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RESEARCH ARTICLES

DNA barcoding of Indian *Alysicarpus* (Fabacae): ITS alone distinguishes species

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Abstract

In India, the genus *Alysicarpus* (Fabaceae) is represented by approximately 18 species and seven varieties. It includes some species of considerable economic importance, including two of therapeutic value. The species of this genus are difficult to identify because of overlapping morphological characters. Moreover, it becomes difficult to establish the botanical identity of a herbal medicine available in powdered/fragmented form. In the present study, DNA barcoding was used to identify 42 accessions belonging to 15 species of *Alysicarpus* and two varieties. The DNA regions that were tested as potential barcodes were *matK* (Maturase K), *rbcL* (Rubisco Large Sub-unit), *rpoC1* (RNA Polymerase-β subunit, the main catalytic subunit) and nrITS (Nuclear Ribosomal Internal Transcribed Spacer). In BLAST search on National Centre for Biotechnology Information (NCBI), ITS and *rpoC1* sequences best matched with the respective sequences of species of other genera belonging to the family Fabaceae with less than 100% similarity, as no corresponding sequences of any *Alysicarpus* species were available. The *rbcL* and *matK* sequences had first match, with the respective sequences of *Alysicarpus* species, however, with less than 100% similarity. Among the loci tested, ITS alone discriminated all species on the basis of genetic distance as well as phylogenetic tree methods.

Keywords *Alysicarpus* · DNA barcoding · ITS · Species discrimination

Introduction

The genus *Alysicarpus* Necker ex Desvaux (family Fabaceae) has been reported to include 25–30 species worldwide (Mabberley 2009). According to the Plant List (The Plant List 2013), there are 34 accepted species of *Alysicarpus*. These are distributed in Africa, Asia, Australia, Polynesia and Tropical America (Pokle 2002). The number of *Alysicarpus* species reported from India vary from 14 to 18, besides infra-specific taxa (Chavan et al. 2012; Pokle 2002). Recently, two new species, *A. gautalensis* (Gholami and Pandey 2016a) and *A. poklianus* (Gholami and Pandey 2016b) have been added to this list. Economically the genus is of considerable importance as some species, such as, *A. vaginalis*, are used as fodder and as cover crop in plantations (Chandrashekar and Sandhyarani 1994). *A. vaginalis* and *A. monilifer* are reported to have many therapeutic properties (Gholami 2015; Kumari et al. 2012). The roots of the former species are used for treatment of cough, leprosy and urinary troubles, while its leaves have purgative effect (Kumari et al. 2012). *A. monilifer*, which has been reported to have anti-inflammatory, analgesic, antipyretic, anti-periodic, expectorant and anti-cancerous properties, is also used as an antidote for snake bite (Kumari et al. 2012). Taxonomy of the genus *Alysicarpus* is controversial due to the variability in vegetative characters and scattered distribution (Pokle 2002). The confusion in naming of the species in this genus has also been highlighted by other taxonomists (Pramanik and Thothathri 1986; Pedley 2001; Sanjappa 1992).

DNA barcoding is an important taxonomic tool for rapid and easy identification of plants up to the species level, even if only a part, fragment or its DNA is available (Ali et al. 2014; Babbar et al. 2012). Based on the species discrimination abilities of seven widely used barcode loci, viz. *matK* (maturase K), *rbcL* (large subunit of the ribulose-bisphosphate carboxylase/oxygenase gene), *rpoB* (RNA polymerase-β subunit), *rpoC1* (RNA polymerase-β'

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subunit) and three non-coding regions, *atpF-atpH* (ATP synthase subunits CFO I- CFO III), *trnH-psbA* (tRNA for histidine- photosystem II protein D1 spacer) and *psbK-psbI* (Photosystem II reaction centre protein K-I spacer), reported from different laboratories for various assemblages of plants, CBOL Plant Working Group (CBOL Plant Working Group 2009) proposed a combination of *matK* and *rbcL* as the core barcode for plants. China Plant BOL Group (Li et al. 2011) have provided compelling experimental evidence for inclusion of nrITS (nuclear ribosomal internal transcribed spacer), in the core barcode of *matK* + *rbcL*. ITS alone discriminated all species of *Dendrobium* and *rpoC1* provided better species resolution than *rbcL* (Singh et al. 2012).

The present study was undertaken with precise objectives of (i) testing the suitability of DNA barcoding for discriminating and identifying species of *Alysicarpus* available in India and (ii) identifying the best locus/loci, from among ITS, *matK, rbcL* and *rpoC1*, for its species identification and discrimination.

Materials and methods

Plant materials

Forty two accessions belonging to 15 species of *Alysicarpus*, available in India, and one accession of *Desmodioastrum belagaumensis*, were collected. The details of the species, their varieties and accessions, places of collection and accession numbers of the herbarium specimens submitted to the Delhi University Herbarium (DUH) are provided in Table 1. Figure 1 depicts flowering branches of some of these species.

