Aligned sequences of ITS1, 5.8S and ITS2 for *Astragalus malacophyllus* and *A. rhizanthus* along with *A. candolleanus* & *A. pindreensis* and out group species (*A. curvipes*). Dots (.) represent identical nucleotides and dashes (-) represent deletions, ( ) represents specific substitution to *A. malacophyllus* and ( ) represents specific substitution and insertion to *A. rhizanthus*. Twenty positions at 5' and eight positions at 3' end were excluded from the analysis

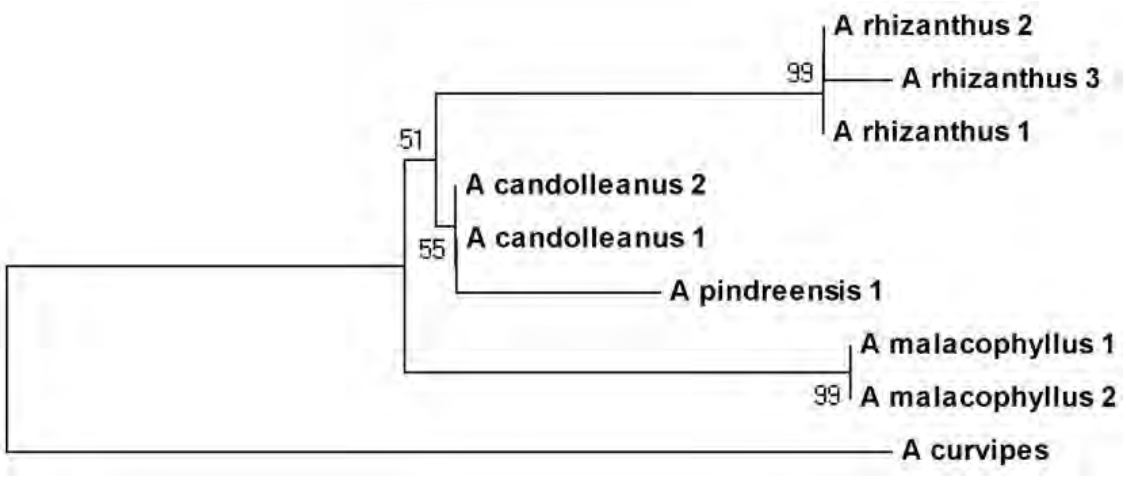

Fig. 6: The neighbour-joining tree of the target species and one outgroup derived from nrDNA ITS sequence. Bootstrap values more than 50% are shown on each branch

Table 2: ITS sequence characteristics of studied species including length, variable nucleotide sites, phylogenetic informative sites and percentage of GC content

ITS Characteristics	ITS 1	5.8S	ITS 2
Length (in base pairs)			
A. candolleanus 1	229	164	229
A. candolleanus 2	229	164	229
A. malacophyllus 1	236	164	226
A. malacophyllus 2	245	164	227
A. pindreensis 1	237	164	228
A. rhizanthus 1	248	164	232
A. rhizanthus 2	243	164	232
A. rhizanthus 3	229	164	232
GC content (%)			
A. candolleanus 1	54.56%	50.64%	51.96%
A. candolleanus 2	54.56%	50.64%	51.96%
A. malacophyllus 1	53.81%	50.64%	52.40%
A. malacophyllus 2	53.00%	50.64%	52.40%
A. pindreensis 1	53.81%	50.64%	52.42%
A. rhizanthus 1	54.43%.	50.64%	53.94%
A. rhizanthus 2	54.32%	50.64%	53.94%
A. rhizanthus 3	54.58%	50.64%	53.44%
Variable nucleotide sites			
(Alligned sequences of *A. candolleanus, A. malacophyllus, A. pindreensis,* and *A. rhizanthus*)	18	0	26
Potentially phylogenetically informative sites			
A. candolleanus	3	0	3
A. malacophyllus	4	0	14
A. pindreensis	6	0	4
A. rhizanthus	7	0	6

