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# Evolution in *Aeschynanthus* (Gesneriaceae) inferred from ITS sequences

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**Abstract.** *Aeschynanthus* Jack, an epiphytic genus with c.160 species, is widespread in SE Asia. We selected 50 species for ITS nrDNA sequencing, to include all biogeographic areas and all infrageneric groupings, which are currently based on seed morphology. Some species were sequenced directly from PCR product; others cloned because of ITS length polymorphisms. The clone sequences were analysed individually and combined in an elision matrix. Results extend earlier findings that *Aeschynanthus* is divided into two clades, one occurring primarily in mainland SE Asia and the other in Malesia. This pattern is interpreted as indicating an ancient vicariance event followed by dispersal and plate fusion. Clade I has straight or clockwise spiral orientation of the testa cells and clade II anticlockwise spiral orientation. In clade I some species of section *Microtrichium* form a basal group with other sections being polyphyletic or paraphyletic. In clade II the monophyletic section *Aeschynanthus* is nested within the paraphyletic basal *Microtrichium*.

**Key words:** *Aeschynanthus*, biogeography, Gesneriaceae, internal transcribed spacers, molecular phylogeny, seed morphology, nuclear ribosomal DNA, Southeast Asia.

In the forests of Southeast Asia the brightly coloured (usually red or orange) flowers of *Aeschynanthus* species are a characteristic part of the epiphytic flora. The genus has some 160 species distributed from Sri Lanka and the Himalayas to New Guinea and the Solomon Islands. The genus therefore crosses Wallace's Line and it thus makes an interesting biogeographical case study. A previous study based on limited sampling (Denduangboripant and Cronk 2000) suggested that the genus was divided into two main clades: one centred west and one centred east of Wallace's line but with extensive overlap in the Sunda shelf islands. We have now more than doubled the sampling and can present a much more detailed analysis.

Most species appear to be bird-pollinated with scarlet tubular flowers (but occasional green-flowered species occur with unknown pollination syndrome). Typically, after pollination the gynoecium elongates into a very long (up to 43 cm) thin capsule containing large numbers of wind-dispersed seeds. Dispersal is aided by the presence of one or more usually hair-like seed appendages (although in those species with short seed appendages, these might be as important in substrate attachment as in dispersal). The appendages, one at the apical end of the seed and one or more at the hilar end, have been used to subdivide the genus into five sections (Bentham 1876, Clarke 1883, Burtt and Woods 1975). Wang

(1984) added a sixth section based on corolla characters. Recent SEM studies of seed and appendage morphology of 99 taxa (Mendum et al. 2001) found that differences in testa cell orientation and papilla and appendage structure enable the genus to be divided into two groups, A and B, within which the existing sections may be placed. Group A (testa cell orientation spiral or rarely straight, papillae formed from single cells, hilar appendage one, short and smooth) contains sections *Microtrichium*, *Aeschynanthus* and *Haplotrichium* s. str. Group B (testa cell orientation always straight, papillae formed from the junction of two cells, hilar appendages one or more, long and papillose) contains sections *Polytrichium*, *Diplotrichium* and a third assemblage of species with a single appendage at each end and previously placed in section *Haplotrichium*. Seed morphology places the two members of Wang's (1984) section *Xanthanthos* in group B (Mendum et al. 2001) although floral characters conflict with this placement (however, the differences are no greater than those found in the very variable section *Microtrichium*). It may be that this assemblage will be placed in section *Xanthanthos* but until more material of the latter becomes available for DNA sampling and further study, a decision cannot be made. Therefore the assemblage is here referred to as Section *X*. There is strong correlation between seed type and geographical distribution: group A species are essentially Malesian whereas group B species are largely confined to mainland South and Southeast Asia, with the exception of the small section *Polytrichium* which is more widespread.

Because the genus *Aeschynanthus* occurs throughout Southeast Asia but shows a high degree of endemism, the phylogenetic relationships of these species might be expected to have some relevance to the geological evolution of the area. Southeast Asia has a complex geological history, resulting from an intricate pattern of geotectonic movements (De Boer and Duffells 1996, Hall 1998, Metcalfe 1998). Many islands in the region, for instance Sulawesi and New Guinea, are geological composites formed of microcontinents and fragments of island arcs. The evolution and geographical distribution of fauna and flora has been greatly influenced by the geological complexity, and this may go some way towards explaining the high level of biodiversity in Southeast Asia (Gaston et al. 1995, Taylor et al. 1999, Myers et al. 2000). The region has long been attractive to biogeographers. Wallace's Line refers to the boundary proposed by Alfred Russel Wallace in 1860, separating the Asian from the Australasian faunistic region, and marking the point at which the two biotas collided after having been separated since the break-up of Gondwanaland in the mid-Mesozoic. This boundary divides Bali and Borneo from Lombok and Sulawesi, and passes southeast of the Philippines (Wallace 1860). However, many other biogeographic boundaries are evident in Southeast Asia and other lines have been proposed (Wallace 1863, Huxley 1868, Weber 1904, Wallace 1910). The study of the phylogenetic patterns of widespread groups of organisms of different evolutionary ages should reveal patterns explicable as a result of vicariance (geotectonic separation of land masses) and dispersal (geotectonic fusion of land masses or inter-island dispersal) (Nelson and Platnick 1980, Nelson and Platnick 1981). In this context *Aeschynanthus* is a highly suitable subject for study.

As discussed in a previous paper (Denduangboripant and Cronk 2000), many species of *Aeschynanthus* show a higher level of ITS sequence polymorphism than is usual in the Old World Gesneriaceae. In some species the sequence length polymorphism makes PCR consensus sequences unreadable and in these species we have cloned the PCR product and sequenced two clones. Two clones are sufficient as intra-individual clone variation is slight (consisting mainly of length polymorphism rather than base polymorphism) and the clones are all found to cluster together on the tree (Denduangboripant and Cronk 2000). No problems of this kind were encountered during ITS sequencing of *Streptocarpus*, an African genus also in the Gesneriaceae (Möller and

Cronk 1997a) (except for *S. saxorium* with a 4 bp insertion from duplication (Möller, unpublished)). However, Denduangboripant and Cronk (2000) reported that clone variation in *Aeschynanthus*, although significant, appeared to postdate the origin of the species and thus cloned sequences could be used satisfactorily for phylogenetic reconstruction. The approach taken here is to combine cloned sequences with consensus PCR sequences where the latter are not problematic.

