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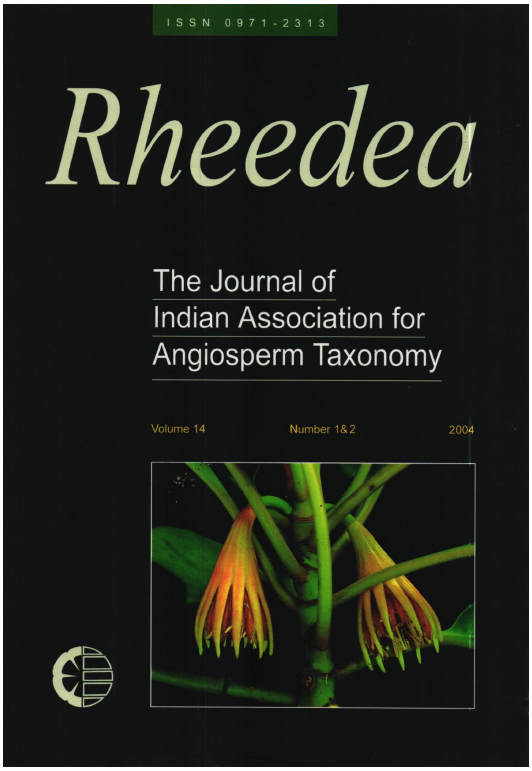
Pandey A.K., Jun Wen & M.K. Pathak



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Relationships in Indian Araliaceae as inferred from Sequences of Internal Transcribed Spacer (ITS) Regions of Nuclear Ribosomal DNA

Arun K. Pandey, Jun Wen[1] and M. K. Pathak[2]

Department of Botany, T.M. Bhagalpur University, Bhagalpur 812007, Bihar, India.
[1]Department of Botany, The Field Museum, 1400 S. Lakeshore Dr., Chicago 60605, Illinois, U.S.A.
wen@fieldmuseum.org
[2]Botanical Survey of India, P.O. Botanic Garden, Howrah 711103, West Bengal, India.

Abstract

The Internal Transcribed Spacer (ITS) regions of nuclear ribosomal DNA were obtained from 42 accessions belonging to nine genera (*Aralia, Brassaiopsis, Eleutherococcus,* , *Heteropanax, Macropanax, Merriliopanax, Panax, Schefflera* and *Tupidanthus*) to assess relationships of Araliaceae in India. The ITS sequence data suggest two major clades of Indian Araliaceae: the *Aralia* - *Panax* clade and the Indian core palmate clade. The close relationship between *Aralia* and *Panax* was strongly supported with a bootstrap value of 89%. The two species of *Panax* (the ginseng genus) form a monophyletic group. Three sections of *Aralia* occur in India and the genus was strongly suggested to be monophyletic with sect. *Dimorphanthus* and sect. *Pentapanax* forming a subclade. Within the Indian palmate clade, the following monophyletic groups are detected: the *Heteropanax-Schefflera-Tupidanthus* group and the *Eleutherococcus-Macropanax* group. *Merrilliopanax* was found to form a clade with *Brassaiopsis-Schefflera-Heteropanax-Tupidanthus*. India is rich in species of *Brassaiopsis*. Our ITS phylogeny recognizes two groups within *Brassaiopsis*: 1. *B. aculeata* - *B. glomerulata* - *B. hainla* - *B. mitis* - *B. shweliensis* and 2. *B. hispida* - *B. griffithii* - *B. simplicifolia*.

