RESEARCH ARTICLE

Molecular systematics of Indian *Alysicarpus* (Fabaceae) based on analyses of nuclear ribosomal DNA sequences

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Abstract

Alysicarpus Necker ex Desvaux (Fabaceae, Desmodieae) consists of ~30 species that are distributed in tropical and subtropical regions of the world. In India, the genus is represented by ca. 18 species, of which seven are endemic. Sequences of the nuclear Internal transcribed spacer from 38 accessions representing 16 Indian species were subjected to phylogenetic analyses. The ITS sequence data strongly support the monophyly of the genus *Alysicarpus*. Analyses revealed four major well-supported clades within *Alysicarpus*. Ancestral state reconstructions were done for two morphological characters, namely calyx length in relation to pod (macrocalyx and microcalyx) and pod surface ornamentation (transversely rugose and nonrugose). The present study is the first report on molecular systematics of Indian *Alysicarpus*.

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Introduction

The genus *Alysicarpus* Necker ex Desvaux (tribe Desmodieae, Fabaceae) includes ~30 species distributed in tropical and subtropical regions of the old world (Lewis *et al.* 2005). In India, the genus is represented by approximately ca. 18 species, of which seven are endemic (Pokle 2002; Dhabe 2013; Gholami and Pandey 2016).

The genus *Alysicarpus* is characterized by its scarious calyx with complex venation and, turgid articles of its indehiscent pods (figure 1). The leaves are generally unifoliolate or rarely pinnately 3-foliolate. Pedley (2001) reported that all species of *Alysicarpus* described from Australia possessed unifoliolate leaves. However, Pokle (2002) observed the sporadic occurrence of trifoliolate leaves in *A. hamosus*, *A. tetragonolobus* and *A. scariosus*, a feature also observed by us in these species (Gholami and Pandey, personal observation). The trifololate condition has also been observed in *A. rugosus* occurring in India, Taiwan (Peng and Chaw 1986) and Africa (Gillett 1971).

The genus was first recognized by Necker in 1790 and was established and validly published by Desvaux in 1813. Baker (1876) divided *Alysicarpus* into two groups: (i) Microcalycinae, characterized by the calyx not longer than the first joint of the pod, and (ii) Macrocalycinae where the calyx is much longer than the first joint of the pod and its teeth imbricate in the fruiting stage. In *Alysicarpus*, the pods may be moniliform or nonmoniliform, glabrous or pubescent, reticulate or transversely rugose (Pedley 2001) and flowers may be monocoloured or bicoloured (Pokle 1998). The analysis of these morphological characters in a phylogenetic context is needed to help to understand their evolution and also to assess their significance at different taxonomic levels. Ancestral trait reconstruction can help in understanding the evolutionary patterns of a particular trait (Maddison 1995; Schluter *et al.* 1997; Swofford and Maddison 1987) by using well-supported phylogenies of extant taxa without reference to potentially relevant documented fossil record (Webster and Purvis 2002; Pagel *et al.* 2004; Webster *et al.* 2004).

The objectives of the present study were to: (i) test the monophyly of *Alysicarpus*, (ii) examine the relationships among Indian species of *Alysicarpus* based on ITS sequence data, and (iii) study the phylogenetic patterns of two significant characters, namely macrocalyx/microcalyx and pod surface ornamentation and to interpret their taxonomic significance at the infrageneric level.

Materials and methods

Taxon sampling

We studied 38 accessions representing 16 species of Indian *Alysicarpus*, corresponding to 88% of the species in India

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Figure 1. Flowering and fruiting twigs of some of the investigated species of *Alysicarpus*. (a) *A. tetragonolobus*; (b) *A. pubescens*; (c) *A. longifolius*; (d) *A. vaginalis*; (e, f) *A. heyneanus*; (g) *A. naikianus*.

(table 1). Author citations are given in table 1. The present study includes seven endemic taxa, namely *Alysicarpus gamblei*, *A. gautalensis*, *A. hamosus*, *A. luteovexialltus*, *A. naikianus*, *A. poklianus* and *A. pubescens* accounting a total of 16 species considered for analyses. All voucher specimens of the species collected by us have been deposited in the Delhi University Herbarium (DUH). Two sequences of *Desmodium gangeticum* (L.) DC. and one of *Uraria picta* (Jacq.) DC. were included as outgroups for the analyses. The sequences of the outgroup taxa were retrieved from GenBank.

