PHYLOGENETIC RELATIONSHIPS AMONG Strobilanthes s.l. (Acanthaceae): EVIDENCE FROM ITS nrDNA, trnL-F cpDNA, AND MORPHOLOGY

ELIZABETH C. MOYLAN,2,6 JONATHAN R. BENNETT,3 MARK A. CARINE,4 RICHARD G. OLMSTEAD,5 AND ROBERT W. SCOTLAND2

2Department of Plant Sciences, University of Oxford, South Parks Road, Oxford, OX1 3RB, UK; 3Harvard University Herbarium, 22 Divinity Avenue, Cambridge, Massachusetts 02138 USA; 4Department of Botany, The Natural History Museum, Cromwell Road, London, SW 5 BD UK; and 5Department of Botany, University of Washington, Box 355325, Seattle, Washington 98195-5325 USA

Chloroplast trnL-F sequence data, nuclear ribosomal internal transcribed spacer (ITS) sequence data, and morphology were used to analyze phylogenetic relationships among members of the subtribe Strobilantheae. Parsimony and maximum likelihood analyses of trnL-F indicate that the Strobilantheae are a monophyletic group. While parsimony analysis of ITS recovers a nonmonophyletic subtribe, maximum likelihood analysis of ITS corroborates results from trnL-F and suggests that systematic error is impacting on ITS parsimony analysis. A combined ITS and trnL-F analysis strengthens the signal and also recovers a monophyletic subtribe. All analyses indicate that Hemigraphis, Sericocalyx, and Strobilanthes are nonmonophyletic. With one exception, all morphological characters included in a combined ITS and morphological analysis are homoplastic. The prospect for a new informative generic classification of the Strobilantheae aiming to recognize and diagnose only monophyletic groups is considered. While some groups can be diagnosed, adequate diagnosis of the majority of groups remains problematic. Consequently, a single expanded genus Strobilanthes sensu lato is proposed at the level of the well-supported and monophyletic Strobilantheae.

Key words: Hemigraphis; ITS; Java; maximum likelihood; parsimony; Philippines; Sericocalyx; southern India; Stenosiphonium; Strobilanthes; trnL-F.

The Strobilantheae (sensu Bremerkamp, 1944) are a morphologically diverse and species-rich group distributed throughout South and Southeast Asia, Malesia, and Northern Australia. The Strobilantheae comprise c. 350 species (Terao, 1983) and can be distinguished from other members of Acanthaceae by a combination of floral characteristics: filaments united to form a membranous sheath, a bident stigma with a reduced posterior lobe, and two bundles or rows of hairs on the inner posterior corolla wall that retain the style (Bremerkamp, 1944; Carine and Scotland, 2000a; Manketelw, 2000). However, this character combination is not found in all species, and the monophyly of the Strobilantheae and their relationship with other taxa remains to be rigorously examined.

Several aspects of the systematics of the Strobilantheae have recently been addressed including species delimitation (Wood, 1994, 1995, 1998; Scotland, 1998; Carine and Scotland, 2000a; Carine et al., 2000, in press; Moylan and Scotland, 2000; Moylan et al., 2002; Bennett and Scotland, 2003; Wood and Scotland, 2003; Wood et al., 2003), investigations of floral anatomy (Manketelw, 2000), pollen morphology (Scotland, 1993; Carine and Scotland, 1998), life history strategy (van Steenis, 1942; Matthew, 1971; Wood, 1994; Carine and Scotland, 2000a), and phylogeny and generic classification (Carine and Scotland, 2000b, 2002).

Generic delimitation within the Strobilantheae has varied dramatically with three main classifications being proposed (Anderson, 1867; Bremerkamp, 1944; Terao, 1983). Anderson (1867) recognized four genera within the subtribe using ovule number as the primary character. Anderson (1867) distinguished Strobilanthes Blume, defined by the possession of 2–4 ovules, from three multiovulate segregates Hemigraphis Nees, Stenosiphonium Nees, and Aechmanthera Nees, each with four to many ovules. A consequence of this approach was that morphologically similar species were referred to separate genera on the basis of ovule number. As a result, Anderson’s system has been criticized as highly artificial and arbitrary (Kurz, 1871; Hallier, 1898; Bremerkamp, 1944).

Bremerkamp (1944) radically reclassified the subtribe and divided Strobilanthes sensu Anderson (1867) into 54 smaller genera using combinations of macromorphological and pollen characters. Bremerkamp (1944) sought to provide a more informative generic classification and overcome the limitations of the Anderson system by describing numerous genera. For example, a new multiovulate genus, Sericocalyx, was described to accommodate species intermediate in morphology between Strobilanthes and Hemigraphis. The majority of newly described genera were small (comprising less than 15 species) or monotypic, and 176 species (more than one-third of species recognized at that time) were left unassigned to genera. Moreover, many generic characters were inaccurately described (Terao, 1983; Carine and Scotland, 1998), and generic boundaries within the subtribe remained ambiguous. Consequently, the diagnosis of groups remains confused and their morphology uncertain. Current researchers have avoided the Bremerkamp system (for example, Wood, 1994; Carine and Scotland, 1998) pending a thorough reinvestigation of the Strobilantheae.

Terao (1983) proposed a third approach and united all genera within an expanded Strobilanthes sensu lato (s.l.). Thus, in Terao’s classification the circumscription of the Strobilan-
thinae and of Strobilanthes s.l. are identical. Although Terao (1983) recognized several newly described or recircumscribed groups within Strobilanthinae s.l., many of Bremekamp segregated continued to be recognized at the infrageneric level. Therefore, Terao’s classification simply transferred many of the problems previously identified with the Bremekamp system from the generic to the infrageneric level.