Keywords: ITS sequences, Relationship patterns, Indian Araliaceae, Two major clades, Monophyletic groups

Introduction

Araliaceae (the ginseng family) include approximately 50 genera and 1350 species (Wen *et al.*, 2001). The family is distributed chiefly in the tropics and the subtropics. In India, Araliaceae are represented by 16 genera distributed mostly in the northern and the north-eastern regions. They are: *Aralia* L. (11 spp.), *Arthrophyllum* Blume (1 sp.), *Brassaiopsis* Decne & Planch. (*c.* 10 spp.), *Dendropanax* Decne & Planch. (1 sp.), *Eleutherococcus* Maxim. (2 spp.), *Gamblea* C.B.Clarke (1 sp.), *Hedera* L. (1 sp.), *Heteropanax* Seem. (1 sp.), *Macropanax* Miq. (3 spp.), *Merrilliopanax* Li (3 spp.), *Panax* L. (3 spp.), *Polyscias* J.R. & G. Forst. (2 spp.), *Schefflera* J.R. & G. Forst. (*c.* 30 spp.), *Tetrapanax* (K. Koch) K. Koch (1 sp.), *Trevesia* Vis. (1 sp.) and *Tupidanthus* Hook. f. & Thoms. (1 sp.).

Aralia in India includes *A. armata* (Wall. ex D. Don) Seem., *A. cachemirica* Decne., *A. foliolosa* Wall. ex C.B. Clarke, *A. gigantea* J. Wen, *A. kingdon-wardii* J. Wen, Lowry & Esser, *A. leschenaultii* (DC.) J. Wen, *A. malabarica* Bedd., *A. parasitica* (D. Don) J. Wen, *A. subcordata* (D. Don) J. Wen, *A. thomsonii* Seem. and *A. tibetana* G. Hoo (Wen *et al.*, 2002). The genus *Pentapanax* has been merged with *Aralia* based on morphological characters and recent phylogenetic data (Wen, 1993, 2001a), and now it is treated as a section of *Aralia* (Wen, 2002). Three of the six sections currently recognized in *Aralia* occur in India: sect. *Aralia*, sect. *Dimorphanthus* Miq. and sect. *Pentapanax* (Seem.) J. Wen.

Panax consists of approximately 18 species, of which about 16 are from Eastern Asia and two from Eastern North America (Wen & Zimmer, 1996; Wen, 2001b; Yoo *et al.*, 2001). Among the Asiatic species , several Himalayan taxa have been problematic due to the existence of occasional morphological intermediates (Wen & Zimmer, 1996). *Panax pseudoginseng* was described by Wallich in 1829 based on specimens

collected from Central Nepal and its circumscription has presented problems to many taxonomists. Hara (1970) recognized four taxa from Nepal based on rhizome, root and leaf characters: *P. pseudoginseng* subsp. *pseudoginseng*, *P. pseudoginseng* subsp. *himalaicus* Hara var. *himalaicus*, *bipinnatifidus* (Seem.) H.L. Li and *angustifolius* (Burkill) H.L. Li. He broadly defined *P. pseudoginseng* as a widespread species in the Himalayas, China and Japan. Hoo & Tseng (1973, 1978) followed Hara's species concept and made a few nomenclatural changes at the varietal level within *P. pseudoginseng*. Zhou *et al.* (1975), however, defined *P. pseudoginseng* narrowly, sensu Wallich (1829). The narrowly defined *P. pseudoginseng* has been supported in Wen and Zimmer (1996), Choi and Wen (2000), Yoo *et al.* (2001) and Lee and Wen (2004). Our recent analysis also supports the recognition of *Panax assamicus* Banerjee from India (Pandey *et al.*, 2002; Lee & Wen 2004). The taxonomy of Indian *Panax* is highly controversial (cf. treatments by Banerjee, 1968; Wen, 2001b) and the relationships of Indian taxa of Araliaceae are not well understood.

