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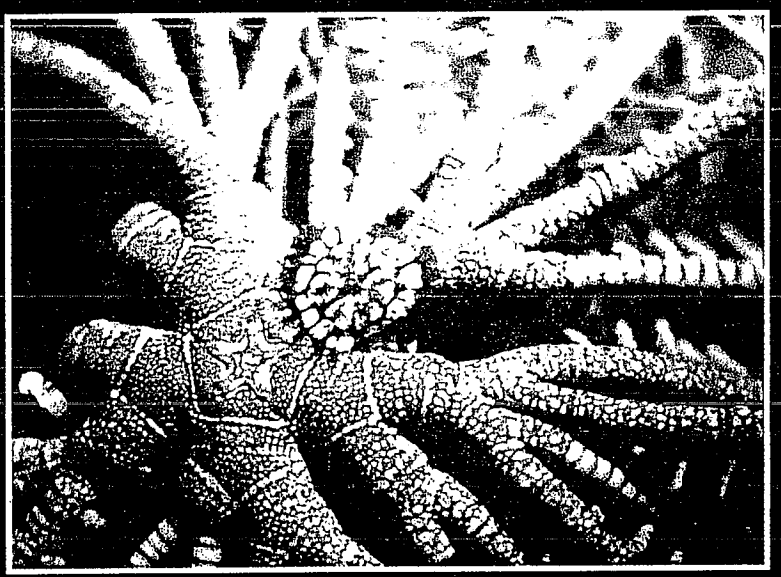

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High intraindividual variation in internal transcibed spacer sequences in *Aeschynanthus* (Gesneriaceae): implications for phylogenetics

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Aeschynanthus (Gesneriaceae) is a large genus of tropical epiphytes that is widely distributed from the Himalayas and China throughout South-East Asia to New Guinea and the Solomon Islands. Polymerase chain reaction (PCR) consensus sequences of the internal transcribed spacers (ITS) of *Aeschynanthus* nuclear ribosomal DNA showed sequence polymorphism that was difficult to interpret. Cloning individual sequences from the PCR product generated a phylogenetic tree of 23 *Aeschynanthus* species (two clones per species). The intraindividual clone pairs varied from 0 to 5.01%. We suggest that the high intraindividual sequence variation results from low molecular drive in the ITS of *Aeschynanthus*. However, this study shows that, despite the variation found within some individuals, it is still possible to use these data to reconstruct phylogenetic relationships of the species, suggesting that clone variation, although persistent, does not pre-date the divergence of *Aeschynanthus* species. The *Aeschynanthus* analysis revealed two major clades with different but overlapping geographic distributions and reflected classification based on morphology (particularly seed hair type).

Keywords: *Aeschynanthus*; Gesneriaceae; internal transcribed spacers; intraindividual variation; molecular phylogeny; nuclear ribosomal DNA

1. INTRODUCTION

The internal transcribed spacers (ITS) of nuclear ribosomal DNA are frequently used for phylogenetic analysis at the species level (Baldwin *et al.* 1995). Although nuclear ribosomal DNA is multicopy in large arrays of repeats, it is useful for phylogenetics as the copies are usually highly homogenous due to concerted evolution. However, there is sometimes persistent intraindividual variation in the rDNA repeats (e.g. Campbell *et al.* 1997), a phenomenon we report here for the genus *Aeschynanthus*.

Aeschynanthus Jack ('lipstick vine') is a genus of climbing or trailing epiphytes from the tropical forests of South-East Asia (*ca.* 160 species). Several species are widely cultivated for their flowers, which are usually scarlet with long corolla tubes and are probably bird pollinated. It has a wide distribution from Sri Lanka and the Himalayas in the west to New Guinea and the Solomon Islands in the east (table 1). It therefore crosses Wallace's Line, which is the biogeographical boundary running between Borneo and Sulawesi dividing the Gondwanic and Laurasian elements in the South-East Asian biota. *Aeschynanthus* belongs to the tribe Trichosporeae (Burtt & Woods 1958), which is characterized by seed appendages. In the case of *Aeschynanthus* these are mostly long and hair like and presumably function in wind dispersal and substrate attachment. Moreover, these seed appendages have been used to divide the genus into five sections (Bentham 1876; Clarke 1883; Burtt & Woods 1975) as shown in table 1. A sixth section, *Xanthanthos*, based not on seed appendages but on the flexuous trailing habit and broad yellow or white corolla, has recently been described (Wang 1984). This small section of two species (from Bhutan, North India, Indochina and China) is not included in the present study. The sections are currently under revision by M. Mendum (Royal Botanic Garden Edinburgh) and the numbers of species given should be regarded as approximate (M. Mendum, personal communication).

As *Aeschynanthus* is an important component of the South-East Asian epiphyte community and is spread over phytogeographically contrasting areas, it is an interesting genus for evaluating ideas about the evolution of plants in a tectonically complex region and, in particular, the importance of Wallace's Line to wind-dispersed epiphytes. We therefore wished to produce a phylogenetic analysis of the genus using sequences of the ITS of nuclear ribosomal DNA, which have proved successful in phylogenetic analyses of the other genera of the Gesneriaceae such as the *Streptocarpus* of Africa and Madagascar (Möller & Cronk 1997*a*) and *Saintpaulia* (Möller & Cronk 1997*b*). For this reason we attempted direct sequencing of polymerase chain reaction (PCR) products as described previously (Möller & Cronk 1997*a*). However, many of the resulting sequences were unreadable or poor, apparently because of significantly more extensive polymorphism at the template DNA level than in *Streptocarpus*. To circumvent this problem we cloned the PCR product and sequenced two clones of each sample.