A recent parsimony analysis of morphological data by Carine and Scotland (2002) demonstrated that Strobilanthes sensu Anderson is paraphyletic and that, of the genera recognized by Bremekamp (1944) and included in their study, only Stenosiphonium comprises a monophyletic group. Carine and Scotland (2002) established that the Strobilanthinae, as with other species-rich taxa (Prance and White, 1988; Wojciechowski et al., 1999), are characterized by a complex and conflicting pattern of morphological variation. They concluded that the prospect for a new generic or infrageneric classification was limited, given the highly homoplastic nature of morphological characters within the subtribe.

While providing some insight into phylogenetic relationships and classification of the Strobilanthinae, the conclusions of Carine and Scotland (2002) were based on analysis of morphological data (32 characters) from a limited sample of southern Indian and Sri Lankan species. Their study did not address the monophyly of the Strobilanthinae or explicitly examine the relationships between Strobilanthes sensu Anderson and those species traditionally referred to the multiovulate genera: Hemigraphis, Aechmatanthera, and Sericocalyx. Other sources of phylogenetic information, such as molecular sequence data, were not considered and their study failed to provide well-supported hypotheses of relationship (as evidenced by bootstrap and Bremer support values), an innate problem of using morphological data to reconstruct phylogeny (Scotland et al., 2003).

This paper aims to investigate the classification of the Strobilanthinae using a morphologically and geographically diverse sample of taxa and data from two gene regions: the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA (nrDNA) and the intron and spacer of the chloroplast DNA (cpDNA) region trnL-trnF (trnL-F), and morphology. The aims of the paper are: (1) to test the monophyly of the Strobilanthinae, (2) to provide robust hypotheses of relationships within the Strobilanthinae, (3) to further evaluate the classifications of Anderson (1867), Bremekamp (1944), and Terao (1983) and determine the monophyly of genera recognized by these authors, (4) to consider patterns of morphological variation in the entire subtribe, and (5) to further evaluate Carine and Scotland’s (2002) assertion that the variation within the subtribe is not amenable to formal classification.

**MATERIALS AND METHODS**

**Nomenclature**—Species names for the most part follow the Anderson (1867) classification, and taxa are therefore referred to as Strobilanthes, Hemigraphis, Stenosiphonium, or Aechmatanthera. The exceptions are Sericocalyx Bremek. and Clarkeasia J.R.I. Wood, a genus created to accommodate a species that cannot currently be placed in either Anderson’s or Bremekamp’s classifications (Wood, 1994).

**Taxon sampling—trnL-F**—Forty-three accessions of Strobilanthinae, representing 42 species, were included in the trnL-F study (Appendix 1; see Supplemental Data accompanying the online version of this article). Fewer taxa were included in the trnl-F study than in the ITS study owing to the lower resolution provided by trnL-F at this level (McDade and Moody, 1999). However, a diverse sample of morphological and geographical variation across the Strobilanthinae was maintained. Outgroup taxa were selected from six genera within the Ruelliaeae on the basis of higher level studies by Scotland et al. (1995) and McDade and Moody (1999).

**ITS**—Eighty-two accessions representing 81 species of Strobilanthinae were included in the ITS study (Appendix 1), including the same six outgroup species as used in the trnL-F analyses. This sample includes a morphologically diverse representation (including multiovulate species) from across the geographic distribution of the subtribe.

**Molecular methods**—Total genomic DNA was extracted from c. 0.1 g of dried leaf material (silica-gel-dried or herbarium specimens) using a modified cetrimidinium ammonium bromide (CTAB) microextraction protocol (Doyle and Doyle, 1987) and purified, without precipitation, using QIAquick spin columns (QIAGEN, Valencia, California, USA) according to manufacturer’s protocols. Standard polymerase chain reaction (PCR) procedures were used to amplify the target gene region. The trnL-F region was amplified (30 cycles of 94°C for 1 min, 45°C for 1 min, 72°C for 2 min) in one fragment or by amplifying this region in two shorter fragments using primers in the trnl 3′ exon (Taberlet et al., 1991). The ITS region was amplified (35 cycles of 96°C for 1 min, 49°C for 1 min, 72°C for 1 min) using primers 4 and 5 (White et al., 1990) or 17S and 26S (Sun et al., 1994). Dimethylsulfoxide (DMSO) (5–10%) was added to the ITS PCR reactions to prevent the formation of secondary structures (Baldwin et al., 1995).

Amplified products were purified using polyethylene glycol (PEG) precipitation or QIAGEN QIAquick columns according to manufacturer’s protocols. Sequences of both strands of the PCR product were determined on an ABI sequencer (Applied Biosystems, Foster City, California, USA). For trnL-F, both strands were sequenced using the amplification primers c and f and the internal primers d and e (Taberlet et al., 1991). In instances where the gene region was amplified in two fragments, the combination of primers trnL 2C and d and primers e and trnF 2F were used (Beardsley and Olmstead, 2002). For ITS, both strands were sequenced using primers 4 and 5 and additional internal primers: 3 and 2 (White et al., 1990). Sequence data were edited and assembled using Sequencer (version 3.1.1; Gene Codes Corporation, Ann Arbor, Michigan, USA).

**Sequence alignment**—Verified sequences were aligned by eye in Se-Al (version 1.0a; Rambaut, 1996), and regions of ambiguous alignment were excluded prior to phylogenetic analysis. Alignments for trnL-F and ITS data sets are available in Appendix 2 (see Supplemental Data accompanying the online version of this article). Indels present in more than one taxon with identical length and position were coded as single, unordered binary characters and appended to the data set.

**Phylogenetic analyses**—Phylogenetic analyses were performed on the following data sets using PAUP* Beta version 4.0b4a (Swofford, 1998): (1) parsimony analysis of 88 taxa using ITS sequence data, (2) maximum likelihood analysis of 88 taxa using ITS data (gaps treated as missing), (3) parsimony analysis of 49 taxa using trnL-F sequence data, (4) maximum likelihood analysis of 49 taxa using trnL-F data (gaps treated as missing), (5) combined parsimony analysis of 43 taxa using ITS and trnL-F sequence data, (6) combined parsimony analysis of 88 taxa using ITS and morphological data.