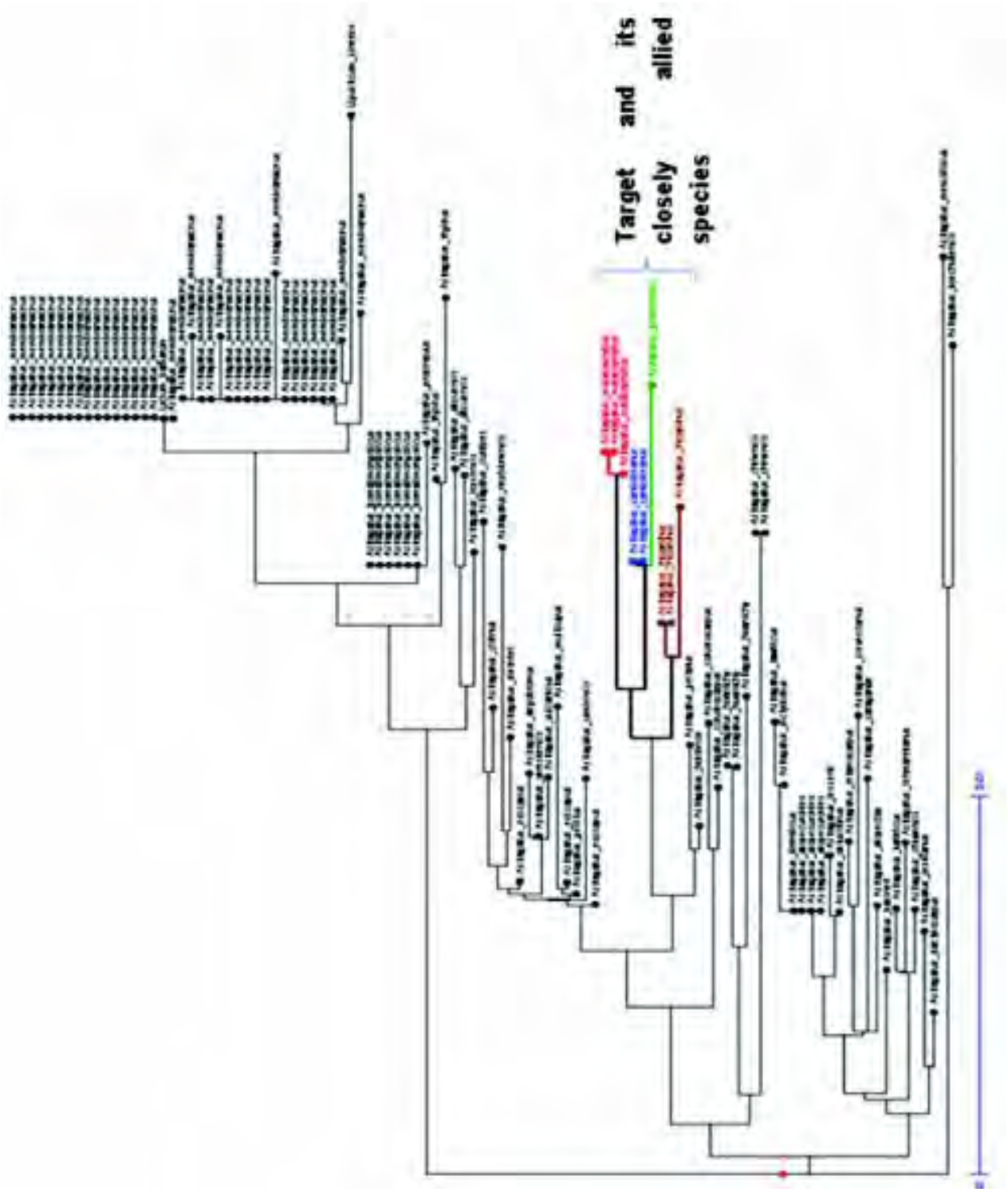

Fig. 7: Phylogenetic tree derived with nucleotide *BLAST* against NCBI database using ITS sequence of *A. malacophyllus* and its closely allied species