2. MATERIAL AND METHODS

(a) *Ingroup and outgroup selection*

We selected *Lysionotus* (Don 1822) of the tribe (Trichosporeae) to which *Aeschynanthus* belongs as an outgroup. We also used a member of another tribe (Cyrtandreae), *Cyrtandra*, as a check in the unlikely event that *Aeschynanthus* proved to be paraphyletic with respect to *Lysionotus*. We chose 23 species of *Aeschynanthus*

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Table 1. *Characteristics of the five main sections of* Aeschynanthus

section name	section morphology	number of species	distribution
Haplotrichium	has seed with one long hair at each end	*ca.* 37	India, Sri Lanka, Nepal, Burma, China, Thailand, Vietnam, Taiwan, Peninsular Malaysia, Sumatra, Java, Borneo and Philippines
Diplotrichium	seed with two long hairs at the hilar end and a single hair at the apical end	*ca.* 10	North India, Nepal, Bangladesh, Bhutan, Burma, China (Yunnan), Thailand and Vietnam
Polytrichium	seed with many hairs at the hilar end and a single hair at the apical end	*ca.* 11	Burma, China (Yunnan), Thailand, Vietnam, Peninsular Malaysia, Sumatra, Java, Borneo, Philippines and New Guinea
Aeschynanthus	seed with one hair at each end but mostly with bubble-like cells at the base of the hilar-end hair. Calyx shallowly lobed	*ca.* 50	Peninsular Malaysia, Sumatra, Borneo, Philippines and New Guinea
Microtrichium	seed with a short flat appendage at each end	*ca.* 55	China (Yunnan), Thailand, Vietnam, Peninsular Malaysia, Java, Borneo, Philippines, New Guinea and Solomon Islands

(all from the living collections at the Royal Botanic Garden Edinburgh) (see electronic Appendix A, which can be found at The Royal Society Web site) representing all sections (except *Xanthanthos*) and geographical areas as the ingroup. Voucher herbarium specimens and spirit material of all these accessions have been prepared and deposited at Edinburgh (E). Although these species represent only a relatively small sample, the choice of taxa is designed to encompass the full morphological variation of the genus.

(b) *DNA extraction, PCR, cloning and DNA sequencing*

The DNA extraction, PCR, sequencing and alignment followed Möller & Cronk (1997*a*,*b*). A leaf sample was taken from a single individual plant for each species. Forward and reverse sequencing reactions were performed for sequence confirmation. The sequencing products were analysed on an ABI 377 prism automatic DNA sequencer (PE Biosystems, Inc., Warrington, UK). ITS RNA secondary structures generated by the program RNAdraw v. 1.1 (Matzura & Wennborg 1996) were used to interpret the deletion in *Aeschynanthus buxifolius* ITS1 and to optimize the alignment in arm 1 of the ITS2 region (data not shown). In order to clarify the intraindividual variation found in the *Aeschynanthus* ITS sequences and solve the problem of reading the PCR consensus sequences, a PCR cloning method was used. We sequenced two clones from each *Aeschynanthus* individual. PCR-amplified DNA of the whole ITS region was gel extracted and purified using the QIAquick gel extraction kit (QIAgen Ltd, Dorking, Surrey, UK) before cloning. The Prime PCR Cloner cloning system (5 Prime-3 Prime, Inc., Boulder, CO, USA) was used to clone the purified PCR products into the pN_0TA/T_7 cloning vector supplied following the manufacturer's instructions. The isolated plasmids were digested with *Xba*I restriction enzyme (Promega, Co., Madison, WI, USA) in order to determine the size of the subcloned PCR fragment in the clones, which could then be compared with the original PCR product. Clones with a correct size insert (*ca.* 700 bp) were sequenced using the normal cycle sequencing methods described above. Both ITS1 and ITS2 were sequenced from each clone. The alignment of all the sequences used in this study is available from the authors' Web site (http://www.icmb.ed.ac.uk/j_matrix.html).

(c) *Phylogenetic analysis*

Phylogenetic trees were reconstructed from the combined ITS1 and ITS2 sequence data using parsimony as implemented in the program PAUP (Swofford 1998) v. 4.0b2 (beta version). Ambiguous regions in the aligned matrix were excluded from phylogenetic analysis. Indels (gaps) were treated as missing data and scored as separate presence–absence characters. Analyses were carried out with and without the gap matrix. Character state changes were weighted equally. We performed heuristic searches in order to find the most parsimonious trees by using SIMPLE addition sequence with tree bisection–reconnection (TBR) swapping, with options MULTREES, steepest descent, COLLAPSE and ACCTRAN optimization. Replication of the searches using 1000 RANDOM addition sequences with TBR swapping failed to find further shortest trees. Bootstrap analysis (full heuristic) (Felsenstein 1985) and jackknife analysis (Lanyon 1985) with 50% deletion ('fast' stepwise addition) were used as indications of branch support for individual clades. The bootstrap and jackknife values were calculated using 100 and 10 000 replicates, respectively. A consistency index (CI) and a retention index (RI) were calculated. The other sequence and tree statistics, i.e. G plus C content, sequence divergence and transition:transversion ratios, were determined as described previously (Möller & Cronk 1997*a*). In order to test for possible effects of the outgroups on the analysis, the searches were rerun without outgroups. Mid-point rooting was then employed. As the topology of the resulting tree and the position of the root were identical, these results are not reported further.