**Parsimony analyses**—All parsimony analyses were simultaneous and unconstrained (Nixon and Carpenter, 1993) with character state changes unordered and weighted equally. The search strategy of Catalán et al. (1997) was designed to search efficiently for most parsimonious trees and ensure that multiple islands of trees are found. Analyses were conducted using an initial heuristic search comprising 1000 replicates of random stepwise addition using tree bisection and reconnection (TBR) branch swapping with MULTREES option on, but saving only five trees per replicate. Multiple most parsimonious trees resulting from this analysis were used to compute a strict consensus tree.
which was then used as a constraint for another round of heuristic searches. Five thousand replicates of random stepwise addition were performed with TBR branch swapping, saving only trees that were incompatible with the strict consensus tree. Trees were rooted on the most distal outgroup (Ruellia), based on knowledge from previous phylogenetic analyses (Scotland et al., 1995). The consistency index (CI) ( Kluge and Farris, 1969) and retention index (RI) (Farris, 1989) were calculated. The robustness of clades in the strict consensus trees was evaluated by nonparametric bootstrap analysis (Felsenstein, 1985) and by computing decay values (Bremer, 1988). Bootstrap values were obtained from 100 pseudo-replicates, each comprising 1000 heuristic searches of random stepwise addition with TBR branch swapping. The following general descriptions for categories of bootstrap support were used: poor, <50%; weak, 50–74%; moderate, 75–84%; strong, 85–100% ( Chase et al., 2000 ). Decay indices (DI) were obtained using AutoDecay (Eriksson, 1998) and those clades with DI greater than or equal to four were considered well supported (Marcilla et al., 2001). Both bootstrap and DI are indicated for clades retained in the strict consensus trees resulting from parsimony analyses. These measures allow an assessment of internal clade support to be made with the recognition that potential sources of bias may lead to erroneously high support values (Hohenbeck et al., 1996; Nei, 1996; Lee, 2000).

Maximum likelihood analyses—A starting tree was first obtained using neighbor joining. Likelihood scores for different models of sequence evolution were calculated for this test topology and statistically compared against a χ2 distribution using ModelTest (version 3.0; Posada and Crandall, 1998). Once an explicit model of sequence evolution was selected for trnL-F and ITS data sets, parameters were adjusted to those estimated during model testing. A final tree topology was then estimated in a new maximum likelihood search implemented in PAUP*.

The robustness of clades in the trnL-F maximum likelihood tree was evaluated using nonparametric bootstrap resampling. One hundred pseudoreplicates using the “fast” reduced-search bootstrap option in PAUP* were performed. Fast bootstrap is known to result in bootstrap values that are less than, and statistically different from, bootstrap values obtained using stepwise addition and branch swapping (DeBry and Olmstead, 2000; Mort et al., 2000). However, given that fast bootstrap is known not to report inflated bootstrap support, it is an efficient method, albeit biased towards lower values, for estimating bootstrap values from maximum likelihood analyses. Robustness of clades in the ITS maximum likelihood tree were evaluated by performing 1000 pseudoreplicates using neighbor joining and implementing the same maximum likelihood model.

Morphological data—Twelve morphological characters were considered to be potentially informative for 88 taxa included in the ITS analysis (Appendix 3; see Supplemental Data accompanying the online version of this article). These include characters previously used to recognize groups within the Strobilanthinae (Anderson, 1867; Bremerkamp, 1944) that do not vary consistently and may be of use in generic classification. All characters were binary. Eleven of the characters form part of a reduced data set that originally comprised 32 characters and 68 southern Indian and Sri Lankan Strobilanthus (Carine and Scotland, 2000a). One character (character 3: superposed, accessory buds present/absent) was included here but was not originally incorporated into the consistency index (CI) and retention index (RI) because it was invariant across taxa sampled in that study. Characters were scored from previously published data in the case of southern Indian and Sri Lankan taxa or from dried herbarium specimens or descriptions given in the literature. In adapting the original matrix of Carine and Scotland (2000a) across a broader sample of taxa, a number of characters became uninformative or inapplicable and were therefore excluded. Other morphologically variable characters were not incorporated because they varied continuously and could be open to subjective and nonrepeatable character conceptualization (Gift and Stevens, 1997) or because morphology assessment and coding of characters were ambiguous (Hawkins et al., 1997; Hawkins, 2000). While Poe and Wiens (2000) advocate coding all possible types of morphological variation for phylogenetic analysis, in this study only those characters that were discrete (Stevens, 1991) and could be repeatedly and objectively scored were used.

Inflorescence—In the Strobilanthinae, a single leaflike bract subtends one or more primary flowers in its axil. Primary flowers are commonly sessile, although pedicellate flowers occur among southern Indian Strobilanthinae. Smaller, leaflike bracteoles may also occur between the bract and calyx, subtending the flower at right angles to the bract, although they are not always present. Occasionally, secondary flowers occur in the axils of bracteoles (Carine and Scotland, 2000b). Because secondary flowers are rarely seen, the inflorescence structure of the Strobilanthinae has frequently been described as a spike with or without the occurrence of so-called “superposed” buds (Bremekamp, 1944), which represent the occurrence of more than one primary flower subtended by a single bract. The variation observed in inflorescence structure was scored as character 1, bracteoles present (1)/absent (0); character 2, flowers pedicellate (1)/sessile (0); character 3, superposed buds present (1)/absent (0); character 4, secondary flowers present (1)/absent (0) or inapplicable (0) if bracteoles are not present.

Corolla—The corolla tube may be resupinate (twisted) at anthesis or nonresupinate. The style is retained against the posterior wall of the corolla throat by two rows of hairs, which may or may not be borne on flaps of corolla tissue. This variation was scored as character 5, corolla tube resupinate (1)/nonresupinate (0); character 6, flaps to retain style present (1)/absent (0) or inapplicable (0) in outgroup taxa in which the style is not retained.