DNA extraction, amplification and sequencing

Genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Amsterdam, The Netherlands). DNA amplification and sequencing of the ITS region were performed using the primers ITS1 and ITS2 (White *et al.* 1990). The polymerase chain reaction (PCR) was performed with standard methods using Bangalore Genei PCR premix (Bengaluru, India) in 20 μL volume containing 2 μL of 10× buffer, 300 μM dNTPs and 1 unit of HF DNA polymerase following the protocol of Subramaniam *et al.* (2013, 2015). PCR products were checked for the presence of appropriate bands on a 0.8% agarose gel, purified, and sequenced at SciGenom, Cochin, Kerala, India. Sequences comprised of ITS1, 5.8S, and ITS2 regions. Forward and reverse sequences were edited and assembled using the computer program DNA Baser Assembler ver. 4 (DNA Sequence Assembler v4 2013 Heracle Biosoft SRL, http://www.DnaBaser.com). All sequences have been deposited in GenBank (table 1). The scale developed by Yanthan *et al.* (2011) was used to infer the proximity of various species within the genus.

Phylogenetic analyses

A total of 38 nucleotide sequences (including outgroups) were aligned using ClustalX ver. 2.0.11 (Thompson *et al.*

Table 1. Plant accessions used for the molecular systematic study of Indian *Alysicarpus* along with their GenBank accession numbers.

Taxon	Voucher	Locality	GenBank accession no.
A. bupleurifolius L.	Gholami & Pandey 4512	Kagal lake, Aurangabad, Maharashtra	KT276183
A. bupleurifolius L.	Gholami & Pandey 4626	Ramling sanctuary, Osmanabad, Maharashtra	KT276184
A. gautalensis Gholami & Pandey	Gholami & Pandey 4549	Gautala forest, Maharashtra	KT276185
A. gautalensis Gholami & Pandey	Gholami & Pandey 8012	Gautala forest, Maharashtra	KT276186
A. gamblei Schindler	Gholami & Pandey 4633	Badami, Karnataka	KT276187
A. hamosus Edgew.	Gholami & Pandey 4523	Aurangabad, Maharashtra	KT276188
A. hamosus Edgew.	Gholami & Pandey 4642	Sinhgarh, Pune, Maharashtra	KT276190
A. hamosus Edgew.	Gholami & Pandey 8003	Aurangabad, Maharashtra	KT276191
A. heyneanus Wight & Arn.	Gholami & Pandey 8010	Kolhapur to Panhala, Maharashtra	KT276192
A. longifolius (Rottl. ex Spreng) Wight and Arn.	Gholami & Pandey 4624	Vanvihar, Solapur, Maharashtra	KT276196
A. longifolius (Rottl. ex Spreng) Wight and Arn. var. *major* Pokle	Gholami & Pandey 8011	Pithan, Maharashtra	KT276197
A. ludens Baker	Gholami & Pandey 4602	Shivaji University, Kolhapur, Maharashtra	KT276193
A. ludens Baker	Gholami & Pandey 4511	Kagal, Maharashtra	–
A. luteovexillatus Naik & Pokle	Gholami & Pandey 8009	Aurangabad, Maharashtra	KT276198
A. luteovexillatus Naik & Pokle	Gholami & Pandey 4628	Ramling sanctuary, Osmanabad, Maharashtra	KT276199
A. monilifer (L.) DC.	Gholami & Pandey 4576	Asola Bhatti, Delhi	KT276200
A. monilifer (L.) DC.	Gholami & Pandey 4630	Badami, Karnataka	KT276201
A. monilifer (L.) DC.	Gholami & Pandey 8004	Aurangabad, Maharashtra	KT276202
A. naikianus Pokle	Gholami & Pandey 4506	Panhala, Maharashtra	KT276203
A. naikianus Pokle	Gholami & Pandey 4608	Shivaji University, Kolhapur, Maharashtra	KT276204
A. naikianus Pokle	Gholami & Pandey 4612	Godal, Dajipur, Maharashtra	KT276205
A. ovalifolius (Schum.) Leonard	Gholami & Pandey 4505	Ratnagiri, Maharashtra	KT276206
A. ovalifolius (Schum.) Leonard	Gholami & Pandey 4619	Madban, Jaitapur, Maharashtra	KT276207
A. ovalifolius (Schum.) Leonard	Gholami & Pandey 4629	Ramling sanctuary, Osmanabad, Maharashtra	KT276208
A. ovalifolius (Schum.) Leonard	Gholami & Pandey 8006	Aurangabad, Maharashtra	KT276209
A. pubescens var. *pubescens* J.S. Law	Gholami & Pandey 4509	Kolhapur, Maharashtra	KT276210
A. pubescens var. *vasavadae* (Hemadri) Sanjappa	Gholami & Pandey 8008	Aurangabad, Maharashtra	KT276211
A. rugosus (Willd.) DC.	Gholami & Pandey 4507	Paithan, Maharashtra	KT276195
A. scariosus (Spreng.) Thwaites	Gholami & Pandey 4532	Aurangabad, Maharashtra	KT276212
A. scariosus (Spreng.) Thwaites	Gholami & Pandey 4622	Vanvihar, Solapur, Maharashtra	KT276213
A. scariosus (Spreng.) Thwaites	Gholami & Pandey 4622a	Vanvihar, Solapur, Maharashtra	–
A. scariosus (Spreng.) Thwaites	Gholami & Pandey 8005	Aurangabad, Maharashtra	KT276214
A. tetragonolobus Edgew.	Gholami & Pandey 4508	Aurangabad, Maharashtra	KT276215
A. tetragonolobus Edgew.	Gholami & Pandey 4640	Sinhgarh, Pune, Maharashtra	KT276216
A. tetragonolobus Edgew.	Gholami & Pandey 8001	Aurangabad, Maharashtra	KT276217
A. vaginalis (L.) DC.	Gholami & Pandey 4631	Badami, Karnataka	KT276218
A. vaginalis (L.) DC.	Gholami & Pandey 4641	Sinhgarh, Pune, Maharashtra	KT276219
A. vaginalis (L.) DC.	Gholami & Pandey 8007	Aurangabad, Maharashtra	KT276220