Brassaiopsis comprises approximately 30 species distributed in Central China to South East Asia and westward to the Himalayan region. Hoo and Tseng (1978) suggested that *Pseudobrassaiopsis* (cited as *Euaraliopsis*) might be closely related to *Trevesia* because (1) they shared leaves whose lobes might have pseudopetiolules (see Hoo & Tseng, 1978, pl.1, f.7) (2) *Brassaiopsis* was perhaps related to or derived from *Schefflera* and (3) *Trevesia* with its 5-12-carpellate ovary, was more 'primitive' than *Pseudobrassaiopsis* (=*Euaraliopsis*). Phylogenetic analysis using nuclear (ITS) and plastid (*trn*L-*trn*F) sequences suggests a close relationship among *Brassaiopsis s.* lat. and *Trevesia* (Wen *et al.*, 2001; Plunkett *et al.*, 2004). India harbours 11 of the approximately 30 species of *Brassaiopsis* and examination of the phylogenetic relationships of Indian *Brassaiopsis* is needed.

Heteropanax includes about five species from South Asia and China and is characterized by bicarpellate ovaries, divided styles and leaves that are 2-5 times pinnately compound. The phylogenetic position of *Heteropanax* within Araliaceae has been highly controversial (see Wen *et al.*, 2001). Based on ITS and combined data, Plunkett *et al.* (2004) have placed *Heteropanax* within the Asian *Schefflera* subgroup with low to moderate bootstrap support but its position is not resolved in the *trn*L-*trn*F phylogeny.

Schefflera includes *c.* 650 species, representing half of the species diversity within Araliaceae. Based on ITS and *trn*L-*trn*F data, it has been concluded that *Schefflera* species form five distinct clades: Asian *Schefflera*, Neotropical *Schefflera*, Pacific Island *Schefflera*, African/Malagasy *Schefflera* and *Schefflera* sect. *Schefflera* (which includes the type species, *S. digitata*) (Plunkett *et al.*, 2001, Wen *et al.*, 2001, Plunkett *et al.*, 2004). All *Schefflera* species in India belong to the Asian *Schefflera* clade and should be placed in *Heptapleurum* Gaertn.

The genus *Merrilliopanax* H.L. Li consists of four or five species although the recent monograph by Shang (1983b) recognized only three species: *M. alpinus* (C. B. Clarke) C.B. Shang, *M. listeri* (King) H.L. Li and *M. membranifolius* (W.W. Smith) C.B. Shang. Sastry (1967) described *Merrilliopanax cordifolius* from Subansiri District, Arunachal Pradesh of India. Shang (1983b) placed *M. cordifolius* under the synonymy of *M. alpinus*. Our recent examination of specimens including the types of *M. cordifolius* suggests that *M. cordifolius* is morphologically highly distinct from *M. alpinus*. *Merrilliopanax cordifolius* differs from *M. alpinus* by the former's subcordate (vs. deeply cordate) leaf base, narrower leaves (vs. 10.5-22 cm wide) and less pubescent leaves and inflorescences. The two taxa also can be distinguished by the almost uniformly trilobed leaves in *M. alpinus* vs. mostly unlobed and sometimes 2-3 lobed leaves in *M. cordifolius*.

Macropanax comprises about 15 species with a wide distribution from Central China westward to the Himalayan region (Sikkim, Bhutan and Nepal) and southward to West Malaysia, with one species in Java (Philipson, 1979; Shang, 1983, 1985; Grushvitzky *et al.*, 1987). The centre of diversity for *Macropanax* is South West China and Vietnam where 12 species occur. Hoo and Tseng (1978) suggested that *Macropanax* might be related to *Schefflera* but Wen *et al.* (2001) indicated a close relationship to *Metapanax* based on ITS sequences, a feature supported by subsequent studies on ITS and *trn*L-*trn*F data (see Plunkett *et al.*, 2004). Three species of *Macropanax* are distributed in India: *M. dispermus* (Blume.) Kurz, *M. meghalayensis* Harid. & R.R. Rao and *M. undulatus* Miq.