3. RESULTS

(a) *Types of consensus sequencing problem*

Direct consensus sequencing of the ITS PCR products of some *Aeschynanthus* taxa proved difficult to interpret. In contrast, cloning prior to sequencing solved these problems by revealing the real sequences hiding behind the ambiguous regions in the consensus sequences. An example is shown in figure 1. There are three kinds of intraindividual variation found in *Aeschynanthus* ITS sequences.

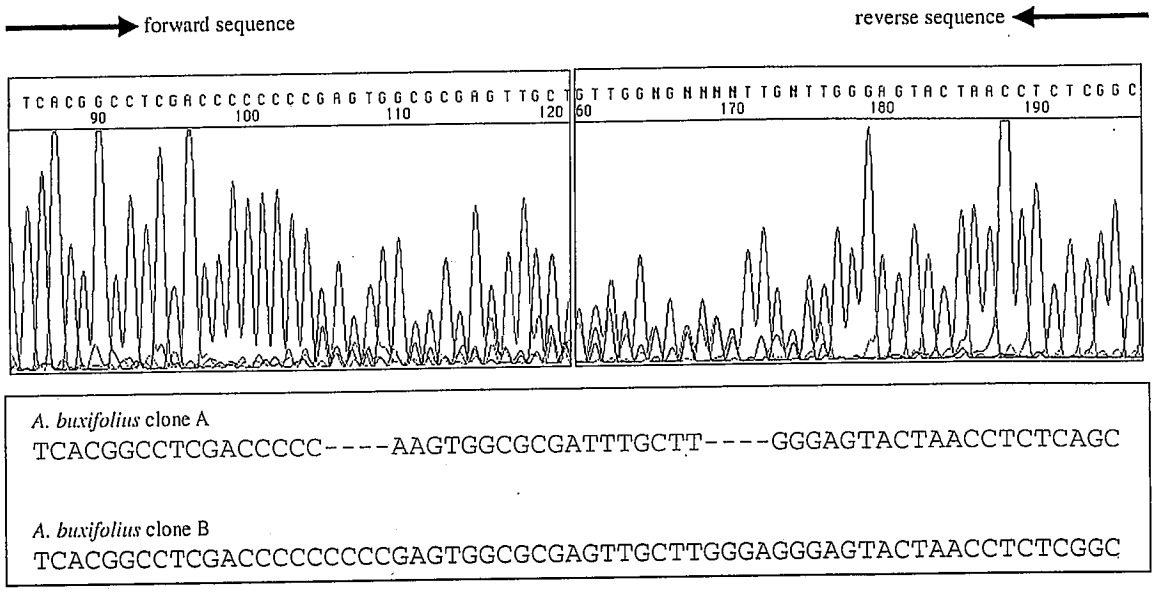

Figure 1. Electropherograms of an ITS1 consensus sequence of *A. buxifolius* compared to the same region sequenced in two clones. When sequenced with primer 5P (left side of the figure), the consensus electropherogram is easily readable until position 104, whereas sequencing with primer 2G for the reverse sequence (right side of the figure) gives a reverse sequence readable to position 176. A comparison of the clones' sequences reveals two four-base-pair indels in this region (clone A). A secondary structure analysis (figure 2) revealed that these indels are complementary insertions in a stem structure.

(i) Single-base-pair sequence polymorphisms showing a double-peak signal of two approximately equal nucleotide signals at a single base position in consensus sequencing (these are distributed apparently randomly through the sequences). This variation, which was found in both the ITS1 and ITS2 regions, was found in clone pairs of all taxa except *Aeschynanthus ceylanicus* and *Aeschynanthus hookeri*. In some cases the signal from one nucleotide was approximately half the magnitude of the other signal.

(ii) Single-base-pair indel (insertion–deletion) polymorphisms showing a clear consensus sequence up to the indel position, at which point the sequence showed double-peak nucleotide signals to the end. As such sequences are the result of a frame shift, the underlying sequences can be reconstructed uneasily from the consensus or from reverse sequences. Such indels are found in the ITS1 region of four *Aeschynanthus* taxa, i.e. *Aeschynanthus guttatus*, *Aeschynanthus bracteatus*, *A. buxifolius* and *Aeschynanthus parvifolius* and in the ITS2 region of three taxa, i.e. *A. guttatus*, *Aeschynanthus gracilis* and *Aeschynanthus mimetes*.

