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PHYLOGENETIC POSITION AND GENERIC DIFFERENTIATION OF EPITHEMATEAE (GESNERIACEAE) INFERRED FROM PLASTID DNA SEQUENCE DATA[1]

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The systematic position and generic differentiation of the morphologically and geographically outstanding tribe Epithemateae (Gesneriaceae) was analyzed using the *rbcL*/*atpB*-spacer and *trnL-F* intron-spacer regions of chloroplast DNA. In our analysis Epithemateae forms a strongly supported monophyletic clade (bootstrap [BS] = 100%; jackknife [JK] = 100%; decay index [DI] = 12) and appears as sister to the rest of the paleotropical Gesneriaceae (= subfamily Cyrtandroideae). The paleotropical Gesneriaceae form a monophyletic group (BS = 88%; JK = 85%; DI = 3) that is sister to the neotropical Gesneriaceae (subfamily Gesnerioideae) plus Austral Gesneriaceae (subfamily Coronantheroideae) (BS = 99%; JK = 98%; DI = 10). Within Epithemateae *Rhynchoglossum* is sister to the remaining Epithemateae (BS = 97%; JK = 96%; DI = 12), in which *Epithema* is sister to a clade of two genera: *Loxonia*/*Stauranthera* (BS = 68%; JK = 64%; DI = 1), which form, together with *Epithema*, a sister clade (BS = 85%; JK = 83%; DI = 2) to *Whytockia* and *Monophyllaea*. While the support for *Loxonia* and *Stauranthera* is moderate, the relationship of *Whytockia* and *Monophyllaea* is very strongly supported (BS = 100%; JK = 100%; DI = 13). Apart from the somewhat surprising (but well-substantiated) isolated position of *Rhynchoglossum*, the results are in perfect accordance with the relationships worked out earlier on grounds of architectural and floral characters. Especially remarkable is the predicted coherence between the morphologically and geographically different genera *Whytockia* and *Monophyllaea*.

Key words: Epithematheae; Gesneriaceae; *rbcL*/*atpB* spacer; systematic position; *trnL-F* intron spacer.

The tribe Epithemateae (= Klugieae including Loxonieae, see below) is a small group of essentially paleotropical Gesneriaceae that merits special interest: (1) Its genera display a wide range of morphological diversity that includes very unusual and strange patterns, e.g., unifoliate habit (*Monophyllaea*) (Fig. 1b), mixture of opposite and alternate leaf arrangement (*Epithema*) (Fig. 1h), strong anisophylly (*Loxonia*, *Stauranthera*) (Fig. 1d–g), alterniphylly (*Rhynchoglossum*) (Fig. 1a), sympodial plant architecture (*Loxonia*, *Stauranthera*, *Rhynchoglossum*), presence of pair- and single-flowered cymes of certain species (*Loxonia*, *Stauranthera*), extremely condensed pair-flowered cymes enclosed by the subtending bract (*Epithema*), reduction of cymes to single flowers giving rise to unilateral, raceme-like inflorescences (*Rhynchoglossum*), bilocular ovary with axile placentae like in Scorphulariaceae (*Whytockia*, *Monophyllaea*), presence of medullary vascular bundles, presence of secretory canals in the vegetative organs and/or sepals (*Monophyllaea*, *Rhynchoglossum*, *Whytockia*), and others (see Weber, 1971–1988; Wilson, 1974; Burtt, 1978; Wang and Pan, 1996). (2) Though clearly centered in tropical southeast Asia, the geographical range is wide and includes (warm-)temperate China (*Whytockia*, *Rhynchoglossum* pro parte), West Africa (one species of *Epithema*), and even the neotropics (one species of *Rhynchoglossum*). Epithemateae is indeed the only part of subfamily (subfam.) Cyrtandroideae that reaches America. (3) In view of the diverse and strange morphological patterns it is very difficult to characterize and delimit the tribe.