Androecium—The androecium consists of either four fertile didynamous stamens (an anterior pair and a posterior pair) or two anterior stamens and two infertile stamnodes or two anterior stamens with no staminodes. However, of the Strobilanthinae taxa included in the ITS study, only species with four fertile stamens or two anterior stamens were represented. Filaments unite to form a filament curtain (Mankelow, 2000) adnate to the inner side of the posterior corolla tube. The filament curtain may be pubescent or glabrous. Variation in the androecium has been scored as character 7, posterior stamens present (1)/absent (0); character 8, filament curtain pubescent (1)/glabrous (0).

Pollen—Pollen morphology in the Strobilanthinae is very diverse (e.g., Carine and Scotland, 1998). However, much of the variation is continuous and impossible to code for phylogenetic analysis across all taxa. The only pollen character that has been coded for this study is the position of apertures on the pollen grain, which may be pantaporate or equatorial, and was scored as character 9, pollen apertures pantaporate (1)/equatorial (0).

Gynoeceum—The style may be pubescent along its length or entirely glabrous. Frequently, the style terminates in a filiform stigma with a toothlike protrusion. This variation has been scored as character 10, style hairy (1)/style glabrous (0); character 11, stigma protrusion present (1)/absent (0).

Seeds—Seeds may be pubescent or entirely glabrous and were scored as character 12, seeds hairy (1)/seeds glabrous (0).

Combining data sets—Two combined analyses were performed; one using ITS and trnL-F data sets, and the other using ITS and morphological data. The analysis combining ITS and trnL-F was conducted to investigate the position of Dyschoriste decumbens in a total evidence analysis since D. decumbens behaved anomalously in the ITS parsimony analysis. The analysis combining ITS and morphological data was conducted to investigate the distribution of morphological characters within the Strobilanthinae. A “total evidence approach” provides the best summary of data to hand and can strengthen phylogenetic signal over nonhistoric patterns of information (Olmstead and Sweere, 1994; Chase and Cox, 1998). However, the smaller size of the trnL-F data set would have significantly compromised sampling in a combined analysis including ITS, trnL-F, and morphological data. For this reason, a pragmatic approach was taken and only ITS and morphological data sets were combined.

Congruence between taxonomically equivalent ITS and trnL-F data sets and ITS and morphological data sets was evaluated by comparing tree topologies. This approach enabled clades in strongly supported agreement or disagreement to be identified before data sets were combined. An incongruence
length difference (ILD) test (Farris et al., 1995) was also conducted to investigate character congruence between taxonomically equivalent ITS and trnL-F partitions. This was implemented as the “partition-homogeneity test” in PAUP®. ITS and trnL-F comparisons were conducted with and without the outgroup taxon Dyschoriste decumbens, which was placed inconsistently in ITS and trnL-F analyses. For this reason, ITS and morphological comparisons were conducted with D. decumbens excluded. All parsimony-uninformative characters were removed from each data set prior to conducting the partition-homogeneity test (Wiens and Hollingsworth, 2000). One hundred replicates were analyzed, with each repartitioned data set subject to a heuristic search of five random stepwise addition replicates and TBR branch swapping.

RESULTS

trnL-F analyses—The length of individual sequences of trnL-F varied from 868 to 892 base pairs (bp). The addition of seven coded gap characters, resulted in an aligned length of 1018 bp, of which 182 were variable and 55 were parsimony informative, thus yielding a total of 55 characters for parsimony analysis (Table 1).

Parsimony analysis of the trnL-F data set and recoded indels resulted in 2626 equally most parsimonious trees of length 222 steps (CI = 0.89; RI = 0.92; Table 1). The strict consensus of all equally most parsimonious trees is shown in Fig. 1A.

From the trnL-F strict consensus (Fig. 1A) the Strobilanthes comprise a strongly supported monophyletic group (96% bootstrap, DI of 5) and within the subtribe, Hemigraphis, Sericocalyx, and Strobilanthus are nonmonophyletic. Dyschoriste decumbens is moderately supported as sister group to the subtribe (76% bootstrap, DI of 1). The majority of Hemigraphis species sampled comprise a strongly supported group (clade A; 89% bootstrap, DI of 2) although resolution within this clade is poor. Clade B, comprising three species of Sericocalyx sampled from Thailand and H. ridleyii, also from Thailand (60%, DI of 1), plus Clade A form a strongly supported clade (96% bootstrap, DI of 4). Hemigraphis confinis from the Malay Peninsula is shown to be distantly related to the majority of Hemigraphis species and, within the Strobilanthinae, is placed as sister group to the rest of the subtribe, which form a weakly supported clade (56% bootstrap, DI of 1). Sericocalyx appears in two separate clades in the analysis. In addition to Clade B comprising the Thailand species, two Javanese species (Sericocalyx crispus + Sericocalyx sp. B49) form a strongly supported group (96% bootstrap, DI of 3), although their position within the Strobilanthinae is unresolved. Strobilanthus species are resolved in three weakly supported clades in the strict consensus tree. However, there is stronger support for small clades comprising two Javanese Strobilanthus (S. repanda + S. autapomorpha) and two Japanese species (S. japonica + Strobilanthus sp. s.c. [sine collegit] s.n. [sine numero]).