(iii) Multiple-base-pair indel polymorphisms. The consensus sequencing results are similar to the single-base-pair indel polymorphism, but the ambiguous region is impossible to interpret from the consensus or reverse sequencing. We detected this phenomenon in only one species: it occurred in two places in the ITS1 region of the *A. buxifolius* consensus sequence. Cloned sequences revealed two four-base-pair insertions in these regions (figure 1). Interestingly, a RNA secondary structure analysis revealed that these two insertions are complementary insertions in a stem structure (figure 2).

(b) *Sequence characteristics: intraindividual clone variation and between species variation*

The 581-base-pair-aligned ITS sequence matrix of *Aeschynanthus* and the outgroup taxa was analysed with 31 ambiguous base pairs excluded. Five of the excluded base-pair positions showed interclonal differences. Each clone could be aligned with its clone pair without adding any gaps (indels) with the exception of five species (*A. guttatus*, *A. bracteatus*, *A. buxifolius*, *A. parvifolius* and *A. mimetes*). However, the alignment between species required numerous gaps. Most of the added indels were found to be shared by both clones and were thus possible species-specific markers. Although the sequences were aligned without much difficulty, the region corresponding to arm 1 of the ITS2 secondary structure (bases 278–385) was particularly variable. This region showed considerably different indel characteristics between clades I and II (see § 3(d)). Furthermore, two large deletions occurred in this region in *A. buxifolius* and *Aeschynanthus argentii* (28 and 61 base pairs, respectively). These deletions occurred in both clones of each species and made alignment difficult. Although a secondary structure analysis was used to improve the alignment, an ambiguous 18-base-pair region was excluded from the analysis at this location (table 2). The intraindividual clone divergences range from no difference to 5.01% between *A. guttatus* clones, which is higher than the sequence divergence between the most closely related species. Approximately 50% of the

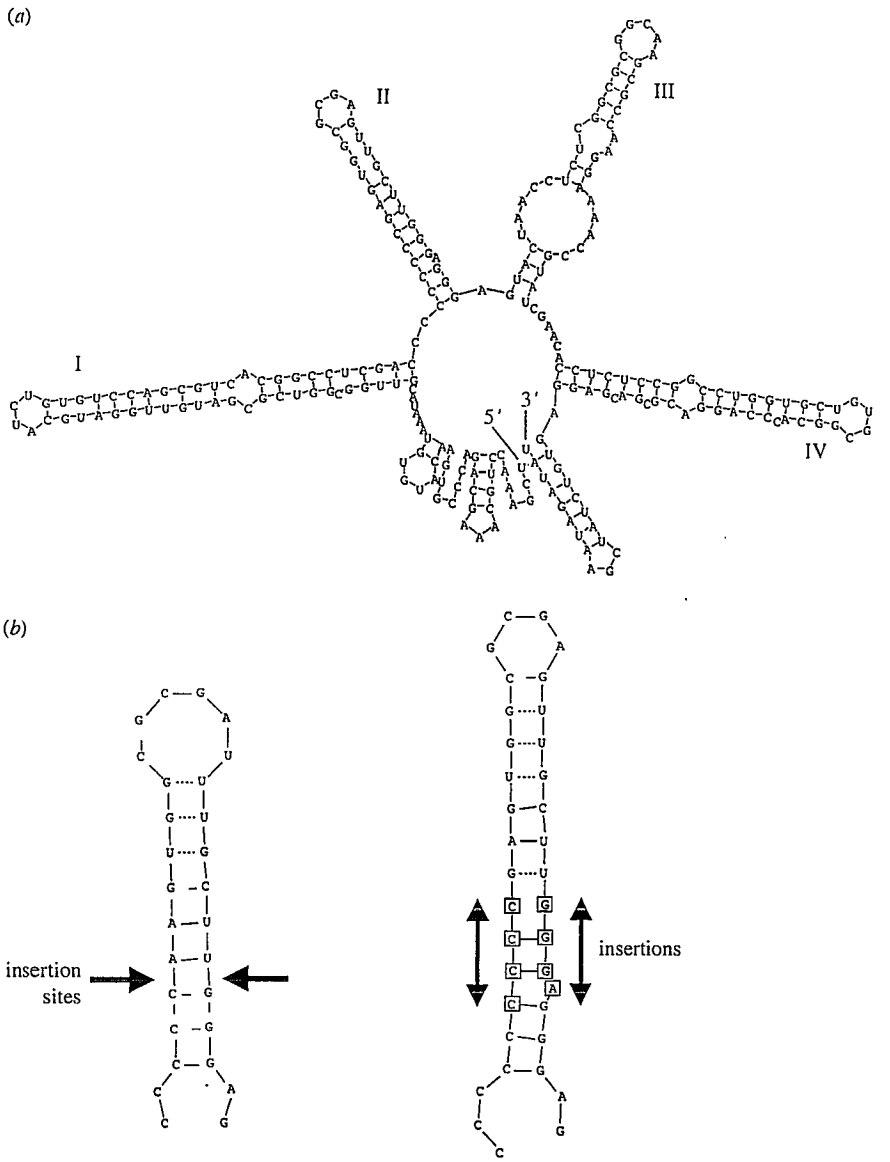

Figure 2. (*a*) Minimum free energy configuration of the ITS1 region of *A. buxifolius*, d$G = -117$ kcal at 20 °C. (*b*) Minimum free energy configuration of arm 2 of the ITS1 region of *A. buxifolius* showing (left) clone A with complementary four-base-pair deletions and (right) clone B with the complementary insert sequence.