In fact, the recognition of Epithemateae as a systematic entity has come about very slowly. While in early classifications the genera appear erratically scattered over the system and partly closely associated with neotropical genera (see Bentham, 1876; Fritsch, 1893–1894), Burtt (1963) filtered out a number of odd genera and classified them into two tribes: Klugieae and Loxonieae. The former contained *Rhynchoglossum* (including *Klugia*; Burtt, 1962), *Monophyllaea*, *Moultonia* (now included in *Monophyllaea*; Burtt, 1978), and *Epithema*. The latter contained *Loxonia*, *Stauranthera*, *Whytockia*, and ''for want of a better place'' *Cyrtandromoea*. Burtt (1965) himself later removed *Cyrtandromoea* from the tribe by transferring it to the family Scrophulariaceae.

In a series of morphological analyses, Weber (1975–1982) showed that the generic affinities run across the two tribes and the separation of Klugieae and Loxonieae no longer appeared tenable. The suggested union into a single tribe (Klugieae) was accepted informally by Burtt (1977) and later formally by Burtt and Wiehler (1995) and Burtt (1997), who also replaced the tribal name Klugieae written Epithemateae due to priority reasons.

Epithemateae stands as a compact group with *Epithema*, *Loxonia*, *Monophyllaea*, *Rhynchoglossum*, *Stauranthera*, *Whytockia*, and *Gyrogyne*, another monotypic, and little-known genus later added by Wang (1981). Its position within Gesneriaceae, however, has recently become a matter of dispute. It became increasingly apparent that the Epithemateae was not a

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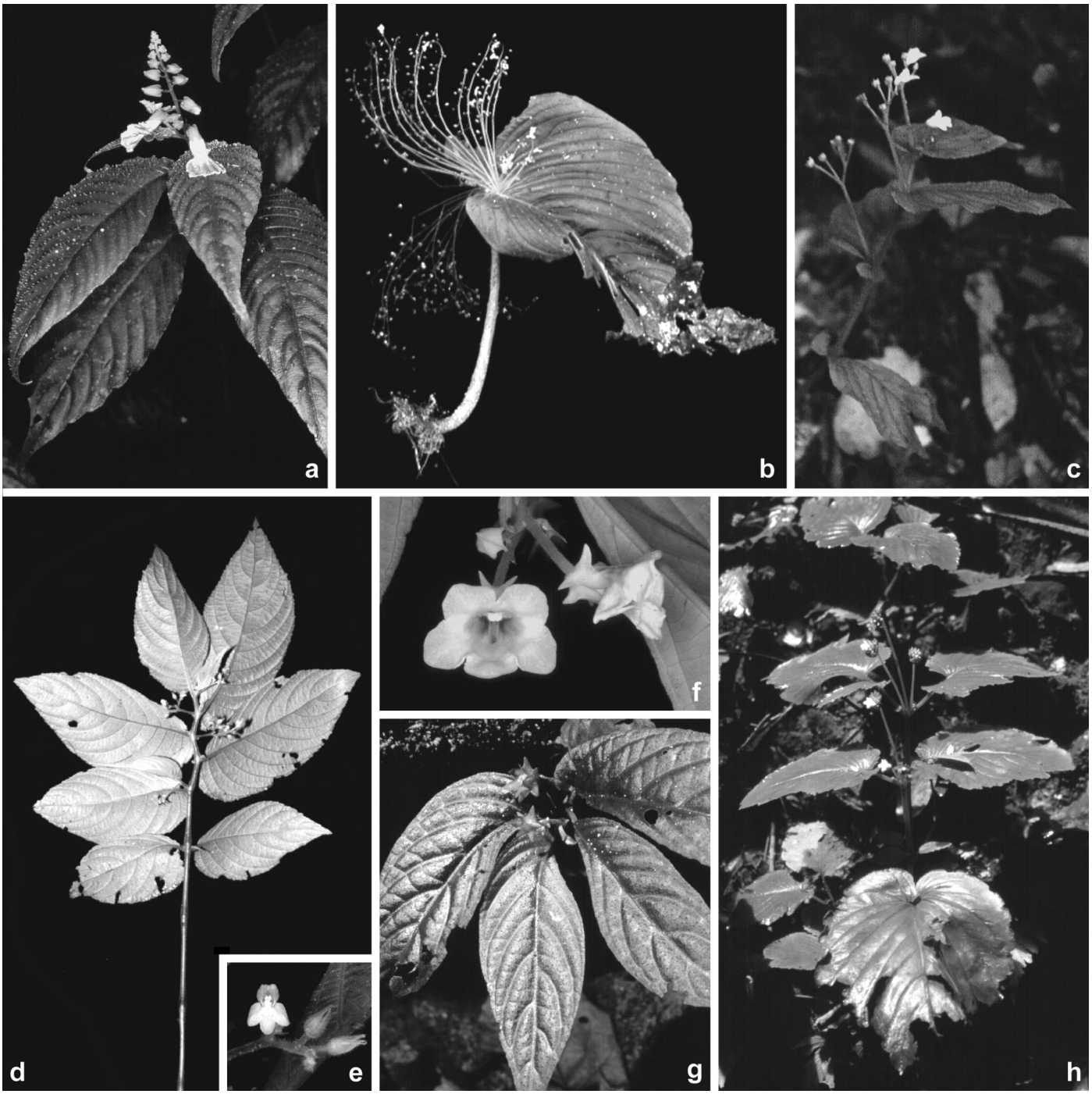