Table 2. Character matrix showing the character states for all the species used in ancestral state reconstruction in Mesquite.

Taxon/character	Calyx type (microcalyx /macrocalyx)	Pod surface ornamentation (transversely rugose/ nonrugose)
A. bupleurifolius	1	1
A. gautalensis	1	1
A. gamblei	1	1
A. hamosus	0	1
A. heyneanus	1	0
A. ludens	1	0
A. longifolius	1	1
A. longifolius var. *major*	1	1
A. luteovexillatus	1	1
A. monilifer	0	1
A. naikianus	1	1
A. ovalifolius	0	1
A. pubescens var. *pubescens*	1	1
A. pubescens var. *vasavadae*	1	1
A. rugosus	1	0
A. scariosus	1	0
A. tetragonolobus	1	1
A. vaginalis	0	1

Coding for the character states is as follows: 0, microcalyx; 1, macrocalyx; 0, transversely rugose; 1, nonrugose.

1997) followed by manual adjustments in Mesquite ver. 2.72 (Maddison and Maddison 2009). All positions containing gaps and missing data were eliminated. Phylogenetic analyses were done using Bayesian (maximum posterior probability, MPP), and maximum likelihood (ML) methods (Stamatakis 2006). Bayesian analyses were done using Mr Bayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquis and Huelsenbeck 2003). The best fit model was determined using Model Test ver. 0.1.1 (Guindon and Gascuel 2003; Posada 2008) by the Akaike information criterion (AIC) and the ML method. It was found to be GTR+G, with the lowest AIC score and highest log-likelihood score. Parameters for the evolutionary model were set to default and the state frequency parameter for stationary nucleotide frequency of the rate matrix was fixed. The number of chains was set to four with three heated and one cold chain. Two runs were executed in parallel. Analyses were run for 700,000 generations until stationarity (standard deviation below 0.01). In each run, trees were sampled every 100 generations with a sample frequency of 10. The parameters were summarized after excluding 25% of the samples (sump burnin command) based on the inspection of log likelihoods of sampled trees after stationarity. The potential scale reduction factor (a convergence diagnostic) approached 1.0 for all the parameters suggesting good sampling from the posterior probability distribution with no spread. Trees were summarized by the sumt burnin command yielding a cladogram showing posterior probabilities and clade credibility for each split and a phylogram with mean branch lengths. The following criteria were used to evaluate the pp's: 0.50–0.80, low; 0.81–0.94, moderate; 0.95–1.0, strong.

ML analyses were performed using RaxML v.1.3 (Stamatakis 2006). For likelihood (ML) analyses, settings were 'ML thorough bootstrap' with 100 (replicate) runs and 1000 (bootstrap) repetitions with the GTR+G model (six general time-reversible substitution rates, assuming gamma rate heterogeneity). The following criteria were used to assess bootstrap support percentages (BP): 50–70%, low; 71–84%, moderate; 85–100%, strong. The results of ML analyses are congruent with those from Bayesian analyses.

Ancestral state reconstruction of morphological characters

The calyx size in relation to pod and pod surface ornamentation were studied in the representative species using our collections, herbarium data and literature. Character states recorded for two characters included calyx type: microcalyx and macrocalyx (0 and 1) and pod surface ornamentation: transversely rugose and nonrugose (0 and 1) (table 2). Variation in these character forms were examined by ancestral state reconstruction using Mesquite ver. 2.72 (Maddison and Maddison 2009). One hundred most probable trees were retrieved from the TRPROBS file produced in the Bayesian analyses and read into Mesquite. Ancestral state reconstruction of characters were done using maximum parsimony and ML methods. A probability of more than 0.85 for a character compiled from the

100 most probable trees at each node was considered to be the most probable state at that node.