Eleutherococcus consists of about 35 species from Eastern Asia (China, Korea, Japan, and Eastern Russia) and the Himalayan region (Harms, 1918; Li 1942; Hoo & Tseng, 1965, 1978; Kim & Sun, 2000). The genus has been highly controversial taxonomically, especially regarding its delimitation, infrageneric classification and species circumscription (eg. Harms, 1918; Nakai, 1924; Li, 1942; Hoo & Tseng, 1978; Ohasi, 1987; Plunkett *et al.*, 2004). Only two species occur in India: *E. cissiflorus* (Griff. ex C.B. Clarke) Nakai and *E. trifoliatus* (L) S.Y. Hu.

With the high diversity of Araliaceae in India, we attempt to examine the phylogenetic relationships of Araliaceae in India. We employ sequences of the nuclear ribosomal internal transcribed spacer (ITS) regions to infer the relationship patterns. This molecular marker has been successfully used for phylogenetic studies of Araliaceae (Wen & Zimmer, 1996; Wen *et al.*, 1998, 2001; Pandey *et al.*, 2002).

Materials and Methods

This study sampled 44 accessions of Araliaceae, including two species of *Osmoxylon* as outgroups (Table 1). *Osmoxylon* was found closely related to the Asian group of Araliaceae, yet is outside the clade within the core Araliaceae. It is thus a good choice as the outgroup for our analysis. Voucher specimens are deposited at the Bhagalpur University Herbarium (BHAG), Central National Herbarium (CAL) and Field Museum Herbarium (F) (Table 1).

Plant materials of most species were collected in nature. Total DNA was extracted by following the CTAB method of Doyle and Doyle (1987) or using the Dneasy Plant Mini kit (QIAGEN Inc.). Some of the ITS sequences were already available from our earlier study (Pandey *et al.*, 2002). DNA amplifications were performed in 100-μl reactions containing approximately 50ng genomic DNA, 20 mM Tris buffer (pH 8.3) with 50 mM KCl, 1.5 mM $MgCl_2$ and 0.1% Tween 20, 0.15 mM of each dNTP, 1 μM of each amplification primer (ITS4 and ITS5, White *et al.*, 1990), and 5 units of Taq DNA polymerase (Promega).

The ITS regions were amplified following Wen and Zimmer (1996) using different primers (for primer sequences see Pandey *et al.*, 2002). Double stranded PCR products were produced via 45 cycles of denaturation (94°C for 1 min.), annealing (50°C for 2 min.) and extension (72°C for 2 min.). A 5-min. final extension cycle at 72°C followed the 45 cycles to ensure the completion of novel strands. The PCR products were purified with the Wizard PCR Preps DNA Purification System (Promega, Madison, WI, USA) prior to sequencing.

The sequencing reaction was performed in a 10 μl final volume with the BigDye Terminator cycle sequencing kit (Perkin-Elmer, Applied Biosystems) following the manufacturer's instructions. Sequenced product was precipitated with 17μl of deioidized sterile water, 3μl of 3M NaOAc and 70 μl of 95% EtOH. Polyacrylamide gel electrophoresis was conducted with Long Ranger Singel packs (FMC BioProducts) and an ABI 3100 automated DNA sequencer (Perkin-Elmer, Applied Biosystems). The resulting sequences were assembled using Sequencher (ver.3.1.1) and aligned manually.

Initial direct sequencing of *Panax bipinnatifidus* (*Pathak & Bhaumik 4115*) revealed polymorphic sequences. We, therefore, closed the PCR product prior to sequencing. TOPO™ (Invitrogen) kits for cloning Taq polymerase-amplified PCR products into a plasmid