Fig. 1. Representative species of the six genera of Epithemateae included in the analysis; (**a**) *Rhynchoglossum azureum* (Schltdl.) B.L. Burtt, Costa Rica, Valle Virgen; (**b**) *Monophyllaea hirticalyx* Franch., Peninsular Malaysia, Kelantan, north of Gua Musang; (**c**) *Whytockia tsiangiana* (Hand.-Mazz.) A. Weber, South China, Taiping river valley; (**d, e**) *Loxonia hirsuta* Jack, Peninsular Malaysia, Pahang, Pulau Tioman; (**f, g**) *Stauranthera grandiflora* Benth., Peninsular Malaysia, Pahang, Pulau Tioman, near Kampang. Tekek, (f) flower, (g) fruiting specimen; (**h**) *Epithema saxatile* Blume, Peninsular Malaysia, Selangor, Gua Batu (Batu Caves); all photos by A. Weber, except (c): by W. T. Wang.

tribe equivalent to the other tribes of Cyrtandroideae, but was rather juxtaposed to all other Cyrtandroideae. Based on molecular *ndhF* data, Smith et al. (1997) even suggested that Epithemateae is sister to all other (including neotropical and austral) Gesneriaceae and considered the group to represent an old and relic assemblage that has evolved and differentiated very early and in isolation from the rest of Gesneriaceae.

In view of the complex morphology of the Epithemateae a broad molecular approach is an appropriate approach to analyze its infra- and intratribal relationships. Chloroplast DNA (cpDNA) sequences such as the *rbcL-atpB* spacer and the *trnL-F* intron-spacer regions were used for our present phylogenetic analysis, as these regions were previously found suitable to resolve generic relationships within Gesneriaceae

(Samuel, Kiehn, and Pinsker, 1997; Samuel, Pinsker, and Kiehn, 1997; Möller et al., 1999).

The intent of the present study is to contribute to a better knowledge of (a) the position of the Epithemateae within the family Gesneriaceae and (b) the relationships and evolutionary differentiation of the genera included in this group.

MATERIALS AND METHODS

Outgroup taxa—As outgroups we used two members of Solanaceae (*Nicandra physaloides* and *Nicotiana tabacum*) and three members of Scrophulariaceae (*Antirrhinum majus*, *Tetranema mexicanum*, and *Verbascum speciosum*). Family delimitation between Gesneriaceae and its closest related families such as Scrophulariaceae is traditionally problematic, and several taxa have been transferred between the families (e.g., *Rehmannia*, *Titanotrichum*, *Cyrtandromoea*, etc., Burtt, 1965). Therefore the trees were rooted on members of the Solanaceae that have been shown to be distant enough from Gesneriaceae/Scrophulariaceae (Chase et al., 1993; Olmstead et al., 1993; Olmstead and Reeves, 1995) but close enough to allow unproblematic sequence alignment. Representative members of Scrophulariaceae have been added to illustrate the monophyly of the ingroup taxa of Gesneriaceae included.