Sequence alignment is generally straightforward with one exception: a short region of ITS2 corresponding to arm 1 of the predicted ITS secondary structure. This arm (stem-loop structure) is notably long in Gesneriaceae and the top of the arm appears to be redundant and is sometimes deleted altogether (Denduangboripant and Cronk 2001). This phenomenon also occurs in *Streptocarpus* and corresponds to the 40 bp deletion found in some species of that genus (Möller and Cronk 1997a). In the present study we have used secondary structure analysis to guide the alignment of this problematic area. Despite these unusual features of ITS evolution in *Aeschynanthus*, ITS appears to have robust phylogenetic utility in this genus.

## Materials and methods

**Plant materials.** Fresh leaf material of one plant representing each species was taken from the living collection held at the Royal Botanic Garden Edinburgh, except for five Sulawesi taxa which were sequenced from wild-collected leaf samples. The allied genera *Cyrtandra* and *Lysionotus* were used as outgroups as in previous analyses. Voucher herbarium specimens of all accessions analysed have been prepared and are lodged at E. For the present study, 27 further species (Table 1) were added to 23 species already sequenced in a preliminary study (Denduangboripant and Cronk 2000) to give a total of 50 *Aeschynanthus* species, about one-third of the genus, representing all morphological variation and geographical distribution of *Aeschynanthus*. *Aeschynanthus mimetes* and *A. hildebrandii* in Denduangboripant and Cronk (2000) have been re-identified as *A. fulgens* Wall. and *A. humilis* Hemsl., respectively. The latter names are used here.

**DNA extraction, PCR, cloning, and DNA sequencing.** Details of DNA extraction, polymerase chain reaction (PCR) amplification, PCR cloning, and DNA sequencing strategies for reconstructing the *Aeschynanthus* phylogeny are provided elsewhere (Möller and Cronk 1997a, b; Denduangboripant and Cronk 2000). The genomic DNA of the newly added 27 *Aeschynanthus* taxa was prepared and used as template for PCR amplification, yielding the complete ITS region (both ITS1 and ITS2 and 5.8S ribosomal DNA). The products were sequenced using either the Amplitaq-FS dye terminator cycle-sequencing kit (Perkin Elmer Biosystems Inc., Warrington, UK) or Thermo Sequenase II (Amersham Pharmacia Biotech UK Limited, Bucks, England), and analysed on an ABI 377 prism DNA sequencer (Perkin Elmer, Applied Biosystem Inc., Foster City, CA, USA). When we found uninterpretable sequence electropherograms caused by ITS length intraindividual variation (Denduangboripant and Cronk 2000), PCR cloning was then used for that species. Otherwise the consensus sequences from forward and reverse reactions were obtained without cloning. For cloning, the PCR products were purified and ligated into plasmid vectors using the Topo TA Cloning kit (Invitrogen Co., Carlsbad, CA, USA). The subcloned plasmids were extracted from transformants. At least two transformed clones were sequenced.

ITS sequence results were analysed and aligned with the previous DNA data matrix. A previous study (Denduangboripant and Cronk 2001) found a problem in sequence alignment of a short region of ITS2. This region corresponds to arm 1 (stem-loop structure) of the predicted ITS secondary structure. Therefore, we used minimum free energy secondary structure analysis to guide the alignment of this problematic area. RNA secondary structures of the arm 1 of ITS2 region were generated by the program RNAdraw version 1.1 (Matzura and Wennborg 1996). This method was also used to guide alignment in this study. However even with this aid, a 20 bp region of the aligned matrix in this region was considered ambiguously aligned and was excluded from the analysis. Full details of the analysis can be found elsewhere (Denduangboripant and Cronk 2001). All sequences and the alignment have been submitted to GenBank (accession numbers AF349153-AF349312). The new alignment of all

**Table 1.** Accessions of 27 additional species of *Aeschynanthus* examined in this study

| Taxon | Locality Collected | Section | RBGE accession No. |
|---|---|---|---|
| (1) *Aeschynanthus acuminatus* Wall. Ex A. DC. | Taiwan | *Haplotrichium* | 19991496 |
| (2) *Aeschynanthus andersonii* C. B. Clarke | Yunnan (China) | Section *X* | 19970465 |
| (3) *Aeschynanthus arfakensis* C. B. Clarke | Irian Jaya (Indonesia) | *Polytrichium* | 19972046 |
| (4) *Aeschynanthus austroyunnanensis* W. T. Wang | Yunnan (China) | Section *X* | 19951561 |
| (5) *Aeschynanthus batakiorum* Mendum & Madulid | Palawan (Philippines) | *Polytrichium* | 19980285 |
| (6) *Aeschynanthus curtisii* C. B. Clarke | Sarawak (Borneo) | *Aeschynanthus* | 19622237 |
| (7) *Aeschynanthus ellipticus* Lauterb & K. Schum | Papua New Guinea | *Microtrichium* | 19972009A |
| (8) *Aeschynanthus garrettii* Craib | Thailand | *Microtrichium* | 19750205 |
| (9) *Aeschynanthus irigaensis* (Merr.) Burtt & Woods | Luzon (Philippines) | *Microtrichium* | 19972532 |
| (10) *Aeschynanthus javanicus* Hook | Cultivated | *Aeschynanthus* | 19971339 |
| (11) *Aeschynanthus lineatus* Craib | Yunnan (China) | *Diplotrichium* | 19970163 |
| (12) *Aeschynanthus musaensis* P. Woods | Papua New Guinea | *Microtrichium* | 19750186 |
| (13) *Aeschynanthus myrmecophilus* P. Woods | Peninsular Malaysia | *Polytrichium* | 19981953 |
| (14) *Aeschynanthus nummularius* (Burkill & S. Moore) K. Schum | Papua New Guinea | *Microtrichium* | 19932365 |
| (15) *Aeschynanthus obconicus* C. B. Clarke | Sarawak (Borneo) | *Aeschynanthus* | 19622987 |
| (16) *Aeschynanthus oxychlamys* Mendum | Irian Jaya (Indonesia) | *Microtrichium* | 19930953 |
| (17) *Aeschynanthus pachytrichus* W. T. Wang | Yunnan (China) | *Diplotrichium* | 19970171 |
| (18) *Aeschynanthus philippinensis* C. B. Clarke | Mindoro (Philippines) | *Microtrichium* | 19972491 |
| (19) *Aeschynanthus pseudohybridus* Mendum | Sarawak (Borneo) | Section *X* | 19971340 |
| (20) *Aeschynanthus rhododendron* Ridl. | Peninsular Malaysia | *Microtrichium* | 20001550 |
| (21) *Aeschynanthus roseoflorus* Mendum | Seram | *Microtrichium* | 19880263 |
| (22) *Aeschynanthus* sp. (001) | Sulawesi | *Microtrichium* | Mendum, Argent & Hendrian 001 |
| (23) *Aeschynanthus* sp. (0025) | Sulawesi | *Microtrichium* | Mendum, Argent & Hendrian 0025 |
| (24) *Aeschynanthus* sp. (00171) | Sulawesi | *Polytrichium* | Mendum, Argent & Hendrian 00171 |
| (25) *Aeschynanthus* sp. (00293) | Sulawesi | *Microtrichium* | Mendum, Argent & Hendrian 00293 |
| (26) *Aeschynanthus vinaceus* P. Woods | Sarawak (Borneo) | *Microtrichium* | 19672118 |
| (27) *Aeschynanthus* cf. *viridiflorus* Teijsm & Binn | Sulawesi | *Polytrichium* | Mendum, Argent & Hendrian 00228 |

sequences used in this study is also available at http://www.icmb.ed.ac.uk/J_matrix2.pdf.