Using ModelTest (Posada and Crandall, 1998), the K81uf model (Kimura, 1981) of sequence evolution with a discrete gamma rate distribution was selected for the trnL-F data set. Maximum likelihood analysis using these model parameters resulted in a single maximum likelihood tree of score −ln 2809.8103 (Fig. 1B). The tree generated from maximum likelihood analysis of the trnL-F data is highly congruent with the topology of the strict consensus from the parsimony analysis. In general, relationships supported in the parsimony strict consensus (i.e., >75% bootstrap) are also supported in the maximum likelihood tree. Within the strongly supported group comprising the majority of Hemigraphis taxa (clade A) relationships among species are further resolved with the Indian H. hirta placed as sister to the rest (92% bootstrap). This relationship is found in 52% of the parsimony bootstrap replicates, although not found in all most parsimonious trees (results not shown). However, apart from groups from the Himalaya (S. aprica var. pedunculata + S. clavulicata; 79% bootstrap) and Japan (S. japonica + Strobilanthus sp. s.c. s.n; 61% bootstrap), much of the additional resolution in the maximum likelihood analysis is poorly supported.

ITS analyses—Individual sequences across the ITS region showed length variation from 647 to 699 bp. For the final alignment, 48 bp corresponding to regions of ambiguous alignment were removed from the data set. This resulted in a final data set of 748 aligned characters of which 362 were variable and 230 potentially informative for parsimony analysis (Table 1). Shared gaps introduced into the sequences when aligned provided an additional 22 binary characters.

Parsimony analysis of the ITS data set resulted in 5592 equally most parsimonious trees of length 1133 steps (CI = 0.49 and RI = 0.78; Table 2). The strict consensus tree is shown in Fig. 2A. From the strict consensus, it is apparent that the ITS data broadly corroborate the results from trnL-F analyses in that Hemigraphis, Sericocalyx, and Strobilanthus are nonmonophyletic. Moreover, the majority of strongly supported groups found in the ITS analysis, i.e., those with greater than 85% bootstrap, are congruent with those in the trnL-F analyses. The sampling of Hemigraphis in the ITS analysis is denser than that of the trnL-F analyses with taxa from the Philippines, India, Java, China, and Thailand included. The majority of these species together with four species of Sericocalyx comprise a monophyletic group (clade C; 79% bootstrap, DI of 4). Hemigraphis griffithiana and H. confinis are resolved as a separate monophyletic group (clade D; 100% bootstrap, DI of 10), distantly related to the majority of Hemigraphis species and sister to the Strobilanthinae. The ITS data also support the findings from trnL-F in the placement of Sericocalyx species: those from Thailand (Sericocalyx schom-
Fig. 1. (A) The strict consensus tree resulting from parsimony analysis of trnL-F data. (B) The maximum likelihood tree resulting from analysis of trnL-F data with branch lengths indicated. Bootstrap values are above nodes in both trees, decay values are below nodes in the parsimony strict consensus tree. Capital letters denote groups referred to in the text. Acanthopale = Ac., Aechmanthera = A., Blechum = B., Clarkeasia = C., Dyschoriste = D., Hemigraphis = H., Hygrophila = Hy., Ruellia = R., Sanchezia = Sa., Sericocalyx = Se., Stenosiphonium = St., and Strobilanthes = S.

... burgkii, Sericocalyx glaucescens, Sericocalyx sp. P973) and Burma (Sericocalyx fluviatilis) are more closely related to Hemigraphis than to a clade comprising species from Java and China that form a distinct group (clade E, 98% bootstrap, DI of 6). However, unlike trnL-F, ITS does not find a monophyletic group composed of the four Thailand species (three of Sericocalyx and H. ridleyii).

The ITS data resolve a monophyletic Stenosiphonium (clade F, 100% bootstrap, DI of 9) nested within a clade of southern Indian Strobilanthes. This result confirms previous findings of Carine and Scotland (2000a). Two large clades comprising southern Indian Strobilanthes clade G (59% bootstrap, DI of 2) and clade H (92% bootstrap, DI of 5) and a further clade comprising Javanese Strobilanthes clade I (79% bootstrap, DI of 3) are resolved. However, the spine of the strict consensus tree is unresolved and there is strong support mostly for small groups of 13 or fewer species (with the exception of clade C). Although results of parsimony analysis of ITS data are consistent with parsimony analysis of trnL-F data in the placement of Hemigraphis and Sericocalyx and yield increased resolution among species of Strobilanthes, the ITS topology is in conflict with the results of trnL-F analyses with respect to the position of Dyschoriste decumbens. In contrast to the trnL-F strict consensus in which D. decumbens is sister group to the Strobilantheae, the ITS tree resolves D. decumbens within Strobilantheae as sister group to S. aprica var. pedunculata + S. attenuata. However, the branch leading to D. decumbens is long (46 steps) and the placement of D. decumbens in the ITS strict consensus (and resulting nonmonophyly of the Strobilantheae) is only weakly supported (42% bootstrap, DI of 1).
Fig. 2. (A) The strict consensus tree resulting from parsimony analysis of ITS data. (B) The maximum likelihood tree resulting from analysis of ITS data with branch lengths indicated. Bootstrap values are above nodes in both trees; only bootstrap values > 50% are indicated above nodes in the ML tree for clarity. Bootstrap values for the ML tree were obtained by 1000 pseudoreplicates using neighbor joining to implement the ML model. Decay values are indicated below nodes in the parsimony strict consensus tree. Capital letters denote groups referred to in the text. Acanthopale = A., Aechmanthera = A., Blechum = B., Clarkeasia = C., Dyschoriste = D., Hemigraphis = H., Hygrophila = H., Ruellia = R., Sanchezia = S., Sericocalyx = Se., Stenosiphonium = St., and Strobilanthes = S.
A search constraining Strobilanthinae to monophyly resulted in trees only three steps longer, and ITS 1 sequences alone support monophyly of the subtribe (results not shown).

Using ModelTest (Posada and Crandall, 1998), the GTR model (Rodriguez et al., 1990) with a proportion of invariant sites and a discrete gamma rate was chosen as the model of sequence evolution for the ITS data set. Maximum likelihood analysis with estimated parameters resulted in a single maximum likelihood tree of score −ln 6966.9052 (Fig. 2B).