Ingroup taxa—With the exception of *Gyrogyne*, representatives of all genera of Epithemateae could be included in the analysis (See table at http://ajbsupp.botany.org/v90/). *Gyrogyne subaequifolia* W. T. Wang, the only species of *Gyrogyne*, could not be investigated due to the lack of adequate material. The species is only known from the type collection, and the isotype available to us did not yield successful DNA extracts.

For establishing the position of the Epithemateae within Gesneriaceae, representatives of all tribes of subfamilies Cyrtandroideae, Coronantherae, and Gesnerioideae were included in the analysis (http://ajbsupp.botany.org/v90/). In a few cases (marked by an asterisk) *atpB-rbcL* spacer sequences of Samuel, Kiehn, and Pinsker (1997) and Samuel, Pinsker, and Kiehn (1997) were used. Voucher specimens are deposited in Edinburgh (E), Vienna (WU) and Genève (G).

DNA extraction, polymerase chain reaction, and sequencing procedures—For DNA extraction fresh leaf material, leaves dried in silica gel (from plants cultivated at the HBV = Hortus Botanicus Viennensis and RBGE = Royal Botanic Garden Edinburgh), or leaves from herbarium specimens were used. The material collected by A. Weber in Peninsular Malaysia was quickly dried with a simple transportable ventilator system and proved to be very suitable for DNA extraction.

DNA extraction was made with DTAB/CTAB after Savolainen et al. (1995) or Doyle and Doyle (1987). Of the several attempts made with DNA extracts of herbarium specimens ~60% gave genuine amplification products in polymerase chain reactions (PCR) (as judged from the fragment length on agarose gels). The *atpB* primers used were the forward primer ''JF31'' (TTT CAA GCG TGG AAA CCC CAG) and the reverse primer ''JF5'' (TAC AGT TGT CCA TGT ACC AG); for manual sequencing also the internal primers ''JF9'' (GTC TAT GAT TAT AGA CAA TCC) and ''JF7'' (CCC TAC AAC TCA TGA ATT AAG) were also used (Manen, Natali, and Ehrendorfer, 1994).

The *trnL-F* region was PCR amplified using primers ''C'' and ''F'' (Taberlet et al., 1991), amplifying the *trnL* (UAA) intron and the intergenic spacer between the *trn*L (UAA) 3′ exon and the *trn*F(GAA)5′ exon.

For the *atpB* spacer region PCR was performed in volumes of 50 μL with the following final concentrations: 1× standard PCR reaction buffer (10 mmol/L Tris-HCl pH 8.3, 50 mmol/L KCl), 2.0 mmol/L $MgCl_2$ (2.5–3.0 mmol/L for herbarium extractions), 0.004% BSA, 0.2 μmol/L of each dNTP, 0.2 μmol/L each primer, and 0.7 units Taq-Polymerase (Boehringer, Ingelheim, Germany). Each sample was subjected to an initial cycle of 5 min denaturation at 95°C, 3 min annealing at 46°C, and 5 min extension at 72°C. Thereafter, 35 cycles, each consisting of a denaturation step of 1 min at 95°C, an annealing step of 1 min at 55°C, and an extension step of 1 min at 72°C followed. A final extension of 10 min at 72°C completed the amplification. The PCR products were purified using a Quiagen QuiaQuick Gel Extraction Kit (Crawley, West Sussex, UK).

Sequencing was performed in the forward and reverse directions. At the beginning of the study T7 polymerase was used manually and later an ABI 377 automated sequencer using a dye terminator cycle-sequencing ready-reaction kit (Perkin Elmer, Applied Biosystems Division, Foster City, California, USA) was used, with 4 μL and later 2 μL Dye Terminator (with equal results) in a total of 10 μL reaction used.

For the *trnL-F* region, the PCR reaction mixture and PCR cycle parameter, amplicon purification, quantification, and sequencing procedures are described elsewhere (Möller et al., 1999).