**Phylogenetic analysis.** Phylogenetic analyses by parsimony, branch support analyses, and other sequence and tree statistics were performed as described previously (Denduangboripant and Cronk 2000), with the program PAUP* (Swofford 1998) version 4.0b4a and MacClade version 3.01 (Maddison and Maddison 1992). Heuristic searches were used to find the most parsimonious trees by using RANDOM sequence addition with TBR swapping for 10 000 replicates with Multrees and Steepest Descent options. Decay Indices (Bremer support values) were calculated using the program AutoDecay version 4.0 (Eriksson 1998). Three methods of combining PCR consensus sequences and multiple clone sequences were used: (1) Clones analysed as separate individual sequences, plus the consensus PCR sequences; the problem here is the relatively large number of terminal items for analysis (80 items representing 52 species). (2) Clones combined as a consensus sequence, plus the consensus PCR sequences. Where clones differ by substitutions, the new consensus sequence is coded as both nucleotides (e.g. A and G coded R etc.); where they differ as an indel, the available sequence is used (gaps ignored). (3) Clones analysed sequentially in an elision matrix. Both clone sequences for each species are analysed in combination, while PCR consensus sequences are included twice to give a matrix of uniform length. Elision matrices are commonly used in two gene studies to combine data sets, and the method is used in an analogous way here.

A reweighting parsimony analysis was also carried out by weighting characters according to mean values of their rescaled consistency indices (RC). Successive reweighting was carried out four times, at which point no further topological changes occurred. The results of parsimony analyses were compared to a maximum likelihood (ML) analysis. To find the optimum model for the likelihood analysis, the program Modeltest version 3.0 (Posada and Crandall 1998) was firstly used to compare the likelihood score results and associated P-values between 56 ML DNA-evolution models. The program then provides a choice of the model that best fits the data by nested likelihood ratio tests and the Akaike information criterion (minimum theoretical information criterion, AIC; Akaike 1974). The model selected here was TrN + G. Appropriate substitution values, base frequency parameters and Gamma distribution shape parameter (1.0051) determined by Modeltest were then used for the maximum likelihood analysis in PAUP* with TBR swapping.

## Results

**ITS sequence characteristics.** Of the 27 additional species sequenced here, six had to be cloned (for reasons discussed in Denduangboripant and Cronk 2000). A further four species showed ITS length polymorphism resulting from single 1–2 bp deletions between different intraindividual ITS copies, but these could be interpreted satisfactorily by comparison of forward and reverse sequences, with the indel bases coded as missing data. This suggests that nearly 40% of *Aeschynanthus* species show some evidence of significant intragenomic polymorphism in their ITS sequences (22% with severe polymorphism, and 15% with minor polymorphism).

When all the sequences are aligned, a matrix of 603 aligned positions results in 213 (37.4%) potentially informative sites (Table 2). In addition, 92 indels were coded of which 64 are informative. The cause of the ITS polymorphism was usually evident from inspecting the differences between the respective cloned sequences. Most striking was *A. pachytrichus* in which the clones differ by a 9 bp indel event. The highest intraindividual clone divergence yet recorded in *Aeschynanthus* is between the two highly divergent (7.68%) clones of *Aeschynanthus* sp. (0025) from Sulawesi. However, when analysed separately, even these clones fall together on the tree.

**Phylogenetic analysis.** Three different types of matrix were analysed (see methods): (1) the full matrix with all clones analysed as separate entities (81 terminals); (2) the clones combined as a consensus matrix; and (3) combined as an elision matrix. Strict consensus trees resulting from the three methods of matrix assembly have no conflict, being fully congruent at the species level. Minor differences in resolution are noticeable between trees. Between two and three nodes supported in

**Table 2.** Sequence characteristics of ITS1 and ITS2 regions of 81 sequences (representing 52 species) of Gesneriaceae. Characteristics of the aligned matrix excluding ambiguous sequence sites

| Parameter | ITS1 | ITS2 | ITS1 and ITS2 |
|---|---|---|---|
| Length range (bp) – Ingroup + outgroup | 217–237 | 206–254 | 430–491 |
| – Ingroup | 217–233 | 206–247 | 430–477 |
| – Outgroup only | 225–237 | 243–254 | 468–491 |
| Length mean (bp) – Ingroup + outgroup | 225.7 | 239.6 | 465.0 |
| – Ingroup | 225.3 | 239.3 | 464.6 |
| – Outgroup only | 231.0 | 248.5 | 479.5 |
| Aligned length (bp) | 289 | 314 | 603 |
| G + C content range (%) (complete matrix) | 48.12–59.66 | 49.57–59.51 | 48.90–59.58 |
| G + C content mean (%) (complete matrix) | 54.71 | 55.24 | 54.98 |
| Sequence divergence (%) – Ingroup to outgroup | 13.87–22.36 | 16.00–24.38 | 15.96–23.38 |
| – Ingroup (between spp.) | 0.00–19.20 | 0.00–17.18 | 0.00–16.95 |
| – Ingroup (within spp.)[a] | 0.00–8.04 | 0.00–7.33 | 0.00–7.68 |
| Number of indels – Ingroup + outgroup | 34 | 58 | 92 |
| – Ingroup (total) | 29 | 46 | 75 |
| – Ingroup (within spp.) | 5 | 15 | 20 |
| Size of indels (bp) – Ingroup + outgroup | 1–4 | 1–9 | 1–9 |
| – Ingroup (total) | 1–4 | 1–9 | 1–9 |
| – Ingroup (within spp.) | 1, 2, 4 | 1, 2, 9 | 1, 2, 4, 9 |
| Number of excluded sites | 15 | 18 | 33 |
| Number of sites after exclusion | 274 | 296 | 570 |
| Number of variable sites | 140 | 171 | 310 |
| Number of constant sites (%) | 134 (48.9 %) | 125 (42.2 %) | 260 (45.6 %) |
| Number of potentially informative sites (%) | 94 (34.3 %) | 119 (40.2 %) | 213 (37.4 %) |
| Number of autapomorphic sites (%) | 46 (17.1 %) | 52 (17.6 %) | 97 (17.0 %) |
| Transitions on tree (unambiguous) | 124 | 173 | 314 |
| Transversions on tree (unambiguous) | 59 | 106 | 166 |
| Transitions/Transversions | 2.10 | 1.63 | 1.89 |

[a] Divergence between clone pairs

one tree collapse in the other and vice-versa. We found that the elision matrix was fastest to run and gave fewest trees (1440 trees), whereas the full matrix and the consensus combination matrix gave more than 37 600 trees (trees exceeded a computer memory used by PAUP*) and 10 073 trees respectively. The results presented here use the elision matrix, but none of the conclusions reached is affected by the type of analysis.