DNA isolation, amplification and sequencing

Genomic DNA was extracted using Quiagen DNeasy Plant Mini Kit (Qiagen, Amsterdam, Netherlands). The targeted loci were amplified using forward and reverse primers, details of which are provided in Table 2. ITS was amplified using primers ITS1F and ITS4R or ITS4R and ITS5F (White et al. 1990). The thermal cycle used for the amplification of all the loci was 5 min at 94 °C, followed by 35 cycles of 30 s

Fig. 1 **a–f** Flowering and fruiting twigs of some of the investigated species of *Alysicarpus* **a** *A. tetragonolobus*, **b** *A. heyneanus var. ludens*, **c** *A. luteovexillatus*, **d** *A. hamosus*, **e** *A. bupleurifolius var. gracilis*, **f** *A. vaginalis*

Table 1 The list of species of *Alysicarpus* and *Desmodioastrum belagaumensis*, analysed in the present study, along with the details of their voucher specimens

S.No.	Name of species (No. of accessions)	Collector name and collection	Herbarium accession nos	Locality
1	*A. bupleurifolius* (L.) DC (3)	Gholami & Pandey 4512, 8002, 4618	DUH 14151, DUH 14165, DUH 14338	Kagal lake, Aurangabad, Maharashtra
2	*A. gamblei* Schindler	Gholami & Pandey 4633	–	Badami, Karnataka
3	*A. gautalensis* Gholami& Pandey (2)	Gholami & Pandey 4549, 8012	DUH 14154, DUH 14164	Gautala forest, Maharashtra
4	*A. hamosus* Edgew. (2)	Gholami & Pandey 4523, 4652	DUH 14152, DUH 14339	Aurangabad, Maharashtra
5	*A. heyneanus* Wight &Arn. (2)	Gholami & Pandey 4507, 8010	DUH 14147, DUH 14160	Kolhapur to Panhala, Paithan, Maharashtra
6	*A. ludens* Baker (3)	Gholami & Pandey 4511, 4604, 4634	DUH 14150, DUH 14342, DUH 14343	Kagal lake, Maharashtra
7	*A. longifolius* (Rottl. ex Spreng) Wight and Arn. var. *major.* Pokle (3)	Gholami & Pandey 8011, 4624, 4623	DUH 14161, DUH 14344, DUH 14345	Paithan, Maharashtra
8	*A. luteovexillatus* Naik & Pokle (2)	Gholami & Pandey 8009, 4628	DUH 14159, DUH 14346	Aurangabad, Maharashtra
9	*A. monilifer* (L.) DC (2)	Gholami& Pandey 8004, 4576	DUH 14155, DUH 14347	Aurangabad, Maharashtra
10	*A. naikianus* Pokle (3)	Gholami & Pandey 4506, 4612, 4639	DUH 14146, DUH 14348, DUH 14349	Panhala, Maharashtra
11	*A. ovalifolius* (Schum.) Leonard (5)	Gholami & Pandey 8006, 4505, 4629, 4619, 4620	DUH 14156, DUH 14162, DUH 14350, DUH 14351, DUH 14352	Aurangabad, Maharashtra. Ratnagiri, Maharashtra
12	*A. pubescens* var. *pubescens* J.S. Law	Gholami & Pandey 4509	DUH 14149	Kolhapur, Maharashtra
13	*A. pubescens var. vasavadae* (Hemadri) Sanjappa	Gholami & Pandey 8008	DUH 14158	Aurangabad, Maharashtra
14	*A. scariosus* (Spreng.) Thwaites (4)	Gholami& Pandey 4532, 8005, 4622, 4617	DUH 14153, DUH 14163, DUH 14354, DUH14340	Aurangabad, Maharashtra
15	*A. tetragonolobus* Edgew.(3)	Gholami & Pandey 4508, 8001, 4627	DUH 14148, DUH 14166, DUH 14355	Aurangabad, Maharashtra
16	*A. vaginalis* (L.) DC. (5)	Gholami & Pandey 8007, 4641, 4631, 4631A, 4614	DUH 14356, DUH 14357, DUH 14358, DUH 14359, DUH 14360	Aurangabad, Maharashtra
17	*Desmodioastrum belgaumensis*	Gholami & Pandey 4616	DUH 14275	Sindhudurg, Maharashtra

*Delhi University Herbarium

Table 2 The primers used for the amplification and sequencing of the candidate loci

Locus	Primer name	Primer sequence
rpoC1	rpoC1 2F	GGCAAAGAGGGAAGATTTC[13]
	rpoC1 4R	CCATAAGCATATCTTGAGTTGG[13]
rbcL	rbcLa for	ATGTCACCACAAACAGAGACTAAAGC[40]
	rbcLa rev	GTAAAATCAAGTCCACCCRCG[41]
matK	3F KIM A	CGTACAGTACTTTTGTGTTTACGAG[13]
	1R KIM	ACCCAGTCCATCTGGAAATCTTGGTTC[13]
ITS	ITS1F	TCCGTAGGTGAACCTGCC[16]
	ITS 4F	TCCTCCGCTTATTGATATGC[16]
	ITS 5R	GGAAGTAAAAGTCGTAACAAGG[16]

each at 94 °C and 1 min at 72 °C, annealing temperature that varied for each primer pair according to the melting temperature (Tm) of the primer having lowest Tm, with a final extension of 72 °C for 7 min. All the PCR amplified products were cleaned using Exo-Sap method (Bell 2008; Parveen et al. 2012). The cleaned PCR products were sequenced with the same primers which were used for amplification. Sequencing was done at Chromdx Solutions Private Limited and Research Services and Consultancy Corporation, NOIDA, Uttar Pradesh, India.