Table 1. Accessions of Araliaceae and outgroup sampled for ITS study

Taxon	Voucher	Geographic origin in India	GenBank Accession No.
Aralia cachemirica Decne.	*Anzar 501* (BHAG)	Kashmir	AY725107
A. foliolosa Wall. ex C.B. Clarke	*Pandey 5009* (BHAG)	West Bengal	AY233312
	Pathak & Bhaumik 4401 (CAL)	Arunachal Pradesh	AY725108
A. gigantea J. Wen	*Pandey 5001* (BHAG)	West Bengal	AY233313
	Pandey 5099 (BHAG)	Sikkim	AY725138
A. kingdon-wardii J.Wen, Lowry & Esser	*Pathak & Bhaumik 4470* (CAL)	Arunachal Pradesh	AY725109
A. leschenaultii (DC.) J. Wen	*Pandey 5002* (BHAG)	West Bengal	AY233318
Brassaiopsis aculeata Seem.	*Pathak & Bhaumik 4447* (CAL)	Arunachal Pradesh	AY725110
B. glomerulata Regel	*Pathak & Bhaumik 4406* (CAL)	Arunachal Pradesh	AY725111

B. hainla Seem.	*Pathak & Bhaumik 4405* (CAL)	Arunachal Pradesh	AY725112
	Pathak & Bhaumik 4412 (CAL)	Arunachal Pradesh	AY725113
B. mitis C.B. Clarke	*Pandey 5005* (BHAG)	West Bengal	AF551726
B. hispida Seem.	*Pathak & Bhaumik 4419* (CAL)	Arunachal Pradesh	AY725115
	Pathak & Bhaumik 4424 (CAL)	Arunachal Pradesh	AY725117
B. griffithii C. B. Clarke	*Pathak & Bhaumik 4473* (CAL)	Arunachal Pradesh	AY725116
B. shweliensis W.W. Smith	*Pathak & Bhaumik 4497* (CAL)	Arunachal Pradesh	AY725114
B. simplicifolia C.B. Clarke	*Pathak & Bhaumik 4404* (CAL)	Arunachal Pradesh	AY725118
Eleutherococcus trifoliatus (L.) S.Y. Hu	*Pathak & Bhaumik 4425* (CAL)	Arunachal Pradesh	AY725119
Heteropanax fragrans (Roxb.) Seem.	*Pathak & Bhaumik 4442* (CAL)	Arunachal Pradesh	AY725120
Macropanax dispermus (Blume) Kuntze	*Pandey 5057* (BHAG)	Meghalaya	AY725121
	Pathak & Bhaumik 4411 (CAL)	Arunachal Pradesh	AY725123
	Pathak & Bhaumik 4415 (CAL)	Arunachal Pradesh	AY725139
	Pathak & Bhaumik 4423 (CAL)	Arunachal Pradesh	AY725124
	Pathak & Bhaumik 4448 (CAL)	Arunachal Pradesh	AY725122
	Pathak & Bhaumik 4469 (CAL)	Arunachal Pradesh	AY725140
M. undulatus Miq.	*Pathak & Bhaumik 4421* (CAL)	Arunachal Pradesh	AY725125
Merrilliopanax alpinus (C.B.Clarke) C.B.Shang	*Pandey 5008D* (BHAG)	West Bengal	AY233309
M. cordifolius Sastry	*Pathak & Bhaumik 4500* (CAL)	Arunachal Pradesh	AY725137
Panax assamicus Banerjee	*Pandey 5000H* (BHAG)	Darjeeling	AY233320
	Pandey 5056 (BHAG)	Meghalaya	AY725136
	Pandey 5058 (BHAG)	Meghalaya	AY725135
P. bipinnatifidus Seem.	*Pathak & Bhaumik 4115* (CAL)	Arunachal Pradesh	AY725134
Schefflera hypoleuca (Kurz) Harms	*Pandey 5051* (BHAG)	Meghalaya	AY725127
S. hypoleuca (Kurz) Harms	*Pathak & Bhaumik 4410* (CAL)	Arunachal Pradesh	AY725128
	Pathak & Bhaumik 4413 (CAL)	Arunachal Pradesh	AY725129