All phylogenetic trees produced in this study, by whatever method, confirm the division of *Aeschynanthus* into two major clades both of which have high Bootstrap support (97% clade I; 99% clade II) (Fig. 1). The maximum likelihood tree (Fig. 2) does not show any incongruence with the maximum parsimony strict consensus tree, and provides further support for the two-clade division of the genus. Many well-supported nodes on the MP tree allow us to conclude with a high degree of certainty that the existing sections, based largely on seed-appendage types, are paraphyletic or polyphyletic. An exception is section *Aeschynanthus* which is monophyletic (Bootstrap support = 99%). In clade I, section *Microtrichium* is basal with the other sections polyphyletic or paraphyletic. In clade II most

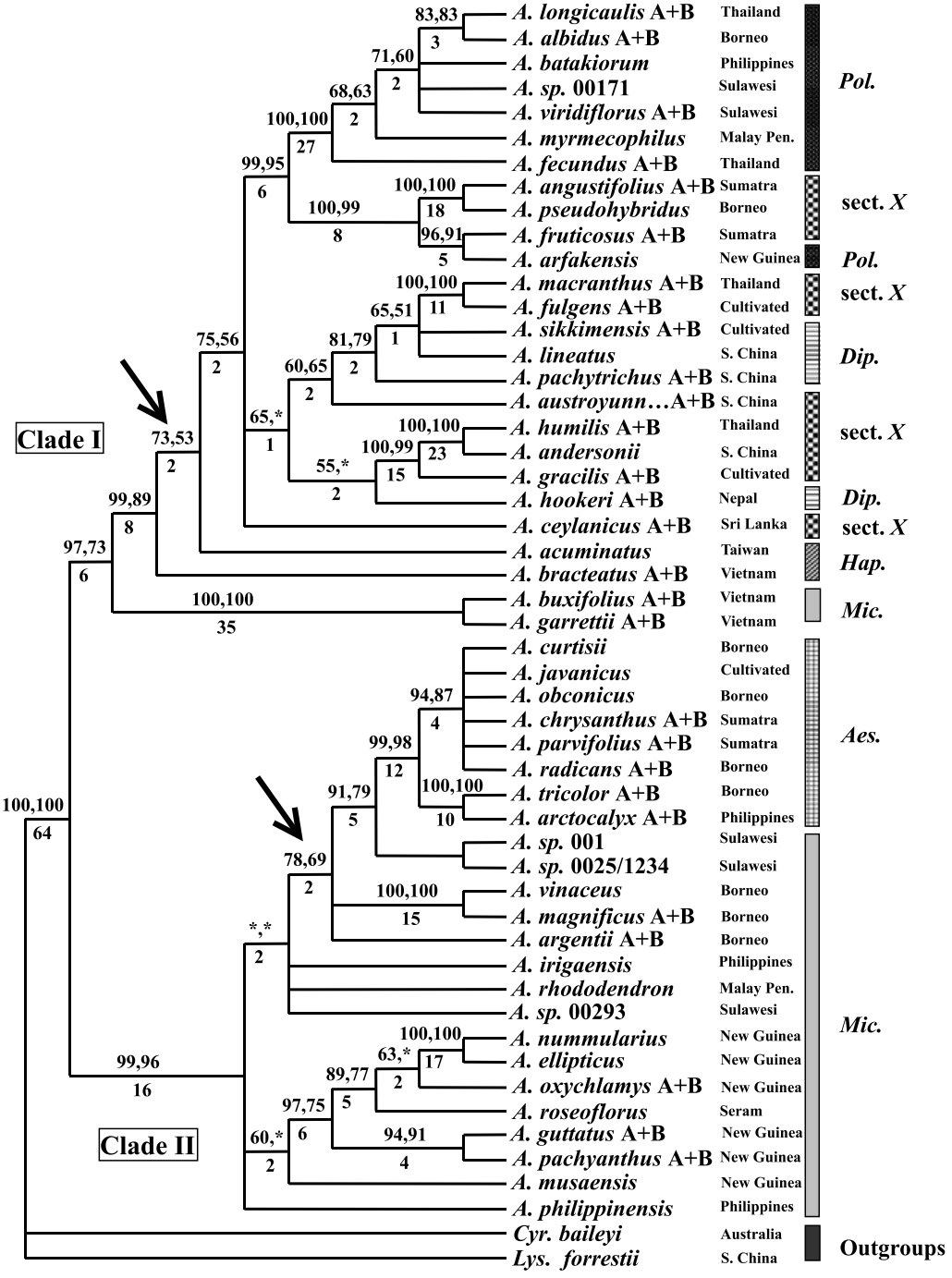


**Fig. 1.** Strict consensus of 1440 most parsimonious trees for 50 *Aeschynanthus* species and two outgroup Gesneriaceae taxa (1557 steps in length) based on parsimony analysis of an elision matrix of the combined ITS1 and ITS2 sequence data plus the alignment gap matrix. The first values of upper numbers are full heuristic Bootstrap percentages of 100 replicates. The second values of upper numbers are 50% deletion Jackknife percentages ("fast" stepwise-addition) of 10 000 replicates. Lower numbers are decay indexes. The two arrows indicate branches that collapse when the gap matrix is excluded and the analysis rerun. The country of origin of the specimen is indicated. [CI = 0.62, RI = 0.79, RC = 0.49] The two clones per species are designated A and B, except for *Aeschynanthus* sp. 0025 for which the four clones are designated 1234