Data analysis and species identification

The chromatograms obtained from the sequencer were base-called, using Phred (Codon-code aligner version 5.0.1).

Only the sequences with Phred score more than 20 were considered for further analysis. The forward and reverse sequences were trimmed and assembled using the same software. A total of 84 sequences from forty two individuals belonging to fifteen species were generated and submitted to NCBI GenBank and accessions numbers were obtained (KT222191–KT222263). Nucleotide sequences of all the four loci were aligned using BioEdit version 7.0.9.0 (Hall 1999).

All the four loci were compared individually for their amplification, sequencing and species discrimination rates. The sequences of all the loci were analyzed for their identity using BLAST method (Altschul et al. 1990) to ascertain that (i) the sequence was only of the targeted locus and (ii) it had best match with the sequences of the respective locus of the same species, genus or family. The species discrimination rate for each locus was determined using (i) the K2P (Kimura 2 Parameter) genetic distances obtained among the sequences of each locus by utilizing MEGA 6.0 (Tamura et al. 2013) and (ii) Neighbour-Joining (NJ) trees were constructed with the sequences of the selected loci. The selected parameters were; Bootstrap method with 1000 replications, K2P model, transitions and transversions included under substitutions, and complete deletion selected for gaps/missing data treatments. Only those species not having zero distance estimates with any of the other species were considered as successfully discriminated. Likewise, in NJ trees if an accession of species clustered along with the accession of any other species, both were not considered distinct. ITS sequences of all species belonging to allied genera, *Desmodium, Uraria, Pseudarthria and Desmodiastrum*, available in GenBank, NCBI, were also included for constructing ITS NJ phylogenetic trees.

Results

Amplification and sequencing success

Twenty one accessions belonging to 15 species were used for the comparison of amplification, sequencing and species discrimination rates of the selected loci individually, except for ITS, number of accessions for which were increased to 42 for the reason described later. Amplification rate for ITS and *rpoC1*was 100%, whereas for other two loci, *matK* and *rbcL*, it was 95.7%. The sequencing success rates for *rbcL* and *rpoC1* were 100%, while for ITS it was 82.1%. The sequencing success rate for *matK* was only 55%, and moreover, the sequences, which were obtained from the accessions of only 10 species, were short in length (400–500 bp; Table 3).

Identification using BLAST

BLAST analysis revealed that all sequences generated in the present study were of the targeted loci. The first hits of all the *matK* sequences, generated in the present study, were only with the respective species. None of the *matK* sequence matched 100% with any other species on NCBI. BLAST results obtained with the *rbcL* sequences of the eight species showed that *rbcL* could not be used as a barcode marker for the identification of *A. naikianus*, *A. bupleurifolius*, *A. gautalensis*, *A. pubescens*, *A. ludens*, *A. scariosus*, *A. tetragonolobus*, and *A. longifolius* var. *major*) as their *rbcL* sequence had 100% match with other species too. The *rpoC1* sequences of most of the *Alysicarpus* species matched 100% with the sequences of other species and hence were not species specific.

Intra- and inter-specific genetic distances and species discrimination

Intra-specific distances, based on ITS, could be calculated among 10 species of *Alysicarpus*, which were represented by two or more accessions. Intra-specific distances in six of these were zero, whereas, among the accessions of other four it varied from 0.002 to 0.007. Thus, intra-specific distances of 0.002, 0.005 and 0.000–0.007 were obtained for *A. heyneanus*; *A. naikianus, A. tetragonolobus* and *A. vaginalis*, respectively. The maximum inter-specific distance of 0.088 among ITS sequences was between A. *bupleurifolius* and A. *vaginalis*. In NJ tree of ITS that also had downloaded

Table 3 The amplification and sequencing success rates, aligned lengths, numbers of variable and Parsimony informative sites and mean inter-specific divergences of the four tested loci

	ITS	*matK*	*rbcL*	*rpoC1*
Amplification (%)	92.8	95.7	95.7	100
Sequencing (%)	82.1	55	100	100
Aligned length (bp)	689	757	596	529
No of Parsimony informative site	92	9	6	2
No of variable sites	127	47	16	7
Mean inter-specific divergence (Range)	0.047 (0.000–0.088)	0.025(0–0.071)	0.002 (0–0.004)	0.002(0–0.007)