Aeschynanthus clone pairs have low character divergences, from zero to two changes (figures 4 and 5). However, *A. guttatus* was remarkable in having 23 character changes (substitutions) between the two clones sampled.

(c) *Phylogenetic analysis*

A heuristic search using the 46 *Aeschynanthus* sequences (23 species with two clones per species) with two outgroups yielded 18 equally most parsimonious trees of 550 steps in length when the gap matrix was included. The strict consensus tree is shown in figure 3. None of the clone pairs are separated from each other on different clades and mostly they occur as sister sequences. When the gap matrix was excluded from the analysis, 96 maximally parsimonious trees were obtained with a tree length of 452 steps and lower resolution. However, the branch lengths shown in figure 4 were calculated using only substitutions (no indels) for simplicity. The bootstrap and jackknife support values were similar although the jackknife percentages were lower, giving less than 50% values to those nodes that collapse in the analysis without the gap matrix.

(d) *Morphological and geographical correlates with the putative phylogeny*

These 23 *Aeschynanthus* species form a monophyletic group with two well-supported major clades. Clade I is comprised of the *Haplotrichium*, *Diplotrichium* and *Polytrichium* sections. Clade II is comprised of the *Aeschynanthus* and *Microtrichium* sections. There are only

Table 2. *Sequence characteristics of the ITS1 and ITS2 regions of 48 taxa of Gesneriaceae*

(The characteristics of the aligned matrix exclude ambiguous sequence sites.)

parameter	ITS 1	ITS 2	ITS 1 and ITS 2
length range (bp)			
total	217–237	206–254	430–491
ingroup	217–229	206–246	430–491
outgroup	225–237	243–254	468–491
length mean (bp)			
total	225.2	239.9	465.1
ingroup	225.0	239.5	464.5
outgroup	231.0	248.5	479.5
aligned length (bp)	277	304	581
G plus C content range (%)	49.34–59.32	49.39–57.43	49.37–57.92
G plus C content mean (%)	54.01	54.28	54.15
sequence divergence (%)			
in-outgroup	13.87–20.18	16.41–23.91	15.96–21.00
ingroup (between species)	0.45–15.26	0.41–15.57	0.44–14.16
ingroup (within species)[a]	0.00–4.48	0.00–5.52	0.00–5.01
number of indels			
total	26	47	73
ingroup (total)	20	33	53
ingroup (within species)	3	4	7
size of indels (bp)			
total	1–4	1–6	1–6
ingroup (total)	1–4	1–6	1–6
ingroup (within species)	1 and 4	1	1 and 4
number of excluded sites	13	18	31
number of sites after exclusion	264	286	550
number of variable sites	111	139	250
number of constant sites (%)	153 (58.0)	147 (51.4)	300 (54.5)
number of potentially informative sites (%)	84 (31.8)	96 (33.6)	180 (32.7)
number of autapomorphic sites (%)	27 (10.2)	43 (15.0)	70 (12.7)
transitions (unambiguous)	79	89	205
transversions (unambiguous)	41	61	109
transitions/transversions	1.93	1.46	1.88

[a] Divergence between clone pairs.

two species which deviate from this arrangement: *A. buxifolius* (*Microtrichium* section) in clade I and *A. argentii* (*Haplotrichium* section) in clade II. Both the *Polytrichium* section (with the most elaborate seed hairs) and the *Aeschynanthus* section (with characteristic bubble cells on the hilar seed hairs) are monophyletic. The two main clades show different but overlapping geographic distributions (figure 6). Clade I contains species mainly from the continental western area, whereas the clade II species occur mainly in eastern Malesia, particularly on the Sunda Shelf islands and east of Wallace's Line.

4. DISCUSSION

(a) *Failure of molecular drive in* Aeschynanthus

Significant ITS polymorphism within individuals is generally considered to be unusual in plants (Baldwin *et al.* 1995). Nevertheless, intraindividual variation has been reported in the ITS region of Winteraceae (Suh *et al.* 1993), peonies (Sang *et al.* 1995), conifers (Karvonen & Savolainen 1993), *Zea* (Buckler & Holtsford 1996), *Amelanchier* (Campbell *et al.* 1997), *Cucurbita* (Jobst *et al.* 1998), *Gilia* (Morrell & Rieseberg 1998), *Castilleja* (Mathews & Lavin 1998), *Allium* (Mes *et al.* 1999) and *Larix* and *Pseudotsuga* (Gernandt & Liston 1999). It seems that, in these examples, the homogenization of concerted evolution ('molecular drive') is not fast enough to nullify the sources of variation in the ribosomal DNA repeats. Simple nucleotide polymorphism derives from point mutation at a particular site, while indels result from slippage replication events (Dover 1986; Stephan 1989) and/or short mispairings during replication (Jobst *et al.* 1998). Most reported ITS polymorphisms are associated with a high ploidy level (Suh *et al.* 1993) or allopolyploidy (interspecific hybridization) and/or multiple nucleolar organizing regions on non-homologous chromosomes (Karvonen & Savolainen 1993; Suh *et al.* 1993; Jellen *et al.* 1994). rDNA pseudogenes, as in *Zea*, represent another source of variation (Buckler & Holtsford 1996). A long generation time (Sang *et al.* 1995), loss of sexual recombination (Campbell *et al.* 1997) and a high frequency of introgression during domestication (Jobst *et al.* 1998) have also been suggested to retard molecular drive or to provide an opportunity for different rDNA arrays evolving independently. However, in *Aeschynanthus*, at least in our sample, the intraindividual variation in the ITS region appears not to be associated with polyploidy. For instance, the ITS sequences of tetraploids, such as *A. parvifolius* ($2n=64$) (Ratter 1975) are no more polymorphic than those of the diploid *Aeschynanthus albidus*