S. bengalensis Gamble	*Pandey 5054* (BHAG) *Pathak &* *Bhaumik 4438* (CAL)	Meghalaya Arunachal Pradesh	AY725130 AY725131
S. impressa Harms	*Pathak &* *Bhaumik 4495* (CAL)	Arunachal Pradesh	AY725132
S. roxburghii Gamble	*Pandey 6004* (BHAG)	Jharkhand	AY725126
Tupidanthus calyptratus Hook. f. & Thoms.	*Pathak &* *Bhaumik 4408* (CAL)	Arunachal Pradesh	AY725133

vector were used according to the methods described in Mitchell and Wen (2004). A total of 8 white colonies were then chosen per plate for PCR. Sterile 10 μl pipette tips were used to remove individual colonies which were then placed directly into individual PCR

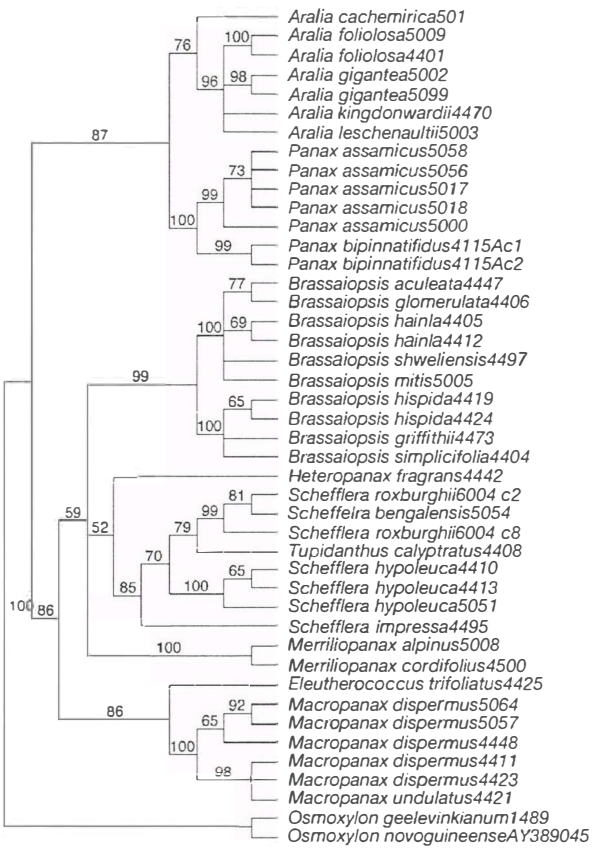

Figure 1. The strict consensus of 72 maximally parsimonious trees of Indian Araliaceae based on the ITS data set with gaps being treated as missing data (377 steps, CI = 0.68, and RI = 0.89). Bootstrap values greater than 50% in 1000 replicates are shown above the lines.