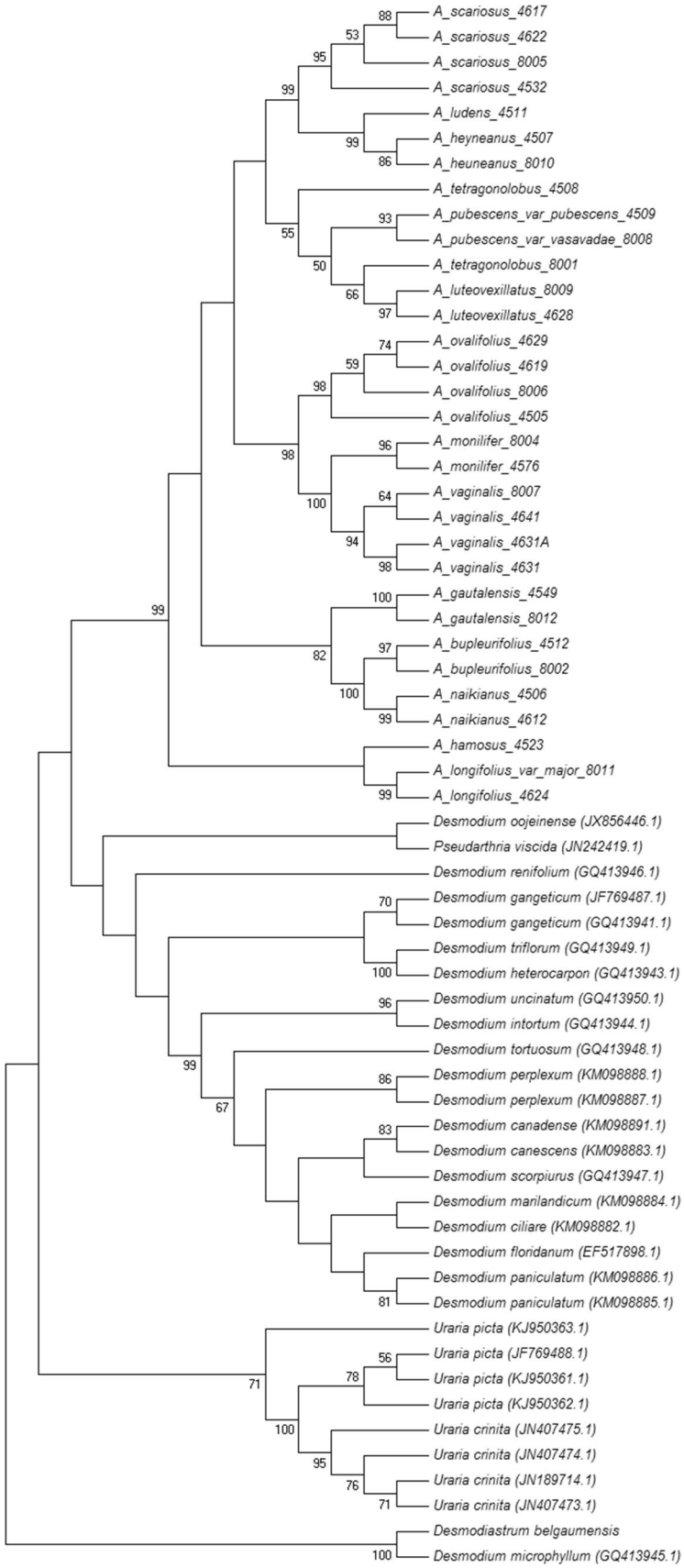

Fig. 2 Neighbour-Joining tree of ITS sequences *Alysicarpus* and those of the species of the allied genera downloaded from GenBank, NCBI. Accession numbers of the downloaded sequences are given in parenthesis after name of the species

Table 4 Percent species resolution of *Alysicarpus* species using four candidate loci

Locus	No. of accession analysed	No. of species	% Species resolution	
			K2P Distance method	NJ tree method
ITS	32	14	100	100
matK	11	10	70	70
rbcL	20	14	0	0
rpoC1	21	14	7.1	7.1

sequences of species of the allied genera, all the species of *Alysicarpus*, the lone species of *Pseudarthria* and those of *Desmodium*, *Desmodiastrum* and *Uraria* segregated in three different clades (Fig. 2). The species discrimination rate based on ITS genetic distances as well as NJ tree was 100% for *Alysicarpus* (Fig. 2; Table 4). The two varieties of *A. longifolius* had zero distance estimates between them, and thus were on the same clade (Fig. 2). Two varieties of *A. pubescens* had a distance of 0.007 between them but were on the same node though with slightly different branch length. The two accessions of *A. heyneanus* formed a clade with *A. ludens* on different branch.

The *matK* sequences of only 11 accessions belonging to ten species and a variety of *A. pubescens* could be generated. Intra-specific distance between the two varieties of one of these, *A. pubescens* was zero. On the other hand, three species, *A. heyneanus*, *A. pubescens* and *A. luteovexillatus*, could not be discriminated with one or the other because of zero distances between them. Thus, species discrimination rate afforded by *matK* was 70% by both distance based and NJ tree methods (Fig. 3; Table 4). The maximum K2P distance of 0.071 on the basis of *matK* was between *A. naikianus* and *A. ludens.*

The maximum genetic distances based on *rbcL* and *rpoC1* among the species were 0.004 and 0.007, respectively. These were much lower than the genetic distances that separated the species on the basis of ITS and *matK*. On the basis of the genetic distances as well as NJ trees of *rbcL* and *rpoC1*, 15 and 13 of the 15 investigated species had zero distances with one or the other, consequently, yielding abysmally low species discrimination rate of zero and 7.1% (Figs. 4 and 5; Table 4).