It is apparent that the topology of the maximum likelihood ITS tree is in conflict with the strict consensus tree for parsimony analysis of the ITS data with respect to the position of D. decumbens. In the maximum likelihood analysis, D. decumbens is sister to a monophyletic Strobilanthinae, a relationship also indicated by both trnL-F analyses. Apart from the position of D. decumbens in the parsimony strict consensus tree, which, as we have shown, is a weakly supported difference, the parsimony strict consensus tree and ML tree are consistent. The results of the maximum likelihood analysis provide some additional resolution among other species of Strobilanthinae. For example, two southern Indian clades together with the largest clade of Hemigraphis and Sericocalyx species (clade J) although bootstrap support for this relationship is <50%. A further southern Indian clade (S. pulcherrima, S. habracanthoides + S. rubicunda) (clade K; 100% bootstrap) is placed as sister group to a clade comprising species from both Japan and the Himalaya, although this relationship also has low bootstrap support. While terminal groups within the Strobilanthinae have increased resolution and support, groups along the spine of the Strobilanthinae are resolved, but remain poorly supported by bootstrap.

**Combined trnL-F and ITS**—The ILD tests on the taxonomically equivalent trnL-F and ITS data sets, both with and without D. decumbens, revealed no significant difference between these partitions (P = 0.26 with D. decumbens, P = 0.62 without D. decumbens), suggesting that incongruence between ITS and trnL-F data sets is not significant. Given that the ILD test demonstrates no significant incongruence between data sets and that conflicting relationships are weakly supported, the disparity observed between ITS and trnL-F topologies is not considered to be evidence of hard incongruence between data sets. Taxonomically equivalent data sets were therefore analyzed simultaneously.

The combined matrix of 43 taxa comprised 1776 characters, of which 488 are variable and 245 parsimony informative. Parsimony analysis resulted in 546 trees of length 1016 steps, (CI = 0.63 and RI = 0.78; see Table 1). The strict consensus tree is shown in Fig. 3A and one most parsimonious tree is shown in Fig. 3B. The strict consensus indicates that Strobilanthinae is monophyletic (68% bootstrap, DI of 5). Dyschoriste decumbens is weakly supported as sister group to the Strobilanthinae (71% bootstrap, DI of 2). Hygrophila corymbosa is weakly supported as sister group to (Strobilanthinae + D. decumbens). The group comprising (Strobilanthinae + D. decumbens + Sanchezia speciosa + Hygrophila corymbosa) is strongly supported (98% bootstrap, DI of 6).

The overall pattern of relationships within the Strobilanthinae in this analysis shows increased resolution and support over the taxonomically equivalent trnL-F parsimony analysis. For example, the majority of Hemigraphis species form a strongly supported group (clade A; 92% bootstrap, DI of 7) in which a further seven internal groups are resolved. The position of H. confinis as sister to the rest of the subtribe is weakly supported by bootstrap but strongly supported by decay values (68% bootstrap, DI of 5). Relationships among species of Sericocalyx are also supported and further resolved. The Thai species Sericocalyx schomburgktii, S. glaucescens, and Sericocalyx sp. P973 and H. ridleyii form a clade sister to the majority of Hemigraphis (clade B; 65% bootstrap, DI of 1), whereas S. crispus and Sericocalyx sp. B49 are sister group to S. andamanensis (clade C; 78% bootstrap, DI of 1). Relationships among the spine of the tree are also further resolved. Clarkeasia parvifolia is sister to Aechmanthera sp. (91% bootstrap, DI of 6), and these species, together with S. aprica var. pedunculata, form a poorly supported group with the majority of Hemigraphis and Sericocalyx species (29% bootstrap, DI of 1). A further eight Strobilanthus species representing taxa from geographically diverse regions of Java, Southern India, and Japan form a weakly supported monophyletic group (50% bootstrap, DI of 1) sister to the rest of the Strobilanthinae.

**Combined ITS and morphology**—Parsimony analysis of the morphological data set (Appendix 3) resulted in 6718 equally most parsimonious trees of length 41 steps (CI = 0.29, RI = 0.82; Table 1) and the resulting strict consensus tree was almost completely lacking in resolution (tree not shown). For the purposes of a combined ITS and morphological analysis to investigate character distribution, taxonomically equivalent ITS and morphological data sets were compared without D. decumbens (due to the apparently anomalous placement of Dyschoriste in the ITS strict consensus tree resulting from parsimony analysis). Five taxa were included as outgroups (Sanchezia speciosa, Hygrophila corymbosa, R. californica, B. pyramidatum, and Acanthopale sp.).

An ILD test on the taxonomically equivalent ITS and morphological data sets detected a significant difference (P < 0.01) between these partitions. However, comparison of the poorly resolved morphological strict consensus with the ITS tree showed no strongly supported topological incongruence. Lack of resolution is interpreted by some authors to be lack of evidence for combining data (Cunningham, 1997); however, it may simply be evidence of insufficient information and signal (Pennington, 1996). The latter may be the case in the morphological data set, in which there is an obvious deficit of discrete characters suitable for parsimony analysis. Because each data set showed no strongly supported conflicting groups, ITS and morphological data sets were analyzed simultaneously.

The combined morphological and ITS matrix comprised 760 characters, of which 374 are variable and 242 parsimony informative (Table 1). The topology of the combined strict consensus tree (Fig. 4) was congruent with the ITS topology and relationships among species of Hemigraphis and Sericocalyx were identical in each tree. In general, the combined strict consensus shows increased resolution for terminal clades within the Strobilanthinae while the very few deeper clades that were resolved lacked strong support.