Results

Characteristics of the ITS region in Alysicarpus

The multiple alignment of the ITS region (with 5.8S) comprised a total of 610 sites including INDELS. Of the total aligned sites, the ITS1 region contained 168 variable and 190 conserved sites, while the ITS2 region contained 201 variable and 96 conserved sites. Hundred and thirty five and 143 sites were parsimony informative in ITS1 and ITS2 regions, respectively. Of the 610 nucleotide sites, more variable sites occurred in ITS2 than in ITS1. The 5.8S region is same in size (160 bp) in all the taxa with no gaps. A high proportion of sites (78.7%) are conserved in 5.8S region. All positions containing gaps and missing data (41 nucleotides) were eliminated from the analyses. The overall mean pairwise distance is 0.116. Multiple sequence alignment of these sites required the inclusion of 35 gapped positions (0.05% of all sites).

Phylogenetic results

In the Bayesian analyses, a total of 2986 trees were obtained from both the runs with 1493 in each run. The 50% majority rule consensus trees resulting from the two searches are similar in topology (ML tree) (figure 2). The ML analyses recovered one ML tree. Similar results were obtained from both the analyses (figure 2). Further, we describe the results of likelihood analyses, with additional remarks regarding Bayesian analyses.

The trees were rooted using the outgroups *Desmodium gangeticum* and *Uraria picta* following the work of Bailey *et al.* (1997). The monophyly of *Alysicarpus* is strongly supported (100 bs/1.00 pp) (figure 2). All species with more than one accession were found to be monophyletic with strong support (>0.90 pp: *A. bupleurifolius*, *A. gautalensis*, *A. hamosus*, *A. longifolius*, *A. luteovexillatus*, *A. monilifer*, *A. naikianus*, *A. ovalifolius*, *A. scariousus*, *A. tetragonolobus* and *A. vaginalis*) or weak support (*A. ludens* and *A. pubescens*). Only single accessions were sequenced from each of *A. gamblei*, *A. heyneanus* and *A. rugosus*. The multiple accessions of *A. hamosus* show branch length and nucleotide variation.

The genus is resolved into four major clades (figure 2). Some of the clades of particular taxonomic or biogeographic interest are discussed below.

***Clade 1: A. scariosus, A. heyneanus, A. rugosus and A. ludens*:** Clade 1 (85 bs/0.99 pp) consists of two nested clades where *A. scariosus* (98 bs/1.00 pp) is monophyletic and is sister to *A. heyneanus–A. rugosus–A. ludens* clade (89 bs/0.99 pp). Both are strongly supported sister clades to each other. In the latter nested clade, *A. rugosus* is sister to *A. heyneanus* and *A. ludens* but with no support. *A. heyneanus* and *A. ludens* form sister clades with a poor support (60 bs/0.86).

***Clade 2: A. pubescens, A. luteovexillatus and A. tetragonolobus*:** This clade consists of *A. pubescens*, *A. luteovexillatus* and *A. tetragonolobus* nested into a strongly supported group (99 bp/1.00 pp) (figure 2). This clade is further divided into two nested-clades, one comprising of *A. luteovexillatus* and its sister *A. tetragonolobus* (96 bs/0.98 pp), and the other *A. pubescens* (66 bs/0.59 pp). Branch length variation for multiple accessions of *A. tetragonolobus* may indicate population variation and opens up the possibility for further analysis within this species.

***Clade 3: A. ovalifolius, A. vaginalis and A. monilifer*:** The clade is characterized by a synapomorphy, namely calyx shorter or slightly longer than first joint of pod and hence encompasses the species *A. ovalifolius*, *A. vaginalis* and *A. monilifer* (100 bs/1.00 pp) (figure 2). These three species belong to the group Microcalycinae (Baker 1876; John and Thengane 1994). The clade is further subdivided into two nested-clades, one comprising *A. vaginalis* as sister to *A. monilifer* (99 bs/1.00 pp) and the other, *A. ovalifolius* as sister to the former clade (100 bp/1.00 pp).

***Clade 4: A. longifolius, A. naikianus, A. gamblei, A. bupleurifolius and A. gautalensis clade ± A. hamosus*:** The five species of clade 4 are well supported (bs/pp), but their relationship with *A. hamosus* is poorly supported. Within clade 4, *A. longifolius* var. *longifolius* and *A. longifolius* var. *major* form a subclade (98 bs/1.00 pp). This subclade is sister to subclade comprising *A. gamblei*, *A. naikianus* and *A. bupleurifolius* (100 bs/1.00 pp). *A. gamblei* forms a sister to *A. naikianus* (74 bs/0.94 pp). This clade (*A. gamblei*, A. naikianus) forms a sister to *A. bupleurifolius* and *A. gautalensis* (100 bs/1.00 pp). Both the species form two clades confirming their distinctness, the latter being a new species to science (Gholami and Pandey 2016).

Phylogenetic pattern of morphological characters

Evolutionary trends in two characters (calyx type: microcalyx and macrocalyx and pod surface ornamentation) were inferred from ancestral state reconstructions (figures 3 and 4; tables 3 and 4). Calyx may be shorter than the first joint of the pod (microcalyx) or longer (macrocalyx), and pod surface ornamentation may be transversely rugose or nonrugose. Figures 3 and 4 represent ancestral state reconstruction done by parsimony method and values of likelihood probabilities are depicted for the clades to be discussed.