Discussion

Since the establishment of the genus *Alysicarpus* by Desvaux (Desvaux 2013), its taxonomy has been in continuous flux with the number of species included varying because of the circumscription of new species (Gholami and Pandey 2016a, b; Pedley 2001; Sanjappa 1992), merger of species (Baker 1876; Gholami et al. 2016; Pokle 2002), transfer of species to other genera or even by describing a new genus, *Desmodiastrum*, that includes some of the species earlier included in the genus *Alysicarpus* (Pramanik and Thothathri 1986). Similar to its global status, in India too, the number of species representing this genus has also been revised from time to time. The number of species of *Alysicarpus* found in India has been estimated to range from 14 to 18, by various workers (Chavan et al. 2012; Pokle 2002; Sanjappa 1992; Santapau and Henry 1973). According to Pokle (2002) circumscription of species included in this genus pose several taxonomic problems due to the presence of variable vegetative characters and scattered distribution. According to him, some of the taxa were of doubtful existence, certain needed re-delimitation, while a few other required determination of

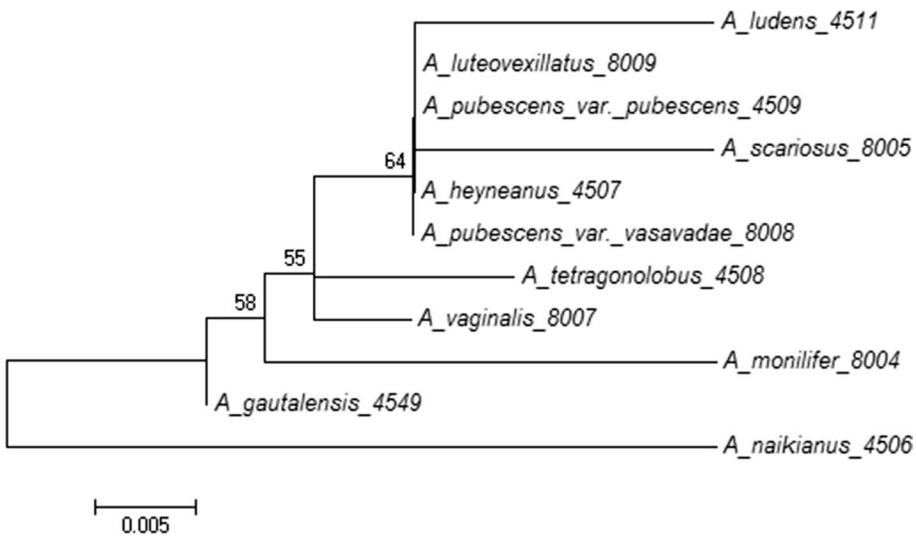

Fig. 3 Neighbour joining tree of *matK* sequences of *Alysicarpus* species

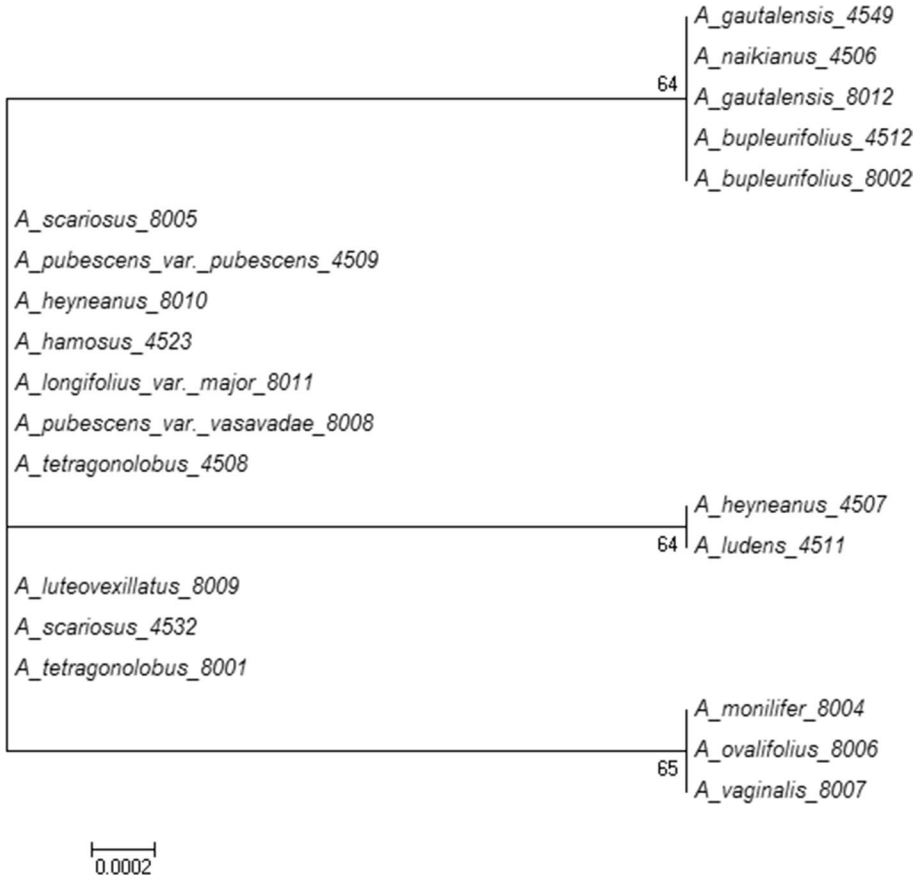

Fig. 4 Neighbour joining tree of *rbcL* sequences of *Alysicarpus* species

their taxonomic status (Pokle 2002). With this background, the present study was undertaken to see if DNA barcoding could resolve some of these problems and provide species specific recognition tags. Of the four tested loci, only ITS could resolve all the investigated species of *Alysicarpus*. The potential to discriminate at species level and easy amplification make ITS a favourable locus for barcoding of plants (Li et al. 2011; Pang et al. 2010; Singh et al. 2012). However, despite of many favourable traits, several characters of ITS, such as, gene duplication, paralogous copies and incomplete concerted evolution, make it a problematic barcode locus (Chase et al. 2007; Starr et al. 2009). Possibly because of these reasons