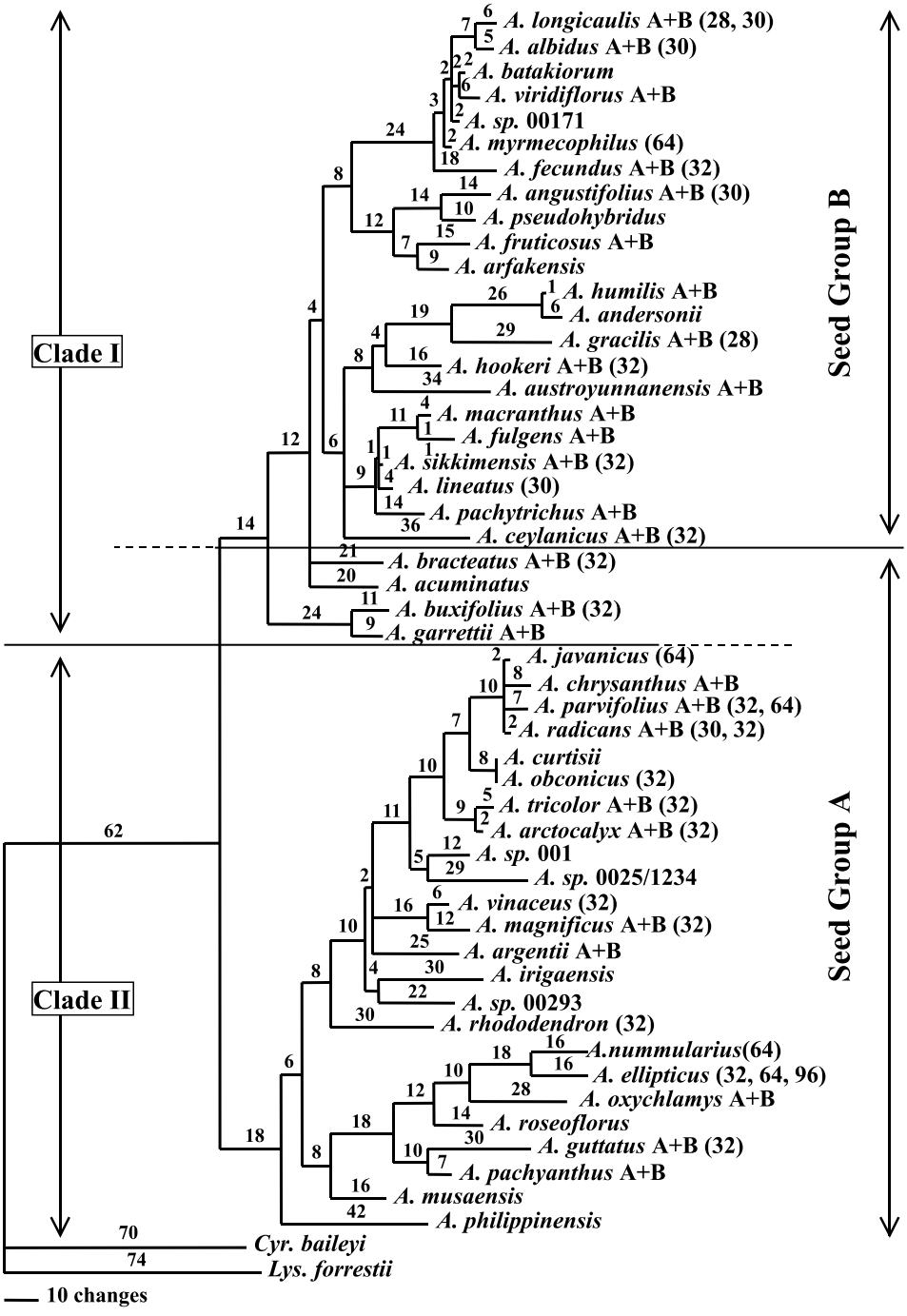


**Fig. 2.** Maximum likelihood (ML) tree for 50 *Aeschynanthus* species and two outgroup Gesneriaceae taxa (−ln likelihood = 8570.14) based on an analysis of the elision matrix of the combined ITS1 and ITS2 sequence data without the gap matrix. As the elision matrix is used, the branches are double their true length. Numbers along branches indicates the amount of character change (branch length). Available chromosome numbers (Rogers 1954, Eberle 1956, Ratter 1963, Ratter and Prentice 1967, Ratter and Milne 1970, Milne 1975, Hellmayr 1989, Kiehn and Weber 1997, Rashid et al. 2001) are given in brackets following the species names (all as 2n for ease of comparison). Vertical lines denote major seed types and the main clades, which are congruent except for the four basal clade I species

of the species belong to section *Microtrichium* with the monophyletic section *Aeschynanthus* nested within this. It is clear from inspection of Fig. 1 that there is considerable biogeographic pattern in the phylogeny. The majority of species in clade I occur in India, Indochina, South China and a few on islands of the Sunda shelf. In contrast species in clade II occur in New Guinea, Sulawesi, Philippines and Seram with some species also occurring on the Sunda shelf islands.

## Discussion

***Aeschynanthus* biogeography: ancient vicariance and recent dispersal.** The extended analysis of the phylogeny of *Aeschynanthus* confirms the division of the genus into two major clades differing in geography. Of the 26 clade I species, 17 occur on mainland SE Asia (including Taiwan) and only nine are Malesian (including Peninsular Malaysia). Six of the nine, including all clade I species east of Wallace's Line, are in section *Polytrichium*. This wider distribution (Fig. 3) is possibly due to the greater effectiveness of a coma (hair-tuft) of many hair-like appendages in wind dispersal. The long filiform appendages possessed by most clade I species are effective for wind dispersal in dry conditions, and the majority of these species occur in the seasonal climates of mainland Southeast Asia. The great majority of clade II species, with shorter less elaborate appendages, occur in the more consistently wet forests of Malesia (Fig. 3 and Fig. 4). The 24 clade II species are all Malesian. The species basal to clade I are from Indo-China and Taiwan, implying a possible ancestral area for clade I in this region. Clade II (Fig. 4) on the other hand has Philippine and New Guinea species as basal with *A. philippinensis* as most basal by ML and reweighting analyses (Figs. 2 and 5).

The geographical difference between the two major clades implies an ancient vicariance event at the time of the origin of the genus between Indo-China and the Philippines. De Boer and Duffells (1996) postulated identical vicariance patterns between the Asian mainland and eastern Malesia for two cicada groups some 20 million years ago. They suggested that the formation of a volcanic island chain at the western Pacific plate margin allowed separation of mainland and island arc clades. The island arc no longer exists, having migrated west as discrete terranes which now form parts of the Philippines, Sulawesi, and New Guinea. The subsequent coming together of the Australasian and Asian plates would result in an overlap of clades along the Sunda shelf margins. This is entirely consistent with the observed geographic patterns in *Aeschynanthus* (Fig. 6, more details presented in Figs. 3 and 4), which would then be the result of ancient vicariance overlain by recent dispersal and coalescence events, as summarised diagrammatically in Fig. 7.