The distribution of each morphological character on several randomly selected most parsimonious trees was investigated by tracing unambiguous character state changes (ACCTRAN and DELTRAN optimization) using MacClade version 3.0 (Maddison and Maddison, 1992). All morphological characters, with the exception of pedicellate flowers, are homoplasic within the Strobilanthinae under a selection of equally most
parsimonious trees and optimization methods (results not shown). The distribution of three unambiguous character transformations is summarized on the combined strict consensus tree together with geographic distribution data (Fig. 4). The clade defined by the single nonhomoplastic character, pedicellate flowers, is highlighted. The distribution of “more than four seeds” historically used to define genera within the Strobilanthinae was not coded in the analysis of morphological data. This character is plotted on to the tree topology in Fig. 4 and is also homoplastic within the subtribe. Of 12 morphological characters assessed, all characters, with the exception of pedicellate flowers, are homoplastic within the Strobilanthinae.

**DISCUSSION**

The conclusion from the molecular analyses is that the Strobilanthinae are a monophyletic group, a finding also supported by evidence from morphological and anatomical data (Bremer, 1994; Manktelow, 2000). Parsimony analysis of ITS data alone failed to recover a monophyletic subtribe, placing Dyschoriste decumbens within the Strobilanthinae. However,
Fig. 4. The strict consensus tree resulting from parsimony analysis of combined ITS and morphological data. Bootstrap values are indicated above nodes and decay values are indicated below nodes in the strict consensus tree. Capital letters denote groups referred to in the text. Morphological characters are optimized onto the tree as indicated (open bars = presence of corolla flaps to retain style, closed bars = more than four seeds, cross-hatched bars = resupinate corolla). Geographic distribution data are also included. Acanthopale = A., Aechmanthera = A., Blechum = B., Clarkeasia = C., Dyschoriste = D., Hemigraphis = H., Hygrophiла = Hy., Ruellia = R., Sanchezia = Sa., Sericocalyx = Se., Stenosiphonium = St., and Strobilanthes = S.
the maximum likelihood analysis of the same data corroborated results from trnL-F and the combined trnL-F and ITS data. Given that ITS parsimony trees with a monophyletic subtribe are only three steps longer and that the terminal branch leading to *Dyschoriste decumbens* is long whether in its most parsimonious position or constrained to fall outside the subtribe, it is possible that systematic error, such as long branch attraction (Felsenstein, 1978) is impacting upon the analysis. Maximum likelihood is better able to place such divergent sequences, so the fact that the ITS ML analysis is consistent with the other analyses in finding a monophyletic Strobilanthinae, leads to our conclusion of monophyly for the subtribe. Simultaneous analysis of trnL-F and ITS strengthened the phylogenetic signal throughout most of the tree relative to either data set individually (Fig. 3A). The combined analysis resulted in fewer most parsimonious trees and a strict consensus with increased resolution (consensus fork index = 0.76). However, support for a monophyletic Strobilanthinae was weakened relative to the trnL-F analysis alone, due to the inconsistent placement of *Dyschoriste decumbens* in the ITS analyses.

**Morphological variation**—Strobilanthinae harbor tremendous diversity among species in their morphology, ecological preferences, and life history strategy. The group includes weedy and herbaceous species in low-lying wet environments (Moylan et al., 2002) and woody shrubs, which often form dense stands of vegetation in tropical upland forests. Some species are polycarpic, whereas others are gregariously monocarpic and flower on a regular (often 12-year) cycle (Wood, 1994; Carine and Scotland, 2000b; Bennett and Scotland, 2003). This diversity in ecology and life history strategy among species is reiterated in the distribution of morphological characters within the Strobilanthinae. The results of this study show that most morphological characters are highly homoplastic within the subtribe (e.g., resupinate corollas and presence of corolla flaps to retain style; Fig. 4). This level of homoplasly is typical of large tropical plant genera (Prance and White, 1988) and may be attributed to repeated reassortment of the same character states in similar allopatric environments (Sanderson, 1998).

The combined ITS and morphological analysis included only those morphological characters that could be unambiguously coded across all Strobilanthinae. Many morphological characters were not coded due to high levels of variation. For example, pollen morphology is impossible to partition into discrete states across the entire group (J. R. Bennett and R. W. Scotland, personal communication). Although Carine and Scotland (1998) scored 32 morphological characters across 68 southern Indian and Sri Lankan *Strobilanthes*, only 12 characters could be coded across 88 taxa from the full geographic range of Strobilanthinae. By increasing the taxon sample by 23%, the number of characters that could be scored was reduced by 63%. This decrease in number of morphological characters can be attributed to a diverse sampling strategy that did not merely increase the density of sampling of the original matrix of Carine and Scotland (1998) but included a broader sample of morphological variation from across the subtribe. This taxon sampling resulted in a number of characters being excluded because they (1) became uninformative or inapplicable, (2) would have involved a prohibitive level of investigation to partition characters into discrete states, or (3) varied continuously across a broader taxon sample. Increased taxon sampling therefore resulted in fewer morphological characters that could be scored across the subtribe, limiting the contribution of morphological data to phylogeny reconstruction to a combined morphological and ITS analysis rather than an independent estimate.

**Implications for classification**—The results of phylogenetic analyses presented here provide broadly consistent estimates of the phylogeny of the Strobilanthinae. The Strobilanthinae comprise a monophyletic group with *Dyschoriste* (represented by *D. decumbens*) as sister group to the subtribe. These results do not support a close relationship between *Acanthopale* and *Strobilanthinae* as advocated by Clarke (1899). *Clarkeasia*, a multi-ovulate genus created to accommodate a new species of *Strobilanthinae* (Wood, 1994), is also nested within *Strobilanthinae*.