Hollingsworth (2011), recommended that ITS, though not a part of the recommended core barcode, could be used whenever required and available. However, in our study it was highly successful, as the amplification and direct sequencing met with 82.1% success. In the NJ tree constructed with ITS sequences of *Alysicarpus* along with those of the species of allied genera, *Desmodium, Desmodiastrum, Pseudarthria* and *Uraria*, all the species of *Alysicarpus* formed an independent clade, thus indicating its monophyletic nature, a fact which has already been highlighted (Gholami et al. 2017). In contrast, *Desmodium, Desmodiastrum* and *Uraria* segregated in one clade, with only *Uraria* forming a distinct sub-clade. The multiple accessions of each species of *Alysicarpus* were on separate nodes. The two varieties of *A. pubescens* segregated on the same branch in ITS tree.

There was a difference of opinions about the taxonomic status of *A. vaginails* and *A. ovalifolius*. Meeuwen et al. (1961) did not accept them as two distinct species. Verdcourt (1974) reported intermediates between them but none was found in the collections made from Australia and India (Gholami, unpublished; Pedley 2001). However, Endo and Ohashi (1990) highlighted reliable distinguishing features of pods of two species. In addition to attributes of pod, open inflorescences and introrsely curved hair on the stems of *A. ovalifolius* distinguishes it from *A. vaginalis* (Endo and Ohashi 1990). The present observations re-affirm the status of *A. vaginalis* and *A. ovalifolius* as distinct species, as the two segregated on different nodes in ITS tree with *A. monilifer* and *A. vaginalis* on one node, and the accessions of *A. ovalifolius* on the other node.

CBOL Plant working Group (2009) had suggested *rbcL* + *matK* as the core barcode for plants. However, in the present study, good quality bidirectional sequences of *matK* locus of only 11 accessions belonging to ten species could be obtained. In the earlier studies too, amplification and sequencing of *matK* have been reported to be problematic in several plant groups (Ford et al. 2009; Sass et al.