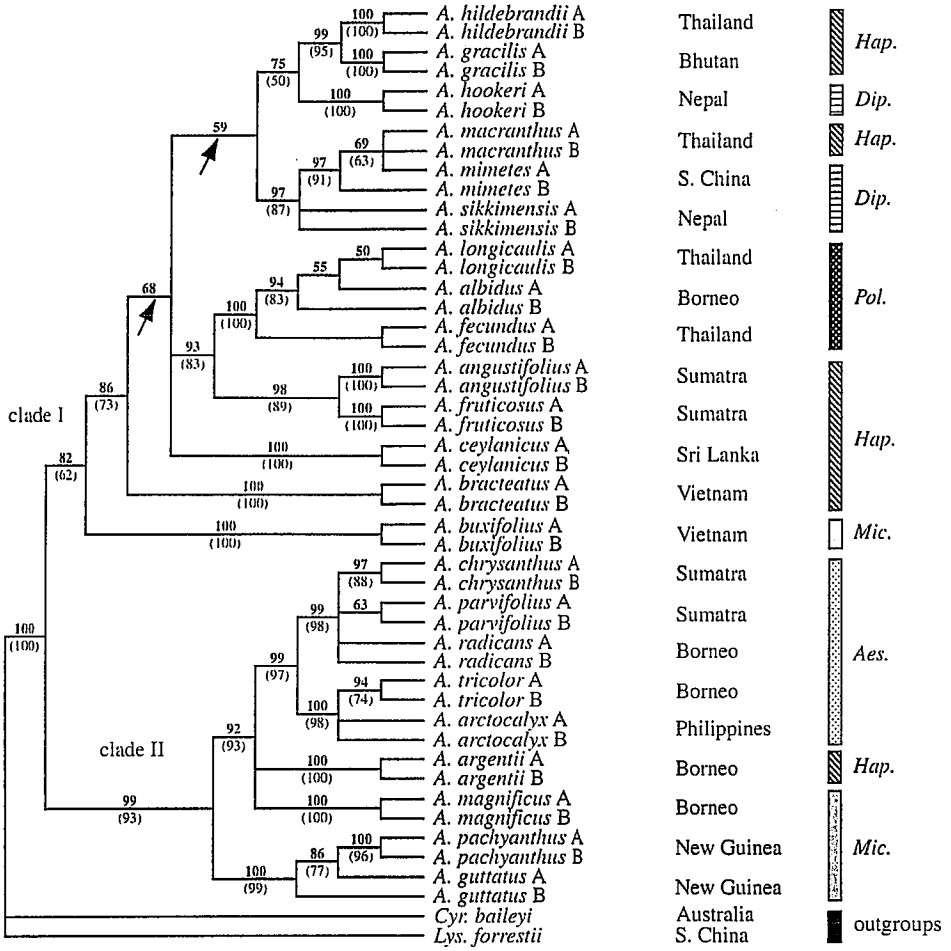

Figure 3. Strict consensus tree based on the 18 most parsimonious trees for 23 *Aeschynanthus* species (two clones per species) and two outgroup Gesneriaceae taxa of 550 steps in length based on parsimony analysis of the combined ITS1 and ITS2 sequence data plus the alignment gap matrix. The upper numbers (in bold type) are the full heuristic bootstrap percentages of 100 replicates. The lower numbers (in parentheses) are the jackknife percentages (fast stepwise addition) of 10 000 replicates. The two arrows indicate branches that collapse when the gap matrix is excluded and the analysis rerun. CI = 0.72, RI = 0.88 and RC = 0.63. *Hap.*, *Haplotrichium*; *Dip.*, *Diplotrichium*; *Pol.*, *Polytrichium*; *Mic.*, *Microtrichium*; *Aes.*, *Aeschynanthus*.

($2n = 30$) (Ratter 1975). Furthermore, *A. guttatus*, which has highest divergence between its clones is diploid ($2n = 32$) (Milne 1975). Further investigation of the cytology of *Aeschynanthus*, in particular the number and position of rDNA loci, would be interesting in this context.

(b) *Use of clones for phylogenetic analysis*

High intraindividual ITS clone variation in *Aeschynanthus* could reduce the phylogenetic use of this region. However, the clones, even the most divergent, are always associated on the trees, usually as sister pairs. Phylogenetic analysis using clones is therefore straightforward. In some cases the clones of one species are paraphyletic with respect to the clones of another (e.g. the clones of *A. albidus* are paraphyletic with respect to those of *Aeschynanthus longicaulis*), but the species relationships are still clear. Although considerable variation can be found within individuals, it is therefore still possible to use these data for reconstructing species phylogeny, indicating that the clone variation in *Aeschynanthus*, while persistent, does not significantly pre-date the divergence of species.