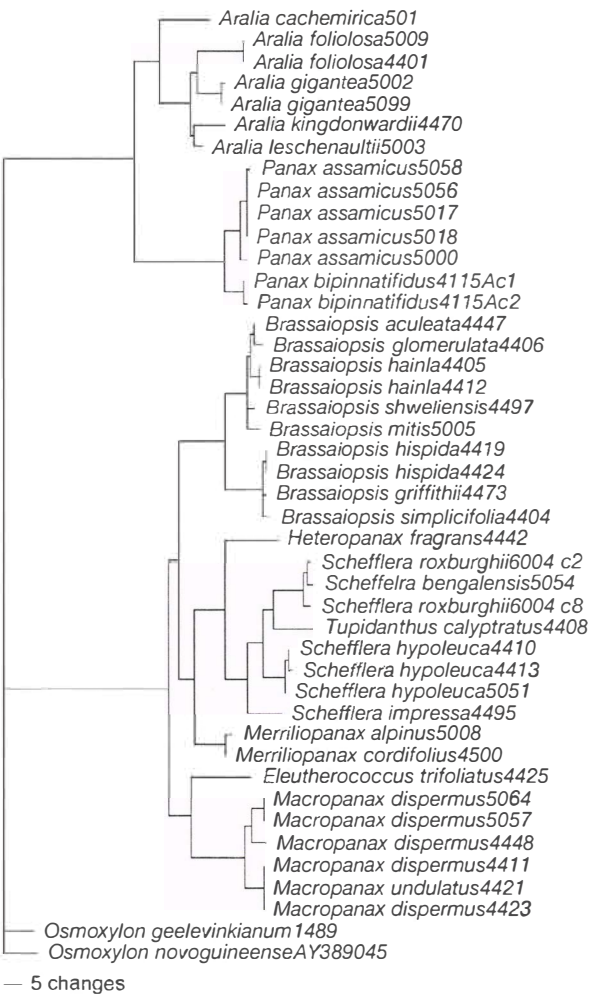

Figure 2. Phylogram of one of 72 maximally parsimonious trees of Indian Araliaceae based on the ITS data set with gaps being treated as missing data.

reaction mixtures (using the same primers as in the original amplification). The subsequent PCR products were then cleaned for sequencing using the GELase™ method.

Phylogenetic analyses were performed with PAUP (Swofford, 2003) using the maximum parsimony (Swofford *et al.*, 1996). Parsimony analysis was performed using branch-and-bound search with MULPARS and furthest addition sequence options. The amount of support for monophyletic groups revealed in the maximally parsimonious tree(s) (MPTs) was examined with 1000 bootstrap replicates (Felsenstein, 1985) with the random addition and the heuristic search options.

Results

Sequence characteristics

The length of the ITS1, 5.8S and ITS2 regions in Araliaceae taxa included in the present study ranged from 604-609 bases with an ITS1 of 218-223 bases, a 5.8S of 164 bases and an ITS2 of 212-223 bases. Insertions and deletions (indels) were necessary to align the sequences. The indels ranged in length from 1–16 bp. Furthermore, eight clones of *Panax bipinnatifidus* from Arunachal Pradesh were successfully sequenced. Initially, our direct sequencing resulted in polymorphic sequences. The cloned sequences indicated that our DNA of this sample was contaminated with that of *Schefflera roxburghii* (*Pandey 6004*). Two clones were indeed from *Panax bipinnatifidus* and the two sequences differed in one nucleotide position. We plan to collect additional material from Arunachal Pradesh and other parts of India to investigate the extent and nature of ITS polymorphism.

Analyses

The parsimony analysis of the entire ITS region resulted in 72 maximally parsimonious trees (MPTs) with a total length of 337 steps, a consistency index (CI) of 0.68 (0.64 excluding uninformative characters) and a retention index (RI) of 0.89. The strict consensus tree and one of the MPTs are presented in Figs. 1 and 2 respectively. The bootstrap values are indicated in Fig. 1 to show the relative support of each clade.

Discussion

All trees resulting from the analysis of ITS sequences resolve two major clades; one includes *Aralia* and *Panax* whereas other consists of *Brassaiopsis, Heteropanax, Schefflera, Merrilliopanax, Eleutherococcus* and *Macropanax* (Figs 1,2).

Aralia consists of six taxonomic sections (Wen, 1993, 2000, 2002). There are 11 species of *Aralia* in India, which belong to sections *Aralia, Dimorphanthus* and *Pentapanax*. In India, sect. *Aralia* is represented by two species (*A. cachemirica* and *A. tibetana*). *Aralia cachemirica* occurs in Kashmir region whereas *A. tibetana* is in Sikkim Himalayas, Nepal and Tibet (see Wen, 2002). *Aralia* sect. *Dimorphanthus* is represented by four species in India (*A. armata, A. foliolosa, A. malabarica* and *A. thomsonii).* Of these, *A. malabarica* is endemic to South India whereas the remaining three species are widely distributed in Asia (Wen *et al.*, 2002). In India, *Aralia* sect. *Pentapanax* is represented by five species (*A. leschenaultii, A. gigantea, A. kingdon-wardii, A. parasitica* and *A. subcordata*). *Aralia gigantea, A.kingdon-wardii* and *A.parasitica* are mainly confined to Northeastern region whereas *A. leschenaultii* is distributed from North East region to Nilgiri hills in South India and many other parts of Eastern Asia. Our ITS phylogeny supports the monophyly of *Aralia.* The ITS sequences suggest that the herbaceous *Aralia cachemirica* is sister to the woody *Aralia* clade. The relationships among the woody species are not well resolved with the ITS data (Figs 1, 2).