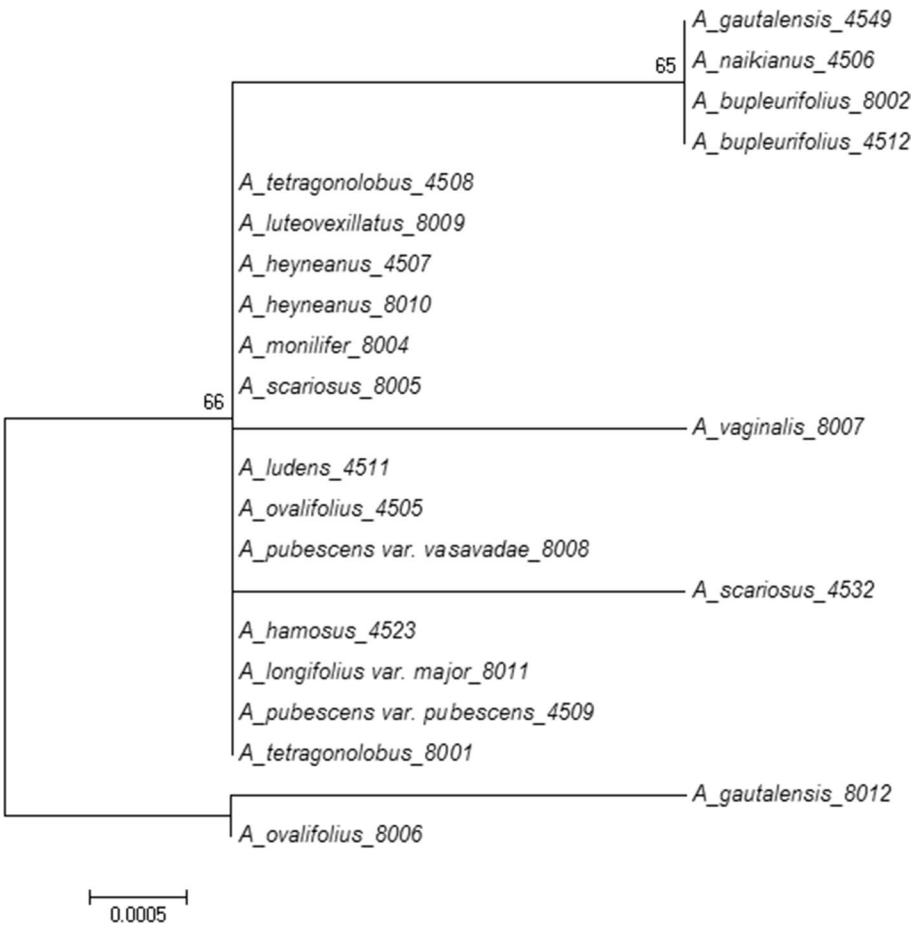

Fig. 5 Neighbour joining tree of *rpoC1* sequences of *Alysicarpus* species

2007). Fazekas et al. (2008) after trying 10 primer pairs could obtain 88% sequencing success in an assemblage of 92 species belonging to 32 genera of higher plants. The reason ascribed for poor sequencing success of *matK* is the presence of mononucleotide repeats which are amplified using the primers designed from within the *matK* (Fazekas et al. 2008). Nevertheless, *matK* provided best species discrimination of 70% among the three tested loci from the chloroplast genome. The other two loci from the chloroplast genome, *rbcL* and *rpoC1*, though were easy to amplify and sequence, yielded abysmally poor species discrimination of zero and 7.1%. Earlier, Ren et al. (2010) in a study on *Alnus*, reported only 10% identification success with *rbcL*. Likewise, *rpoC1* provided fewest informative characters among the seven barcode region compared for 32 genera of the 92 species belonging to different plant groups of land plants (Fazekas et al. 2008). These two loci are known to have poor resolving power at the species level and are used at higher taxonomic levels (CBOL Plant Working Group 2009; Chen et al. 2010; Gielly and Taberlet 1994), as is also evident from the present analysis.

The poor sequencing success of *matK* and almost negligible species discrimination capability of *rbcL* and *rpoC1* did not prove to be an impediment for DNA barcoding of *Alysicarpus* as the ITS provided 100% species resolution among the tested species.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

References

Ali MA, Gyulai G, Hidvégi N, Kerti B, Al Hemaid FM, Pandey AK, Lee J (2014) The changing epitome of species identification—DNA barcoding. Saudi J Biol Sci 21:204–231

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Babbar SB, Raghuvanshi S, Singh HK, Parveen I, Malik S (2012) An overview of the DNA barcoding of plants. Phytomorphology 62:69–99

Baker JG (1876) Leguminosae. In: Hooker F (ed) Flora of British India, vol 2. Reeve & Co., London

Bell JR (2008) A simple way to treat PCR products prior to sequencing using ExoSAP-IT. Biotechniques 44:834

CBOL Plant Working Group (2009) A DNA barcode for land plants. Proc Natl Acad Sci USA 106:12794–12797

Chandrashekar KR, Sandhyarani S (1994) Differences in chemical composition of *Alysicarpus vaginalis* L. (DC.) growing in saline and non-saline habitats Biol. Plant 36:139–143

Chase MW, Cowan RS, Hollingsworth PM, van den Berg C, Madrinan S, Petersen G, Seberg O, Jorgsensen T, Cameron KM, Carine M, Pedersen N, Hedderson TAJ, Conrad F, Salazar GA, Richardson JE, Hollingsworth ML, Barraclough TG, Kelly L, Wilkinson M (2007) A proposal for a standardized protocol to barcode all land plants. Taxon 56:295–299

Chavan S, Sardesai MM, Pokle DS (2012) *Alysicarpus sanjappae* (Leguminosae: Papilionoideae), a new species from the Western Ghats of India. Kew Bull 68:1–4

Chen S, Yao H, Han J, Liu C, Song J, Shi L, Zhu Y, Ma X, Gao T, Pang X, Luo K, Li Y, Li X, Jia X, Lin Y, Leon C (2010) Validation of ITS2 region as a novel DNA barcode for identifying medicinal plant species. PLoS One 5:e8613

Desvaux NA (2013) Précis des caracteres de plusiers genres de la famile des Leguminseuses plants. J Bot Appl Agric Pharm Méd Arts 2:118–125

Endo Y, Ohashi H (1990) New distinctions between *Alysicarpus vaginalis* and *A. ovalifolius* (Leguminosae). Nat Hist Res Chiba no 1:43–48

Fazekas AJ, Burgess KS, Kesanakurti PR, Graham SW, Newmaster SG, Husband BC, Percy DM, Hajibabaei M, Barrett SCH (2008) Multiple multilocus DNA barcodes from the plastid genome discriminate plant species equally well. PLoS One 3:e2802