Sequence alignment and phylogenetic analysis—The first unambiguously alignable sequence of the *rbcL/atpB* spacer was used as sequence boundary at the 5′ end. The 3′ end was determined by the start of the *rbcL* coding sequence in comparison with published *rbcL* sequences of *Streptocarpus* (GBAN-AF170250). For the *trnL-F* region, sequence boundaries were determined in comparison to published *Nicotiana tabacum* sequences (GBAN-Z00044). Obtained sequences were aligned using the CLUSTAL option in the multiple alignment program Sequence NavigatorTM Version 1.0.1 software package (Perkin Elmer, Applied Biosystems Division, Foster City, California, USA), followed by manual optimization. Sequence divergence among taxa was calculated using the DISTANCE MATRIX option in PAUP Version 4.0b8 (Swofford, 2001). For analyses of sequence data, only combined *atpB* and *trnL-F* data of unambiguously alignable positions were used, and gaps (indels) were treated as missing data. Indels were scored as a separate presence/absence character and added to the sequence data matrix in a separate analysis (Wojciechowski et al., 1993; Oxelman and Lidén, 1995).

Maximum parsimony (MP) analyses were performed with characters unweighted and unordered. Two search strategies were pursued. The first was a heuristic search with simple addition sequence, tree bisection-reconnection (TBR) and Multrees on. The second consisted of a modified stepwise search method (Soltis and Soltis, 1997) to optimize the heuristic searches for most parsimonious trees (MPT). In the first round 10 000 replicates of random addition sequence with nearest-neighbour interchanges (NNI) swapping algorithm activated, Multrees and steepest descent deactivated, terminating each replicate after saving not more than two trees of *n* tree length (the *n* tree length was established empirically in the first heuristic search). The stored trees were then subjected to TBR swapping algorithm with Multrees and steepest descent on in the second step of this search process. This strategy is designed to efficiently detect multiple islands of MPT that may exist (Maddison, 1991).

Descriptive tree statistics (consistency index [CI: Kluge and Farris, 1969], retention index [RI: Farris, 1989], rescaled consistency index [RC: Swofford, 2001]) were performed using PAUP. Statistical branch support analyses were performed threefold, as 10 000 replicates of fast jackknifing (JK) with 50% character deletion and as 1000 replicates of heuristic bootstrap (BS) (Felsenstein, 1985) with TBR swapping on and Multrees off (Spangler and Olmsteadt, 1999) under PAUP. Decay indices (DI) were derived from AutoDecay 4.0.2 (Eriksson, 1999) and PAUP on 100 replicates of random addition.

To test for rate heterogeneities in sequence evolution across the matrix, a likelihood ratio test was conducted on a single most parsimonious tree with the highest likelihood value (of the combined data set) (Möller and Cronk, 2001). The maximum likelihood (ML) model chosen using Modeltest 3.06 (Posada and Crandall, 1998) was TVM + G. This uses a tranversion model (TVM, variable base frequencies, variable transversions, transitions equal) with gamma-distributed among-site rate variation (G).

To address the high rate heterogeneity across the matrix (in particular, of members of tribe Klugieae) and to investigate their effect on tree topology, additional parsimony analyses (using the same settings as above), either excluding all Epithemateae or on Epithemateae sequences alone, rooted on all outgroups were executed. Any significant deviation in topology of the reduced data sets would indicate problems of long branch attraction associated with high rate heterogeneities (Siddall and Whiting, 1999).

TABLE 1. The *atpB-rbcL* spacer and *trnL-F* intron-spacer sequence characteristics (plus gap matrix) of 56 taxa of Gesneriaceae and five outgroup taxa analyzed.