The phylogeny of *Panax* has been previously studied using ITS sequences (Wen & Zimmer, 1996), chloroplast DNA restriction sites (Choi & Wen, 2000) and chloroplast trnC-trnD intergenic region (Lee & Wen, 2004). These studies have concluded that *Panax trifolius* is sister to a clade containing the rest of *Panax.* The second diverged group is the *P. pseudoginseng-P. stipuleanatus* clade. *Panax notoginseng* is then basal to a large clade composed of *P. quinquefolius* and the rest of Asian *Panax* taxa. These observations have been supported by morphological and palynological evidences (Wen 2001b; Wen & Nowicke, 1999). The trnC-trnD phylogeny suggests that the eastern North American species *Panax quinquefolius* forms a clade with the Asiatic species *P. ginseng* and *P. japonicus.*

Panax assamicus was described by Banerjee (1968) based on material from the Shillong area, India. Its species status has been questioned by Hara (1970) and Wen (2001b). Morphologically *P. assamicus* is very similar to *P. wangianus* Sun from West Central China and to *P. zhengyianus* J. Wen from Southwestern China. All these three taxa have narrow leaflets, elongated rhizomes with thick and short internodes and fruits turning into black except for a small area near the pedicel. The ITS data suggest that the Indian *P. assamicus* is clearly distinct (Pandey et al. 2002) from the morphologically similar *P. wangianus* from West-Central China, and *P. zhengyianus* from southwestern China. *P. assamicus* and *P. bipinnatifidus* included in the present study form a clade consisting of three subclades represented by *P. assamicus* (collected from Shillong), *P. assamicus* (from Darjeeling) and *P. bipinnatifidus* (from Arunachal Pradesh).

Brassaiopsis species included in the present study form two subclades; one includes *B. aculeata, B. glomerulata, B. hainla, B. mitis* and *B. shweliensis* whereas the other is composed of *B. hispida, B. griffithii* and *B. simplicifolia.* Leaf architecture is also used to divide *Brassaiopsis s. lato* into two genera: *Brassaiopsis s. str.* with palmately compound leaves and *Pseudobrassaiopsis* having simple and palmately divided leaves (Banerjee, 1973). Our ITS study here clearly indicates

that taxa with palmately compound leaves are intermixed with those of simple and divided leaves, as in the clade of *B. aculeata - B. hainla - B. glomerulata - B. shweliensis - B. mitis*. Our ITS data of Indian *Brassaiopsis* support this observation made by Mitchell and Wen (in press).

The phylogenetic position of *Heteropanax* within Araliaceae has been highly controversial (see Wen *et al.*, 2001). ITS (Wen *et al.*, 2001) and combined ITS and chloroplast *trn*L-*trn*F data (Plunkett *et al.*, 2004) placed *Heteropanax* within the Asian *Schefflera* subgroup with low to moderate bootstrap support but its position was not resolved in the *trn*L-*trn*F phylogeny. In the present study, *Heteropanax* is found as sister to the Indian *Schefflera* and *Tupidanthus* clade.

Five accessions of *Macropanax dispermus* were included in the present study and the species forms a clade with *M. undulatus*. The *Macropanax* clade is then sister to *Eleutherococcus*. The specimen of *M. undulatus* in our study has inconspicuous sparse teeth rather than having entire leaflet margin. More field collections are needed to confirm its identity and explore the relationships among the Indian *Macropanax* species.

Our study here has set a solid foundation to understand the relationships and diversification of Araliaceae in India. We plan to further sample additional taxa from South India and Shillong area and incorporate additional species from nearby regions.

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