Ford CS, Ayres KL, Toomey N, Haider N, Stahl JVA, Kelly LJ, Wikstrom N, Hollingsworth PM, Duff RJ, Hoot SB, Cowan RS, Chase MW, Wilkinson MJ (2009) Selection of candidate DNA barcoding regions for use on land plants. Bot J Linn Soc 159:1–11

Gholami A (2015) Biology of *Alysicarpus monilifer* (L.) DC.: an important medicinal plant. Med Plants 7:248–251

Gholami A, Pandey AK (2016a) *Alysicarpus gautalensis* (Leguminosae: Desmodieae), a new species from Maharashtra, India. Phytotaxa 266:141–145

Gholami A, Pandey AK (2016b) *Alysicarpus poklianus* (Fabaceae, Desmodieae), a new species from India. Phytokeys 68:117–124

Gholami A, Pandey AK, Ansari AA (2016) Critical notes on *Alysicarpus monilifer (L.) DC.* [Leguminosae: Desmodieae] and its allies. Pleione 10:71–79

Gholami A, Subramaniam S, Geeta R, Pandey AK (2017) Molecular systematics of Indian Alysicarpus (Fabaceae) based on analyses of nuclear ribosomal DNA sequences. J Genetics 96:353–363

Gielly L, Taberlet P (1994) The use of chloroplast DNA to resolve plant phylogenies: noncoding versus *rbcL* sequences. Mol Biol Evol 11:769–777

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41:95–98

Hollingsworth PM (2011) Refining the DNA barcodes for land plants. Proc Natl Acad Sci USA 108:19451–19452

Kumari KM, Rao BG, Padmaja V (2012) Role of vitexin and isovitexin in hepatoprotective effectof *Alysicarpus monilifer* Linn. Against CCL_4 induced hepatotoxicity. Phytopharmacology 3:273–285

Li DZ, Gao LM, Li HT, Wang H, Ge XJ, Liu JQ, Chen ZD, Zhou SL, Chen SL, Yang JB, Fu CX, Zeng CX, Yan HF, Zhu YJ, Sun YS, Chen SY, Zhao L, Wang K, Yang T, Duan GW (China Plant BOL Group) (2011) Comparative analysis of a large dataset indicates that internal transcribed spacer (ITS) should be incorporated into the core barcode for seed plants. Proc Natl Acad Sci USA 108: 19641–19646

Mabberley DJ (2009) Mabberley's Plant Book. Cambridge University Press, Cambridge

Pang X, Song J, Zhu Y, Xie C, Chen S (2010) Using DNA barcoding to identify species within Euphorbiaceae. Planta Med 76:1784–1786

Parveen I, Singh HK, Raghuvanshi S, Pradhan UC, Babbar SB (2012) DNA barcoding of endangered Indian *Paphiopedilum* species. Mol Ecol Resour 12:82–90

Pedley L (2001) *Alysicarpus* (Leguminosae: Desmodieae) in Australia: a taxonomic revision. Austrobaileya 6:107–116

Pokle DS (2002) Synopsis of *Alysicarpus* Desv. in India, In: *Perspectives of Plant Biodiversity*, edited by Das AP (Bishen Singh Mahendra Pal Singh, Dehradun) 471–481

Pramanik A, Thothathri K (1986) On the status of *Desmodiastrum* Prain (Fabaceae). J Indian Bot Soc 65:373–379

Ren BQ, Xiang XG, Chen ZD (2010) Species identification of *Alnus* (Betulaceae) using nrDNA and cpDNA genetic markers. Mol Ecol Resour 10:594-605

Sanjappa M (1992) Legumes of India. Bishen Singh Mahendra Pal Singh. Dehra Dun, India

Santapau H, Henry AN (1973) A dictionary of flowering plants in India. CSIR, New Delhi

Sass C, Little DP, Stevenson DW, Specht CD (2007) DNA Barcoding in the Cycadales: testing the potential of proposed barcoding markers for species identification of Cycads. PLoS One 2:e1154

Singh HK, Parveen I, Raghuvanshi S, Babbar SB (2012) The loci recommended as universal barcodes for plants on the basis of floristic studies may not work with congeneric species as exemplified by DNA barcoding of *Dendrobium* species. *BMC* Res Notes 5:42

Starr JR, Naczi RFC, Chouinard BN (2009) Plant DNA barcodes and species resolution in sedges (*Carex*, Cyperaceae). Mol Ecol Resour 9:151–163

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA 6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729

The Plant List (2013) Version 1.1. Published on the Internet; http://www.theplantlist.org/ Accessed 28 Aug 2019

van Meeuwen MS, Steenis van CGGJ, Stemmenik J (1961) Preliminary revisions of some Mayaysian Paplionaceae II. *Alysicarpus* Desv Reinwardtia 6:86–89

Verdcourt B (1974) Summary of Leguminosae—Papilionidene—Hedysarae (sensu lato of flora Zambesrana. Kirkia 9:545–546

White TJ, Bruns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A Guide to Methods and Applications*. Innis MA, Gelfand DH, Sninsky JJ, White TJ (Eds). Academic Press, New York. pp. 315–322