An increase in seed appendage length and number (providing a favourable surface area to mass ratio) appears to be a "key innovation" allowing biogeographic transgression of the main clade areas. The *Microtrichium* type is basal in both clades and is probably the ancestral state. The only clade I species known to be present east of the dotted line in Fig. 6 are in section *Polytrichium*, whose derived morphology is extreme in appendage number.

**Seed morphology reflects major clade structure in *Aeschynanthus*.** The recent morphological studies recognising two major groups in *Aeschynanthus* align well with the molecular results. Clade I broadly corresponds to seed group B and clade II corresponds to seed group A (Fig. 2). Those seed group A species that do fall into clade I are those that are basal. *Aeschynanthus buxifolius* and *A. garrettii* (section *Microtrichium*) are two of the only three *Microtrichium* species to have ± straight testa cell orientation (the others have an anticlockwise spiral); they are also the only ones that are not Malesian. *Aeschynanthus acuminatus* and *A. bracteatus* are two members of the very small section *Haplotrichium* which is not known to occur in Malesia. Clade I and clade II differ in the orientation of the testa cells and can therefore be defined morphologically.

| Species in clade I | S India [a] | N India [b] | E Tibet [c] | NE India [d] | Burma | S China [e] | Thai-land | Viet-nam | Laos | Tai-wan | Pen. Malay [f] | Suma-tra | Java | Borneo [g] | Phillip-pines [h] | Sula-wesi | Seram | New Guinea [i] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *A. ceylanicus* | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. hookeri* | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. acuminatus* | 0 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. fulgens* | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. bracteatus* | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. sikkimensis* | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. gracilis* | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. andersonii* | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. pachytrichus* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. austroyunnanensis* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. humilis* | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. buxifolius* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. lineatus* | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. macranthus* | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. garrettii* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. longicaulis* | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. fecundus* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. myrmecophilus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. albidus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 |
| *A. angustifolius* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 |
| *A. fruticosus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. pseudohybridus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| *A. batakiorum* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| *A.* sp. 00171 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| *A. viridiflorus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| *A. arfakensis* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| Total | 1 | 2 | 3 | 6 | 7 | 12 | 11 | 8 | 2 | 1 | 5 | 3 | 2 | 3 | 1 | 2 | 0 | 1 |

[a] South India and Sri Lanka; [b] North India and Nepal; [c] East Tibet (China: Xizang province); [d] Northeast India (Sikkim, Assam, Bengal) and Bhutan; [e] South China (Yunnan, Guizhou, Guangdong, Guangxi); [f] Peninsular Malaysia and Singapore; [g] Borneo (Sabah, Sarawak, Kalimantan and Brunei); [h] the Philippines (Palawan, Mindanao, Luzon, Mindoro); [i] Papua New Guinea and Irian Jaya.

**Fig. 3.** Distribution areas of 26 *Aeschynanthus* species from clade I (0 = absence, 1 = presence)

| Species in clade II | S India [a] | N India [b] | E Tibet [c] | NE India [d] | Burma | S China [e] | Thai-land | Viet-nam | Laos | Tai-wan | Pen. Malay [f] | Suma-tra | Java | Borneo [g] | Philip-pines [h] | Sula-wesi | Seram | New Guinea [i] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *A. nummularius* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| *A. ellipticus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| *A. oxychlamys* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| *A. guttatus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| *A. pachyanthus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| *A. musaensis* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 |
| *A. roseoflorus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| *A.* sp. 001 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| *A.* sp. 0025 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| *A.* sp. 00293 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| *A. arctocalyx* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| *A. irigaensis* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| *A. philippinensis* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| *A. vinaceus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| *A. magnificus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| *A. argentii* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| *A. tricolor* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| *A. curtisii* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 |
| *A. obconicus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| *A. javanicus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| *A. parvifolius* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 |
| *A. radicans* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 | 0 |
| *A. chrysanthus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| *A. rhododendron* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 4 | 4 | 3 | 8 | 3 | 3 | 1 | 6 |

[a] South India and Sri Lanka; [b] North India and Nepal; [c] East Tibet (China: Xizang province); [d] Northeast India (Sikkim, Assam, Bengal) and Bhutan; [e] South China (Yunnan, Guizhou, Guangdong, Guangxi); [f] Peninsular Malaysia and Singapore; [g] Borneo (Sabah, Sarawak, Kalimantan and Brunei); [h] the Philippines (Palawan, Mindanao, Luzon, Mindoro); [i] Papua New Guinea and Irian Jaya.