Previous investigations of southern Indian and Sri Lankan taxa have demonstrated that, of the genera recognized by Anderson (1867) (*Strobilanthinae*, Hemigraphis, Stenosiphonium, and Aechmanthera), *Stenosiphonium* is monophyletic and nested within *Strobilanthinae* (Carine and Scotland, 2000a). These results are confirmed here: *Stenosiphonium* comprises a monophyletic group, and *Hemigraphis* and *Strobilanthinae* are either paraphyletic or polyphyletic. While the majority of *Hemigraphis* species comprise a monophyletic group with the Thai and Burmese species of *Sericocalyx*, a second clade comprising *H. confinis* and *H. griffithiana* is also resolved as sister group to the rest of the Strobilanthinae. This latter relationship is supported in both parsimony and maximum likelihood analyses of trnL-F and ITS data. Clearly, the historic separation of *Hemigraphis* from *Strobilanthinae* on the basis of more than four seeds is artificial (Fig. 4). The possession of more than four seeds cannot be used to distinguish a monophyletic *Hemigraphis* because this character is homoplastic within the Strobilanthinae. Moreover, because four to many seeds occur in a single species (e.g., *H. ciliata*), a distinction between *Hemigraphis* and *Strobilanthinae* on the basis of seed number is impossible. Therefore, the Anderson (1867) classification is in conflict with a phylogenetic approach aiming to recognize only monophyletic taxa.

Bremekamp (1944) divided the Strobilanthinae into 54 segregate genera arranged in 27 informal groups. A new multi-ovulate genus, *Sericocalyx*, was recognized for those species with a sericeous calyx indumentum, which had been variously referred to as either *Strobilanthus* or *Hemigraphis* (Kurz, 1871; Hallier, 1898). However, *Sericocalyx* species comprise two distinct clades, one associated with *Hemigraphis* and one with *Strobilanthinae*. All the traditional multi-ovulate genera (with the exception of *Stenosiphonium*) comprise either para- or polyphyletic taxa.

The classification of Terao (1983), which united all genera previously recognized by Anderson (1867) and Bremekamp (1944) in an expanded *Strobilanthus*, differs only in ranking from that of Bremekamp (1944) because Terao continued to recognize Bremekamp’s segregate genera as infra-generic sections and subsections. For example, section *Aechmanthera* contained subsections *Aechmanthera* sensu Bremekamp), *Hemigraphis* (comprising *Hemigraphis* sensu Bremekamp), and *Sclerostrobilanthinae* (comprising *Sericocalyx* and two additional Bremekamp segregates, *Pleocaulus* Bremek. and *Xanthostachya* Bremek.) (Terao, 1983). Carine and Scotland (2002) have previously demonstrated the nonmonophyly of many of the Southern Indian groups recognized by Terao. The results presented here further demon-
strate that section Aechmanthera and subsections Hemigraphis and Scleroostroblanthes are nonmonophyletic. The combined ITS and morphological analysis illustrates why past generic classifications have been conflicting and inconsistent: of the few characters with discrete variation within the subtribe, almost all are homoplasic. The presence of pedicellate flowers represents the only morphological character that is unique to one group within the Strobilanthinae (Fig. 4). Thus, while molecular data have revealed phylogenetic structure within the Strobilanthinae, morphological evolution has been so highly iterative that no high burden homologues remain (sensu Scotland, 2000) for characterizing groups within the subtribe. Nevertheless, combinations of homoplasic characters can diagnose monophyletic groups within the Strobilanthinae and be locally informative (Wenzel and Siddall, 1999). For example, a clade comprising Stenosiphonium (clade F) can be diagnosed by a combination of “locally informative” homoplasic characters (possession of more than four standards, resupinate corollas, corolla flaps to retain the style; Fig. 4). However, it is apparent that many clades within the Strobilanthinae, and significantly almost all major clades revealed in the molecular analysis, cannot be readily diagnosed, for example, the Hemigraphis clade (clade A) and the Strobilanthes clades from southern Indian (clades G and H). While morphological characters do not diagnose major clades in the combined ITS and morphology tree, it is apparent that there is a strong geographic dimension to relationships. For example, the Hemigraphis clade (clade A) is centered primarily on the Philippine archipelago and the Sericocalyx clade (clade B) comprises taxa from Thailand. Of the remaining clades, one is predominantly Southern Indian/Sri Lankan and one comprises species from Southern India, Java, and China (clade M). This finding contradicts the view that the Southern Indian and Sri Lankan species form a distinct group from the rest of Strobilanthinae (Wood, 1994). The peninsular Indian Strobilanthes flora comprises three distinct elements and the species in this region do not comprise a monophyletic group. Clearly, increased taxonomic sampling will be vital if the biogeography of the subtribe is to be further explored.

The results presented here support the recognition of the Strobilanthinae (sensu Bremekamp, 1944) as a monophyletic group. They reject previously recognized groups within the Strobilanthinae (e.g., Hemigraphis, Sericocalyx, Strobilanthes) and suggest that the circumscription of multiple diagnosable, monophyletic genera within the subtribe is not achievable due to the highly iterative nature of morphological characters within the group. The failure of Anderson’s and Bremekamp’s generic classification and of Terao’s subgeneric classification to define monophyletic groups is directly attributable to high levels of homoplasy in characters traditionally used to delimit higher taxa, for example, seed number and corolla resupination. This finding is in common with other results that cite high levels of homoplasy as one reason for the failure to successfully subdivide “large genera,” for example, Astragalus (Wojciechowski et al., 1999). Generic circumscription within the Strobilanthinae has long proven to be problematic. The results presented here show why this has been the case and demonstrate that the only pragmatic solution to the classification of the Strobilanthinae is to place all segregate genera in synonymy with one large, well-defined genus Strobilanthes within the Ruelliaeae of Scotland and Vollesen (2000). The obvious limitation of this approach is the loss of information of the considerable morphological variation within the group and a failure to accommodate those monophyletic groups that can be diagnosed by a combination of “locally informative” homoplasic characters, e.g., Stenosiphonium. This situation might be partly resolved by informally recognizing monophyletic groups within Strobilanthes s.l. that are well supported by molecular data and have some morphological and geographic coherence (Carine and Scotland, 2002). This pragmatic approach has been adopted by Wood et al. (2003) and will be continued in a checklist providing necessary new combinations within Strobilanthes s.l. that is in preparation.

**LITERATURE CITED**


MOYLAN ET AL.—PHYLOGENY OF STYRIBALANTHES S.L.