clad but shows close proximity with *A. candolleanus* (with a pair wise genetic distance of 0.008) rather than *A. rhizanthus* (with a pairwise genetic distance of 0.013) in analysis. A total of three species-specific substitutions of one base pair (encoded by 231, 242 and 244) were observed which revealed the distinctness from other three species. In addition, one insertion (in ITS1) and one species specific deletion (in ITS2) were also observed in *A. pindreensis*. The difference in overall GC content was also observed in comparison to other species (52.46%). In all analyses *A. candolleanus* also exhibited distinct identity without any substitution in comparison to other species. *A. rhizanthus* also made a distinct cluster with 99% bootstrap value and had clear cut species specific deletion (one bp encoded at 234) and four substitutions in ITS region. A higher overall percentage (53.14%) of GC content with comparison to all species distinguished *A. rhizanthus*. The similar kind of result has been obtained when the nucleotide *BLAST* of other species of *Astragalus* obtained from NCBI database was carried out (Fig. 7).

Fig. 8: Distribution sites of : *Astragalus rhizanthus* and : *A. malacophyllus* in India

4. Discussion

The taxonomic study carried out based on large number of herbarium specimens housed at different herbaria and extensive field survey in the Indian Himalaya reveals that *A. malacophyllus* can easily be differentiated from *A. rhizanthus* in habit, hairs, stem, leaf rachis, terminal leaflet, raceme and style. Generally, the plants grow gregariously with well developed stems (nearly up to one meter long) and forming more or less busy habit in *A. malacophyllus* while in *A. rhizanthus* the plants are almost stem less or occasionally with very short stems which grow singly or in tufts. In comparison to *A rhizanthus*, the hairs are quite dense especially on both sides of leaflets in *A. malacophyllus*. The terminal leaflet in *A. malacophyllus* falls well before lateral leaflets and leave persistent and more or less stiff leaf rachis naked and subspiny contrary to the herbaceous leaf rachis of *A. rhizanthus* where leaflets are not deciduous. Apart from the differences in the vegetative characters, both species also differ in reproductive traits. The flowers are aggregate in a bunch at the tip of stems in *A. rhizanthus*, however, they are axillary on a distinctly peduncled recemes in *A. malacophyllus*. The prominently hairy style of *A. malacophyllus* also makes the species quite distinct from glabrous style of *A. rhizanthus*.

Apart from the morphological characters, the distribution pattern of both species is also quite interesting. *A. malacophyllus* is found in the Himalaya between India and Pakistan (Fig. 8). However, its main concentration lies in India in the N. W. Himalaya from Uttarakhand to Jammu & Kashmir at an altitudes ranging from 2400–4000 m, while very poorly represented in Pakistan as evident from the study of specimens at different herbaria and the number of specimens cited from Pakistan by Ali [9-10]. Further, during our recent field visits in the Indian Himalaya the species was noticed in abundance only from Gangotri to Gaumukh in Uttarakhand province. Earlier also, as evident from the herbarium specimens housed at various herbaria, the species has been collected chiefly from Uttarakhand. On the other hands, *A rhizanthus* is widely scattered in the Himalaya from Nepal to Afghanistan at an altitudes ranging from 3000-5500 m. Interestingly, both species were never seen growing together in the same population in the Himalaya.

Anand *et al*. [13] have examined the genetic differentiation of *A. rhizanthus, A. candolleanus , A. malacophyllus* and *A. pindreensis* using RAPD, ISSR and DAMD and concluded that the accessions of *A. malacophyllus* distinctly form a separate group. Similarly, the present phylogenetic analysis based on nrDNA ITS further confirms the distinct identity of *A. malacophyllus* which is contrary to Podlech [6] but strongly supports the views of Baker [8], Ali [9,10] and Sanjappa [11] as far as the status of the species is concerned.

5. Acknowledgements

The authors are thankful to Dr. C S Nautiyal, Director, CSIR-National Botanical Research Institute (CSIR), Lucknow for facilities and the Department of Biotechnology, Govt. of India, New Delhi for financial support. Thanks are also due to the In-charge of herbaria mentioned in the work for allowing the herbarium consultation as well as loaning the specimens of *Astragalus* for our studies.

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