**Fig. 4.** Distribution areas of 24 *Aeschynanthus* species from clade II

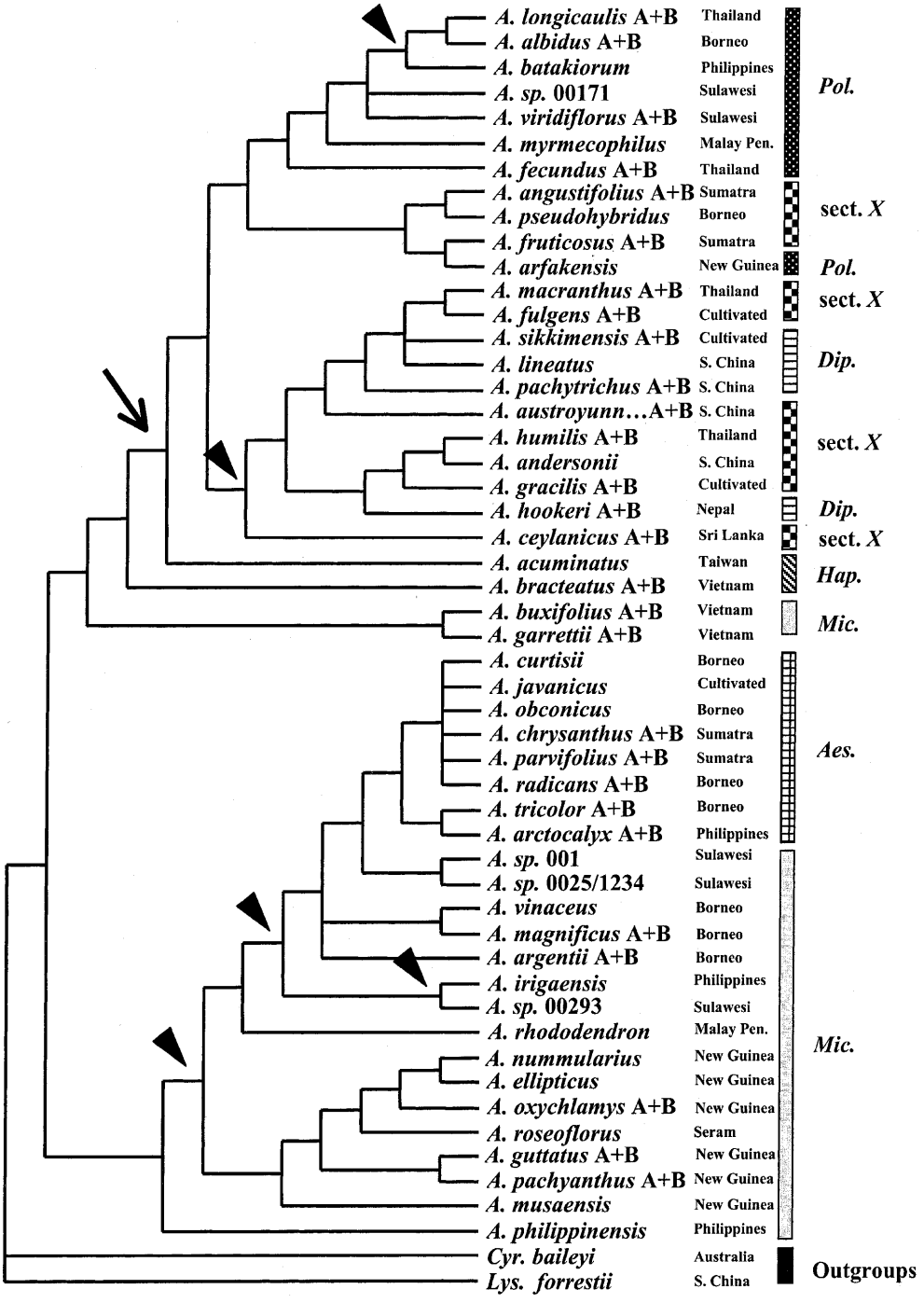


**Fig. 5.** Successive weighting strict consensus tree of 120 most parsimonious trees for 50 *Aeschynanthus* species and two outgroups (733 steps in length) based on parsimony analysis of an ITS elision matrix plus the alignment gap matrix. The 1440 most parsimonious trees of the original elision matrix were used to weight characters by their mean RC value and the analysis rerun. The arrow indicates a branch that collapses when the gap matrix is excluded and the analysis rerun. The five triangles show branches that collapse on the consensus tree of unweighted analysis. *Aeschynanthus philippinensis* is basal in clade I in this analysis as in the ML analysis (Fig. 2). [CI = 0.85, RI = 0.92, RC = 0.78]

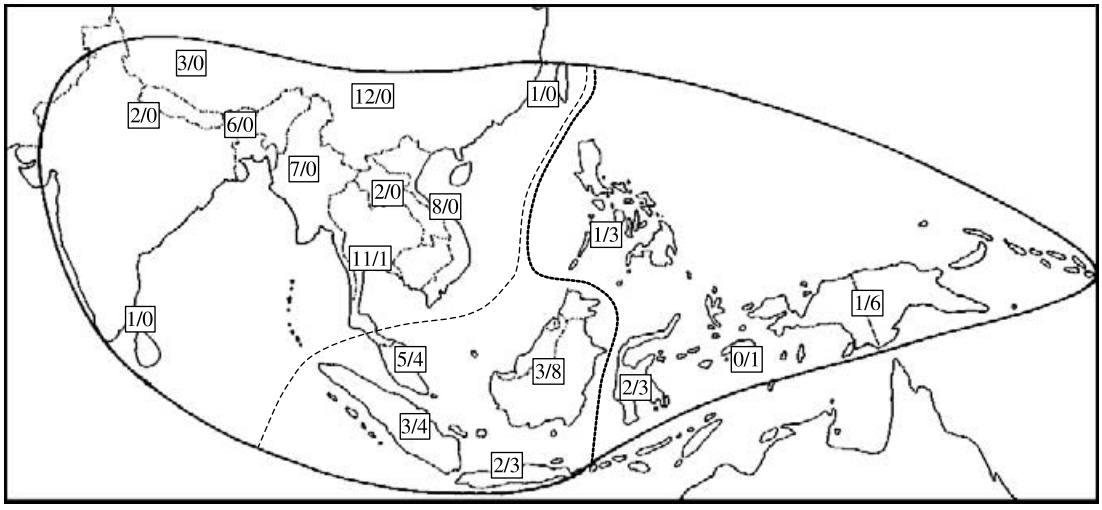


**Fig. 6.** Geographical distribution of sequenced species summarised from Fig. 3 and Fig. 4. The first number in each ratio refers to the number of species from clade I. The second number refers to the number of species from clade II. The solid line shows the geographic distribution of the whole genus. The dashed line indicates an approximate western boundary of clade II species. Clade I species, with the exception of section *Polytrichium*, do not occur east of the dotted line. This line is similar to Huxley's line (Huxley 1868) except that Palawan (Philippines) is to the east of the line

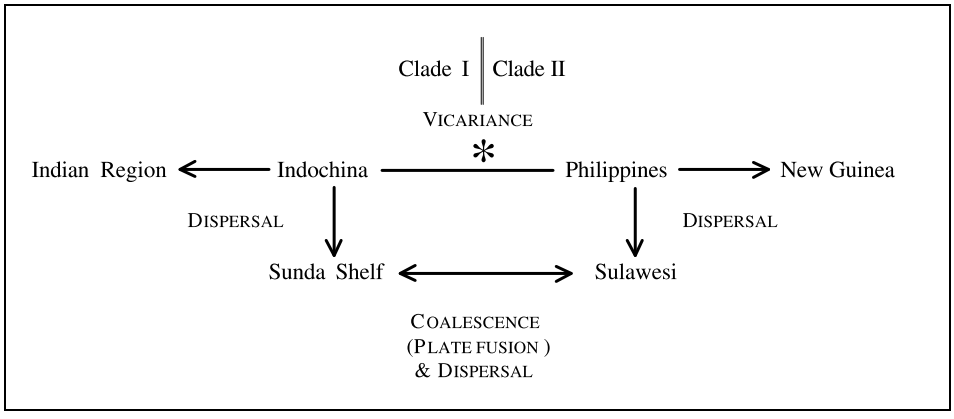


**Fig. 7.** A tentative model of the geographic pattern of *Aeschynanthus* evolution, consistent with the ITS phylogeny, suggesting a combination of ancient vicariance, recent dispersal and coalescence events in the regions. The asterisk indicates a proposed ancient origin of *Aeschynanthus* clades I and II by a vicariance event

Clade I has straight or clockwise spiral orientation and clade II anticlockwise spiral orientation (Fig. 8).