In our analysis, the sequence divergence between clones within the *Aeschynanthus* taxa ranged from 0 to 5.01% (0–0.025 substitutions per site). This variation is similar to that of *Amelanchier* (Campbell *et al.* 1997), in which the within-individual divergence ranges from 0 to 4.2% (0–0.021 substitutions per site). In contrast, highly divergent clones have been found in Winteraceae (Suh *et al.* 1993), in which the sequence divergence is 4.7–7.0% (0.024–0.035 substitutions per site). This is higher than some intergeneric divergence values and might be the result of polyploidization. In *Cucurbita* the intraspecific sequence variation of three cultivated species and three putative progenitors was found to range between 7 and 10% (0.035–0.05 substitutions per site) (Jobst *et al.* 1998). In this case, the polymorphism may be due to introgression during domestication. Clone variation as high as that found in Winteraceae and *Cucurbita* reduces the use of the ITS region for phylogenetic analysis at the species level.

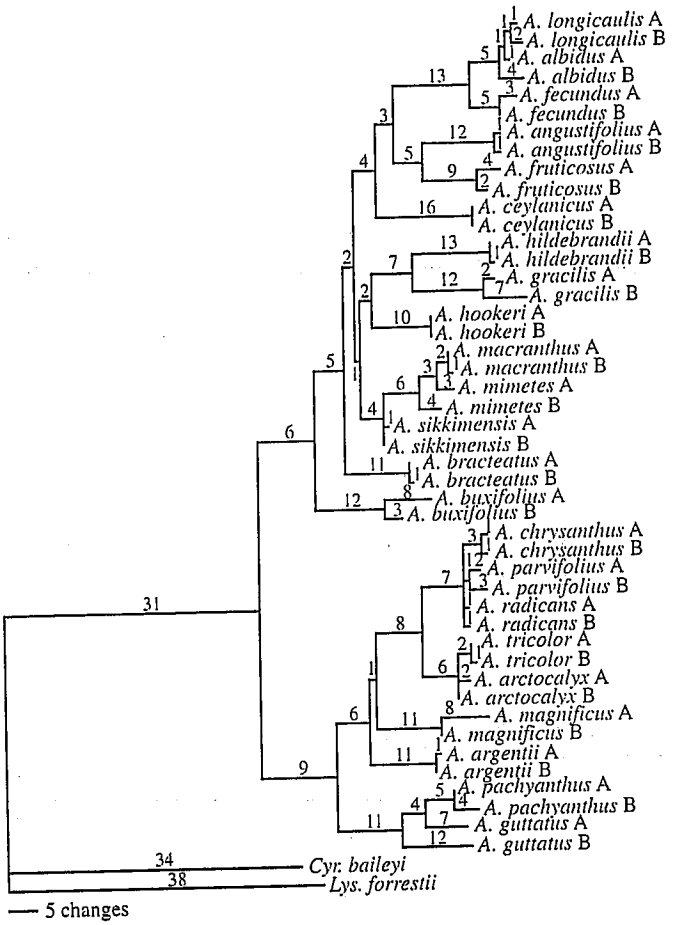

Figure 4. Phylogram of one of the 96 most parsimonious trees for 23 *Aeschynanthus* species (two clones per species) and two outgroup Gesneriaceae taxa of 452 steps in length based on parsimony analysis of the combined ITS1 and ITS2 sequence data without the gap matrix. The numbers along the branches indicate the number of character changes shared among taxa (branch length) including autapomorphic changes. CI = 0.708, RI = 0.869 and RC = 0.615.

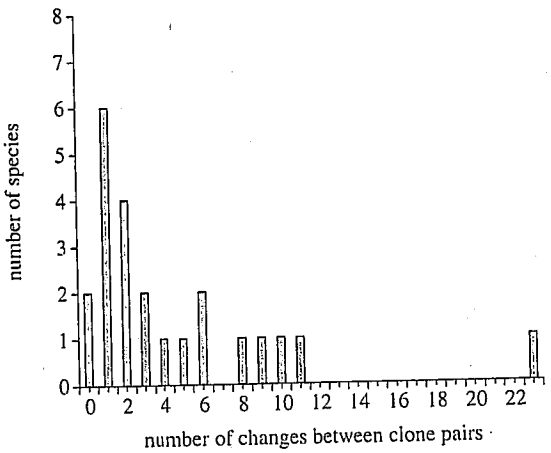

Figure 5. Pairwise clone divergence comparison between the clones of 23 *Aeschynanthus* species. The divergence values are the numbers of substitutions calculated over the tree topology shown in figure 4. The clones of *A. guttatus* show the highest divergence (23 changes), whereas those of both *A. ceylanicus* and *A. hookeri* are identical.