Parameter[a]	*atpB-rbcL* spacer	*trnL-F* intron/spacer	*atpB-rbcL* spacer and *trnL-F* intron/spacer
No. of taxa	61	61	61
Sequence length range (outgroup)(bp)	750–782	795–860	1546–1643
Sequence length range (ingroup)(bp)	677–863	736–839	1354–1664
Total aligned matrix length(bp)	1046	1065	2111
G + C content (%)	30.6	35.1	33.0
No. of excluded sites (bp)	195	122	317
No. of unambiguously aligned sites (bp)[b]	851	942	1793
No. of informative indels[b]	33	47	80
No. of constant sites (%)[b]	468 (55.0)	504 (53.5)	972 (54.2)
No. of variable sites (%)[b]	383 (45.0)	438 (46.5)	821 (45.8)
No. of uninformative sites (%)[b]	155 (18.2)	151 (16.0)	306 (17.1)
No. of informative sites (%)[b]	228 (26.8)	287 (30.5)	515 (28.7)
Sequence divergence (outgroup) (%)[b]	1.9–10.8	1.8–11.8	1.9–11.3
Sequence divergence (ingroup) (%)[b]	0–12.5	0–16.7	0.2–13.6
Sequence divergence (ingroup/outgroup) (%)[b]	4.3–14.2	3.3–18.2	4.1–16.3
Transitions vs. transversions (minimum)	0.75	0.93	0.84

[a] Abbreviations: G + C, guanine and cytosine; bp; base pairs.
[b] Based on alignment matrix excluding ambiguous alignment positions.

RESULTS

Evolution of plastid DNA—The combined *atpB-rbcL* spacer and *trnL-F* intron spacer sequence matrix of the 61 taxa analyzed was between 1546 and 1643 bp long (Table 1). Alignment of the spacer sequences of the accessions resulted in a 2111 bp long data matrix. Due to alignment ambiguities 317 positions were excluded from the analysis (indicated by asterisks in the matrix). These were mainly the consequence of nucleotide duplication events resulting in variable length regions. Unambiguous alignment of all taxa required the insertion of numerous gaps, indicating that the evolution of the spacer is by both base substitution and insertion/deletion events. Out of those indels 80 were potentially informative, coded and added to the sequence matrix. Of the 1793 unambiguously aligned sites, 972 (54.2%) were constant and 515 (28.7%) were potentially informative phylogenetically, whereas 306 (17.1%) were uninformative characters (Table 1). Sequence divergence for outgroup and ingroup taxa ranged from 1.9% (*Nicotiana tabacum-Nicandra physaloides*) to 11.3% (*Nicandra physaloides-Antirrhinum majus*) and 0.2% (*Gloxinia purpurescens-Koellikeria erinoides*) to 13.6% (*Monophyllaea glauca-Petrocosmea nervosa*), respectively. Maximum sequence divergence within individual taxonomic groups was 12.3% for tribe Epithemateae (*Monophyllaea glauca-Rhynchoglossum obliquum*), 5.8% for the rest of subfam. Cyrtandroideae (*Streptocarpus rexii-Chirita longganensis*), 5.9% for subfamily Gesnerioideae (*Napeanthus reitzii-Alsobia dianthiflora*), and 1.1% for subfamily Coronantheroideae (*Lenbrassia australiana-Sarmienta scandens*).

Sequence characteristics for the individual DNA sequence matrices are given in Table 1. Both matrices were very similar and had similar values for length, types of sites, and divergences. Both plastid DNA sequences showed, contrary to nuclear sequences, the typical low value of guanine and cytosine (GC) contents, with 30.6 and 35.1% for the *atpB-rbcL* spacer and *trnL-F* intron-spacer regions, respectively. With respect to the current subfamilial classification, it is interesting to note that the maximum infratribal sequence divergence for both sequence data matrices of Epithemateae is more than two times higher than for subfam. Gesnerioideae and even higher than for the rest of subfam. Cyrtandroideae. These higher values may be the result of an accelerated evolution in a hostile environment imposing increased selection pressures or may simply reflect the older age of this tribe, as suggested by Smith et al. (1997).