Appendage numbers appear to be at least partly homoplastic. There is morphological evidence that the condition of more than one hilar appendage is derived. All other genera in tribe Trichosporeae are reported to have a seed with a single hilar appendage, as do the majority of *Aeschynanthus* species including those basal to clade I in this study. Preliminary studies of *Aeschynanthus* seed ontogeny by Saueregger and Mühlbauer (unpublished) showed that, in section *Polytrichium*, one appendage develops a little before the others and remains somewhat longer and stouter.

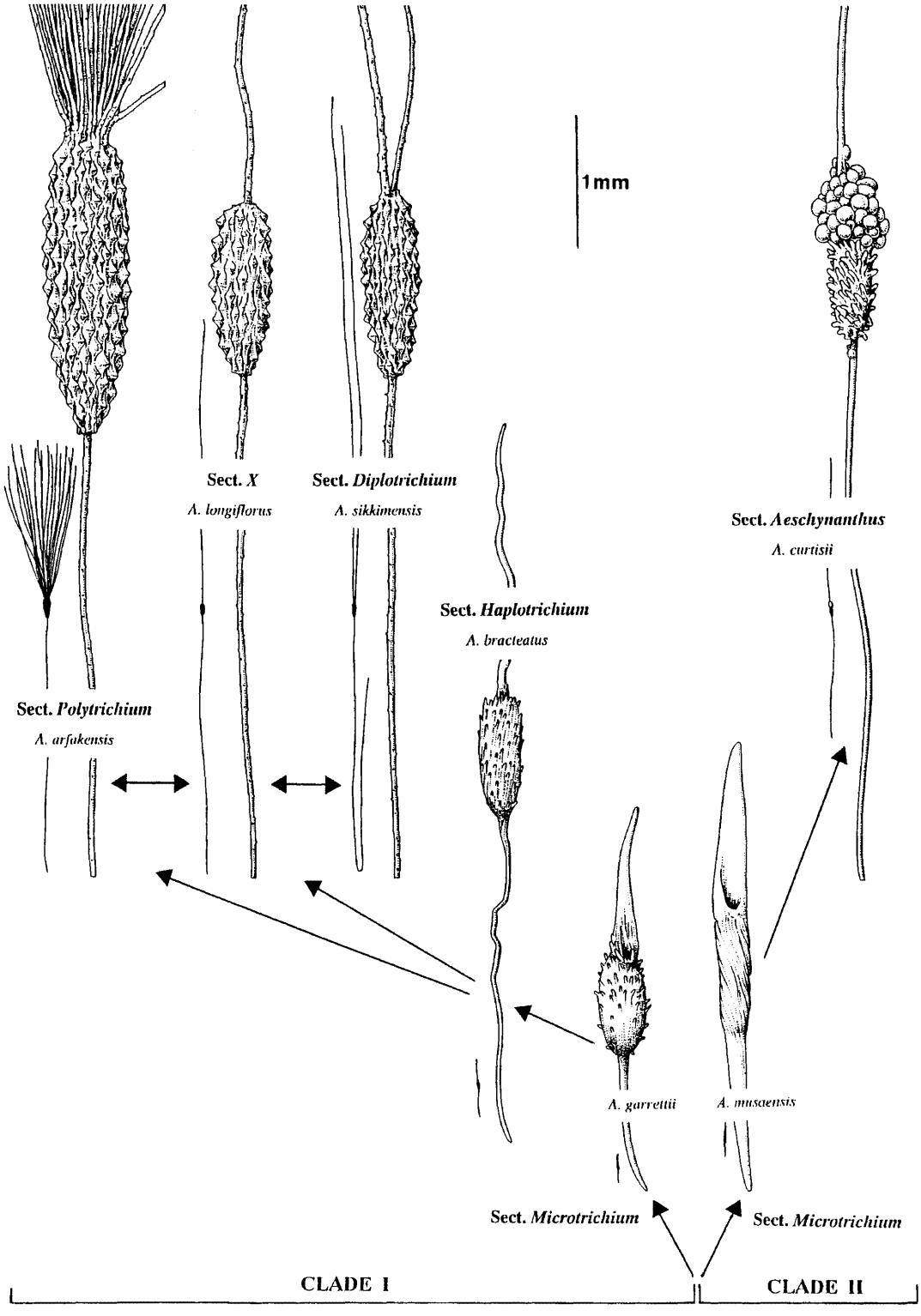


**Fig. 8.** Possible evolution of seed appendage types in *Aeschynanthus* as suggested by the phylogenetic analysis. The morphological types are represented by named exemplar species. The diagram illustrates suggested morphological transitions only, and does not imply transitions between these exemplar species

Current studies by Christie and Mendum (unpublished) confirm this and also show that in section *Diplotrichium* one appendage develops a little before the other. A small percentage of seeds of two Section *X* species show development of a second appendage, and a few seeds of one collection of *A. parasiticus* (section *Diplotrichium*) show development of a short third appendage. Thus the clade I species with Type B seed morphology do appear to be a natural group, notwithstanding the differences in appendage number. The short smooth appendage type (section *Microtrichium*) is basal in both clades, and is thus paraphyletic. Section *Aeschynanthus*, with flexuous trailing habit and tubular or obconic calyx with abscission layer at the base, is a natural group. We propose a possible evolutionary pattern of seed appendage types in *Aeschynanthus*, suggested by the phylogenetic analysis, in Fig. 8.

Available chromosome numbers (Rashid et al. 2001) show a possible slight trend towards dysploid reduction in clade I species, and polyploidy in clade II species. Counts are available for 23 of the sequenced species, 12 in clade I and 11 in clade II (Fig. 2). The commonest number is $2n = 32$, but in clade I five aneuploids occur ($2n = 30$, $2n = 28$), but only one polyploid, *A. myrmecophilus* with $2n = 64$. In clade II by contrast, only one aneuploid occurs, but polyploids are more common. More investigation of *Aeschynanthus* chromosome numbers is required to confirm or reject this possibility.

The present sectional classification, based (with the exception of section *Xanthanthos*) on easily observable appendage characters, has proved to be of considerable practical taxonomic value and it is not our intention to revise it here to reflect the ITS data. However, the existence of two major clades in *Aeschynanthus*, differing in testa cell orientation and in geographical distribution patterns, raises the possibility of dividing the genus into two clearly defined natural subgenera.

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