Analyses on character reconstruction was conducted for calyx type (figure 3; table 3). The states taken into consideration were: microcalyx and macrocalyx. The state in the

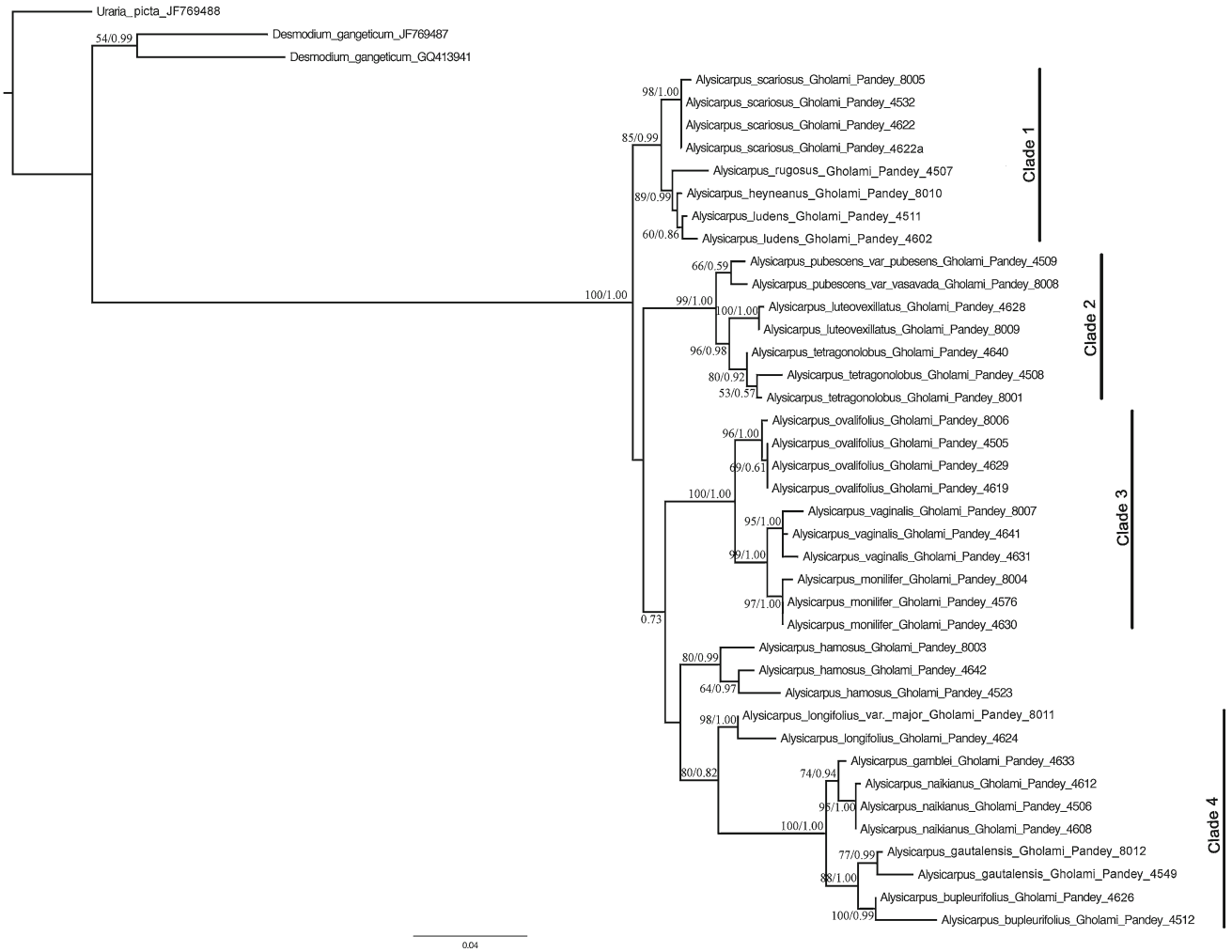

Figure 2. Cladogram constructed from 2986 trees in Mr Bayes. All the clades are labelled. Numbers corresponding to the branches indicate the bootstrap support (BS) and the posterior probabilities (pp).

ancestral node to the genus seems to be ambiguous, since the probability of both the states are equal (microcalyx, p: 0.335; macrocalyx, p: 0.768 in 89 trees). Therefore, nothing can be concluded about the pleisomorphy and apomorphy of the character states. Ancestors to members of clades 1 ($P = 0.999$), 2 ($P = 0.998$ in 90 trees) and 5 ($P = 0.977$ in 92 trees) have strong probabilities for macrocalyx where the calyx is much longer than the first joint of the pod and its teeth imbricate in the fruiting stage. On the other hand, ancestral nodes to members of clades 3 ($P = 0.978$ in 92 trees) and 4 ($P = 0.979$ in 89 trees) have calyx not longer than the first joint of the pod.