(c) *Trangressor species:* A. buxifolius *and* A. argentii

Two species appear to be in the 'wrong' major clades for their sections. Both these species have large deletions in their ITS2 sequences corresponding to arm 1 of the RNA secondary structure. However, clear homology with other taxa in the rest of the sequences indicates that these deletions have not led to the species being misplaced in the phylogeny, but are merely coincidental. *Aeschynanthus argentii*, although classified in the *Haplotrichium* section by Mendum (1999), has seeds which, on subsequent scanning electron microscopy investigation, proved to be of the *Microtrichium* type (M. Mendum, personal communication). If this species is reclassified as a *Microtrichium*, then it falls quite naturally in the tree in the *Aeschynanthus–Microtrichium* clade. *Aeschynanthus buxifolius*, although *Microtrichium*, groups as sister to the *Haplotrichium–Diplotrichium–Polytrichium* clade. This would be expected if the *Microtrichium* seed hair morphology is plesiomorphic and the *Microtrichium* section is paraphyletic with respect to the other sections. *Aeschynanthus buxifolius* is also one of few *Microtrichium* species from continental Asia and its geographical distribution is in accord with its grouping with clade I.

(d) *Ecology of the two major clades and the evolution of* Aeschynanthus

As noted above, the two major clades have eastern versus western geographical tendencies. The eastern Malesian forests tend to be consistently wet, whereas the western continental forests tend to have a marked dry season and monsoonal wet season (Riley & Spolton 1974; Rudloff 1981). The greater frequency of hydathodes in

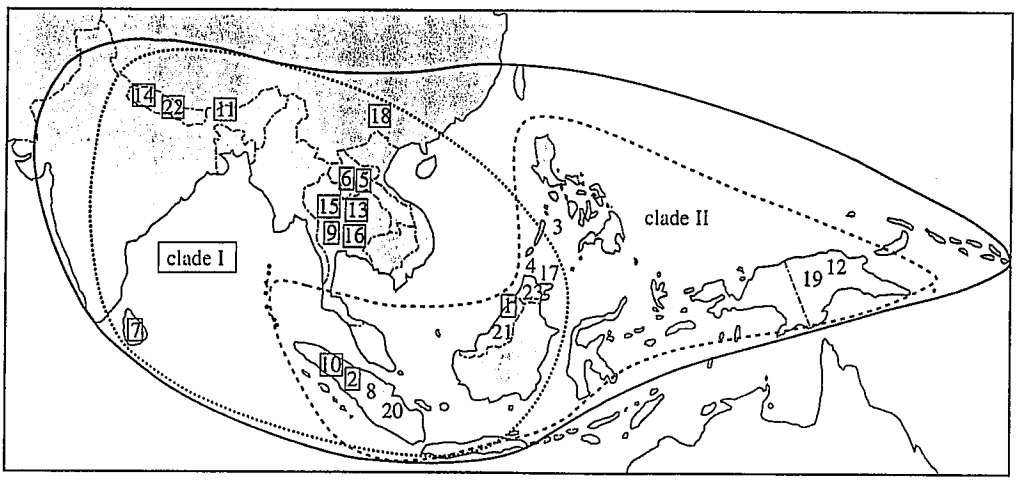

Figure 6. Approximate geographical distribution of the 23 *Aeschynanthus* species sequenced. The numbers refer to the species as listed in table 1. As can be seen from the map, clade I species (boxed, surrounded by a dotted line) are mainly continental in distribution, whereas clade II species (unboxed, surrounded by a dashed line) occur only on Sunda Shelf islands and east of Wallace's Line. The solid line shows the geographical distribution of the whole genus.

clade II (eastern) species (Rosser & Burtt 1969) may result from this. The very thin stems found in some species of the *Microtrichium* section (Rosser & Burtt 1969) with little water-conducting tissue may also reflect this. Other sections usually have stout stems (*Polytrichium–Diplotrichium*). *Aeschynanthus hildebrandii* from Thailand (clade I) has been shown to root under the bark of trees and may derive water in a 'hemiparasitic' way (Burtt & Woods 1975), which could again be seen as a dry season adaptation. A dry season provides ideal conditions for the dispersal of seeds with elaborate seed hairs and the greatest elaboration of seed hairs is found in the clade I species of *Haplotrichium*, *Diplotrichium* and *Polytrichium*. In contrast, the small hairs of *Microtrichium* may be more important for substrate attachment than dispersal, consistent with the wet climate.

If the position of *A. buxifolius* (*Microtrichium*) in clade I is confirmed, then a possible scenario for the evolution of the genus *Aeschynanthus* presents itself. An ancestral lineage is suggested with small seed appendages, as in the *Microtrichium* section, occupying a central Malesian site without a pronounced dry season. The small seed hair type in the west (clade I) has given rise to types with long seed appendages as in the *Haplotrichium* and *Diplotrichium* sections. Further elaboration to the most complex *Polytrichium* types then appears to have occurred. In contrast, in east Malesia the *Microtrichium* seed morphology appears to have been retained and diversified, evolving only once into a long seed hair type: the *Aeschynanthus* section with its characteristic bubble cells. Clade I appears to have spread westwards into seasonal forests in Sri Lanka and India, while clade II has achieved a wide distribution in eastern wet forests. Further species sampling, particularly in Sulawesi and other parts of central Malesia, will be needed to confirm this hypothesis.

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