The pod surface ornamentation in the genus *Alysicarpus* can be either transversely rugose or nonrugose. The ancestral state in the pod type was nonrugose ($P = 0.99$) in the genus and transversely rugose seem to have evolved in the genus in clade 1 (ancestral change of character state: $P = 0.98$ in 96 trees) (figure 4; table 4).

Discussion

This is the first molecular systematic study using ITS sequences to estimate phylogenetic relationships in Indian *Alysicarpus*. The key morphological features that have traditionally been used to support the recognition of *Alysicarpus* species include scarious calyx with upper lobe bifid, complex calyx venation pattern, and jointed indehiscent turgid pod articles (Sanjappa 1992). Reconstructing these informative characters onto the most probable trees clearly shows that transversely rugose pods and microcalyx (given that *A. hamosus* nests with clade 3) are homologous and evolve from a common ancestor. On the other hand, nonrugose pods and macrocalyx are nonhomologoes traits. These traits, in general, have evolved several times in other temperate and subtropical groups of Fabaceae (Dudik 1981; Raven and Polhill 1981).

Based on ITS sequence data, the phylogenetic relationships of the genus *Alysicarpus* are well resolved. Relative to

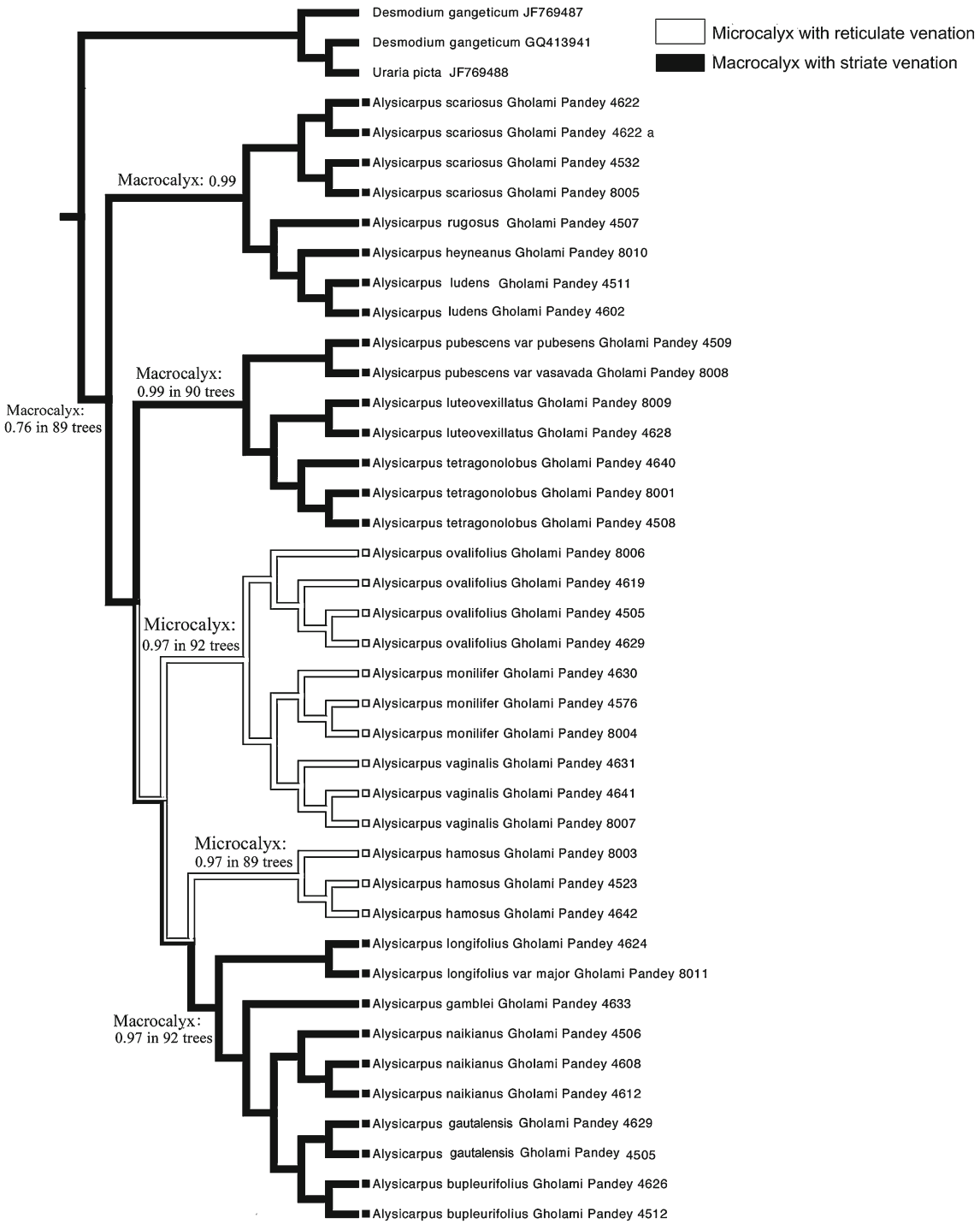

Figure 3. Evolution of calyx in the genus *Alysicarpus*. ML values for reconstruction of calyx type (microcalyx and macrocalyx) plotted at specific nodes of one of 100 most probable parsimonious trees resulting from Bayesian analyses.

the outgroup taxa used in this study, the genus *Alysicarpus* is a well-supported monophyletic group that is unambiguously part of the subtribe Desmodiinae. *Alysicarpus* has been placed as sister to *Desmodium* whereas *Uraria* is more distantly related to *Alysicarpus*. Recent molecular systematic studies place tribe Desmodieae within the core Phaseoleae along with tribe Swartzieae. Based on *rbc*L sequence data, three genera (*Lespedeza*, *Phyllodium* and *Desmodium*) are recognized as comprising tribe Desmodieae (Kajita *et al.* 2001). Dutt *et al.* (1979) conducted a chemosystematic study and concluded that the genus *Alysicarpus* forms a natural and homogeneous group characterized by the conspicuous absence of flavones and many phenolic acids.

Figure 4. Evolution of pod surface ornamentation in the genus *Alysicarpus*. ML values for reconstruction of pod (transversely rugose, nonrugose) plotted at specific nodes of one of 100 most probable parsimonious trees resulting from Bayesian analysis.

Table 3. Summary of evolution of calyx type in the genus *Alysicarpus.*

Calyx type	Microcalyx	Macrocalyx
Common ancestor to genus	0.335	0.768 in 89 trees
Ancestor to *A. pubescens*, *A. luteovexillatus*, *A. tetragonolobus*	0.001 in 93 trees	0.998 in 90 trees
Ancestor to *A. scariosus*, *A. heyneanus*, *A. rugosus*, *A. ludens*	0.0011 in 94 trees	0.999
Ancestor to *A. ovalifolius*, *A. monilifer*, *A. vaginalis*	0.978 in 92 trees	0.021 in 83 trees
Ancestor to *A. hamosus*	0.979 in 89 trees	0.002 in 94 trees
Ancestor to *A. longifolius*, *A. gamblei*, *A. naikianus*, *A. bupleurifolius*, *A. gautalensis*	0.003 in 88 trees	0.977 in 92 trees

ML values for reconstruction of pod hairiness (microcalyx, macrocalyx) at specific nodes of the tree taken from the 100 most probable trees from the results of Bayesian analysis (figure 3).

Table 4. Summary of evolution of pod ornamentation in the genus *Alysicarpus*.

Pod surface pattern	Transversely rugosus	Nonrugose
Common ancestor to genus	4.376 e-5	0.99
Ancestor to *A. scariosus*, *A. heyneanus, A. rugosus, A. ludens*	0.98	0.013
Ancestor to *A. pubescens*, *A. luteovexillatus*, *A. tetragonolobus*	2.345 e-8	0.99
Ancestor to *A. ovalifolius*, *A. monilifer*, *A. vaginalis*	9.877 e-11	0.99
Ancestor to *A. hamosus*	8.886 e-10	0.99
Ancestor to *A. longifolius*, *A. gamblei*, *A. naikianus*, *A. bupleurifolius, A. gautalensis*	4.446 e-4	0.99

ML values for reconstruction of pod surface ornamentation (transversely rugose, nonrugose) at specific nodes of the tree taken from the 100 most probable trees from the results of Bayesian analysis (figure 4).

Infrageneric relationships

The phylogenetic analysis provides several insights into the relationships within the genus, many of which were well supported. There has been considerable confusion in the identification of *A. ovalifolius* and *A. vaginalis* (Pedley 2001; Adema 2003). However, our analyses confirm the distinctness of both species and also suggest that *A. vaginalis* is closer to *A. monilifer* (99 bs/1.00 pp). *A. scariosus–A. heyneanus–A. rugosus–A. ludens* form a strongly supported clade. The morphological traits that support this clade are: plants pubescent, and pods transversely rugose. Backer (1911) treated *A. rugosus* var. *ludens* as a species (*A. ludens*), but Pramanik and Thothathri (1982) treated *A. rugosus* var. *ludens* as a variety under *A. heyneanus*. Based on ITS sequence data, we do not support the views of Backer (1911) in treating *A. rugosus* and *A. ludens* as distinct species. Members of clade 2 (*A. pubescens*, *A. luteovexillatus* and *A. tetragonolobus*) form a strongly supported group. Synapomorphies which characterize this clade are ciliate calyx and reticulate pod surface.

Baker (1876) divided the genus into two groups at the subgeneric level, namely microcalycinae and macrocalycinae. These groups were based on the length of the calyx in relative position to the first article of the lomentaceous pod. Our phylogenetic analyses do not support the groupings suggested by Baker (1876). However, microcalycinae may form a group, as the position of *A. hamosus* is uncer-

tain; if it is allied to clade 3, then it would be a part of microcalycinae characterized by microcalyx. *A. hamosus* is poorly supported as sister to clade 4. This creates a possibility that it forms a sister to clade 3 members. Morphological data (calyx type, flower colour, pod shape, size and surface) support closeness of *A. hamosus* to members of clade 3. Further analyses with additional markers are needed to confirm these relationships.

Clade 3 is composed of *A. vaginalis*, *A. ovalifolius* and *A. monilifer*. Patil *et al.* (2013) studied the leaf architecture pattern and its taxonomic implications in the genus *Alysicarpus*. According to them, *A. longifolius* shows reticulodromous–percurrent–sinuous venation pattern which is different than its allied species, i.e. *A. heyneanus* and *A. scariosus*. The venation pattern of *A. longifolius* is similar to *A. vaginalis*, *A. ovalifolius* and *A. monilifer*. These species along with *A. hamosus* are included in Microcalycinae (Baker 1876). Our study, however, does not support Baker's assumption because *A. longifolius* has been found to be sister to *A. bupleurifolus*, *A. gautalensis*, *A. naikianus* and *A. gamblei*. *A. longifolius* shows venation pattern similar to Microcalycinae members and thus is an added factor to the contention that 'Microcalycinae have evolved from *A. rugosus* complex' (Pokle 2002). Venation pattern is an added feature in the uniqueness of *A. hamosus*, *A. bupleurifolius* and *A. gautalensis* which stand apart in distinct clades supported by their morphologically distinct characters, namely nonmoniloform vs moniliform pods. *A. gautalensis* is a new species discovered from Maharashtra and has close affinities to *A. bupleurifolius* based on glabrous and glossy pods, and calyx shorter than the first joint of the pod (Gholami and Pandey 2016). The groups established by Baker (1876) are not congruent with our molecular analyses.

Systematic significance of morphological characters

The ancestral state reconstructions of calyx type and pod surface ornamentation were done to estimate the ancestral condition of phenotypic traits at internal nodes and make an inference about evolutionary processes in the *Alysicarpus* species in India.

Baker (1876) divided the genus into two major groups: Microcalycinae (calyx not longer than the first joint of the pod) and Macrocalycinae (calyx much longer than the first joint of the pod and its teeth imbricate in the fruiting stage). John and Thengane (1994) correlated calyx type with the DNA content in nine *Alysicarpus* species. The average per chromosome DNA content was found to be lower in members included in Microcalycinae (0.288–0.310 picogram) and in *Desmodiastrum belgaumensis* (=*A. belgaumensis*) (0.300 picogram) and the DNA content was found to be higher in members of Macrocalycinae (0.346–0.390 picogram). Their studies indicated three lines of evolution in the genus, two being with higher nuclear DNA content and one with lower average per chromosome DNA content. These studies signifies the importance of calyx type and necessitated the understanding of evolution of this character in the genus *Alysicarpus*. Since the position of *A. hamosus* is uncertain, it could have been possible that it is a part of clade 3. In that case, microcalyx would be an apomorphy that characterizes the entire clade 3. Macrocalyx is either homoplasious or a symplesiomorphous in the genus. Not much can be said about the evolution of this trait in the genus due to equal likelihood probabilities of either state in the ancestral node of clades 3 and 4.

Alysicarpus scariousus, *A. heyneanus*, *A. rugosus* and *A. ludens* in clade 1 possess rugose pods. This trait is homologous and has evolved from a single common ancestor. On the other hand, members in clades 2, 3 and 4 have nonrugose pods and hence seem to be a nonhomologous trait. The members having microcalyx and nonrugose pods (*A. hamosus*, *A. vaginalis*, *A. ovalifolius* and *A. monilifer*) show easy separation of seeds from the pod whereas *Alysicarpus* species having transversely rugose pods and macrocalyx (clade 1) have seeds which are closely adpressed to the pericarp and are not easily separable.

In conclusion, several relationships are suggested by ITS sequence data analyses: (i) *Alysicarpus* is monophyletic, (ii) molecular data suggest that the genus *Alysicarpus* is closely related to the members of subtribe Desmodinae, (iii) taxa recognized under groups microcalyx and macrocalyx are nonmonophyletic, (iv) macrocalyx and nonrugose pods are pleisomorphies for the genus, (v) microcalyx and transversely rugose pods are apomorphies for the genus, (vi) evolution of macrocalyx and nonrugose pods are nonhomologous and microcalyx and rugose pods evolved from a single common ancestor and pertains to a homologous trait in clades 3 and 1 members, respectively. Further analyses based on more chloroplast and nuclear markers are needed to resolve the relationships within the genus *Alysicarpus*.

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