Phylogenetic analysis—The *atpB-rbcL* spacer and *trnL-F* intron-spacer regions were analyzed independently and combined. The phylogenetic analysis of combined *atpB-rbcL* and *trnL-F* data plus the alignment gap matrix produced 270 most parsimonious trees of 1641 steps, with a consistency index (CI), a retention index (RI), and a rescaled consistency index (RC) (including uninformative character) of 0.7246, 0.8615, and 0.6242, respectively. The average character change per position was 0.88, indicating a relatively low saturation of base substitution and a low potential of multiple substitutions and reversals obscuring the phylogenetic signal. Jacknife and bootstrap values were between 56 and 100% and 51 and 100%, respectively, and decay indices were between 1 and 103. The *atpB-rbcL* spacer region plus alignment gap matrix produced 100 700 most parsimonious trees of 728 steps, the *trnL-F* data plus the alignment gap matrix produced 3863 most parsimonious trees of 898 steps. Jackknife and bootstrap values were between 65% and 100% and 50% and 100%, respectively, for the *atpB-rbcL* spacer region and between 51% and 100% and 53% and 100%, for the *trnL-F* data.

Tree topology—The resulting strict consensus tree of 270 most parsimonious trees of combined data sets showed a monophyletic Epithemateae in the combined tree (bootstrap [BS] = 100%; jackknife [JK] = 100%; decay index [DI] = 12) (Fig. 2), sharing 22 synapomorphic character state changes as depicted in one of the resulting trees, which reproduced as phylogram indicating the respective branch lengths (Fig. 3).

The Epithemateae clade shows that the first bifurcation separates *Rhynchoglossum* (BS = 100%; JK = 100%; DI = 72) from the rest of the tribe (BS = 97%; JK = 96%; DI = 12), both with high branch support. As discussed later, the isolated position of *Rhynchoglossum* is a surprise, but can be well corroborated by morphological characters. The next split separates *Whytockia* (BS = 100%; JK = 100%; DI = 36) and

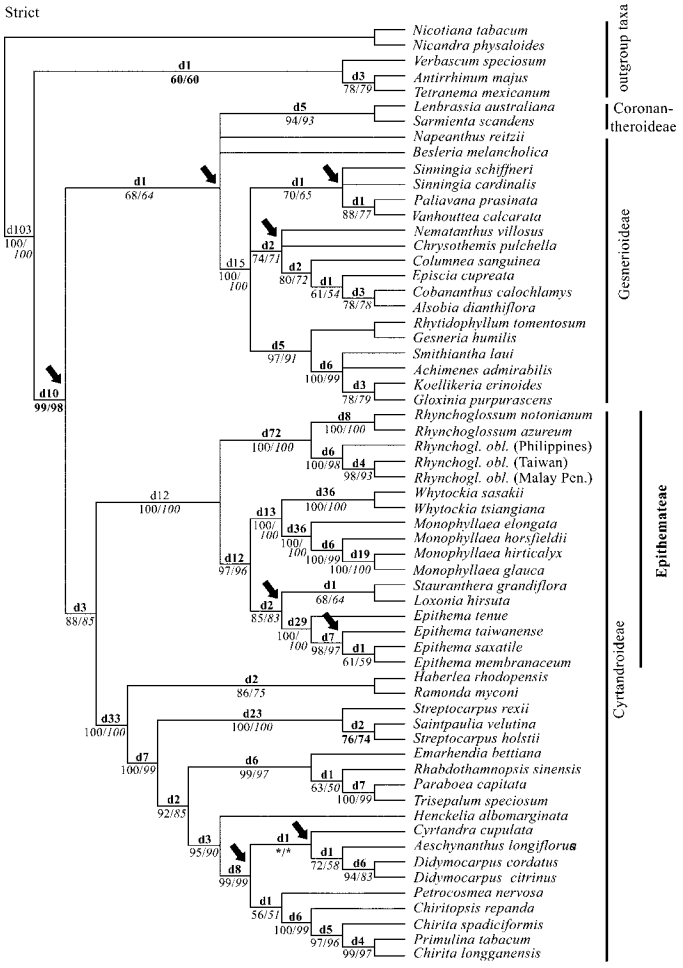

Fig. 2. A single presentative phylogram of 270 most parsimonious trees of 1306 steps, based on combined *atpB-rbcL* spacer and *trnL-F* intron-spacer sequences plus alignment gap matrix (CI = 0.6325, RI = 0.7744, RC = 0.4898, including uninformative characters).

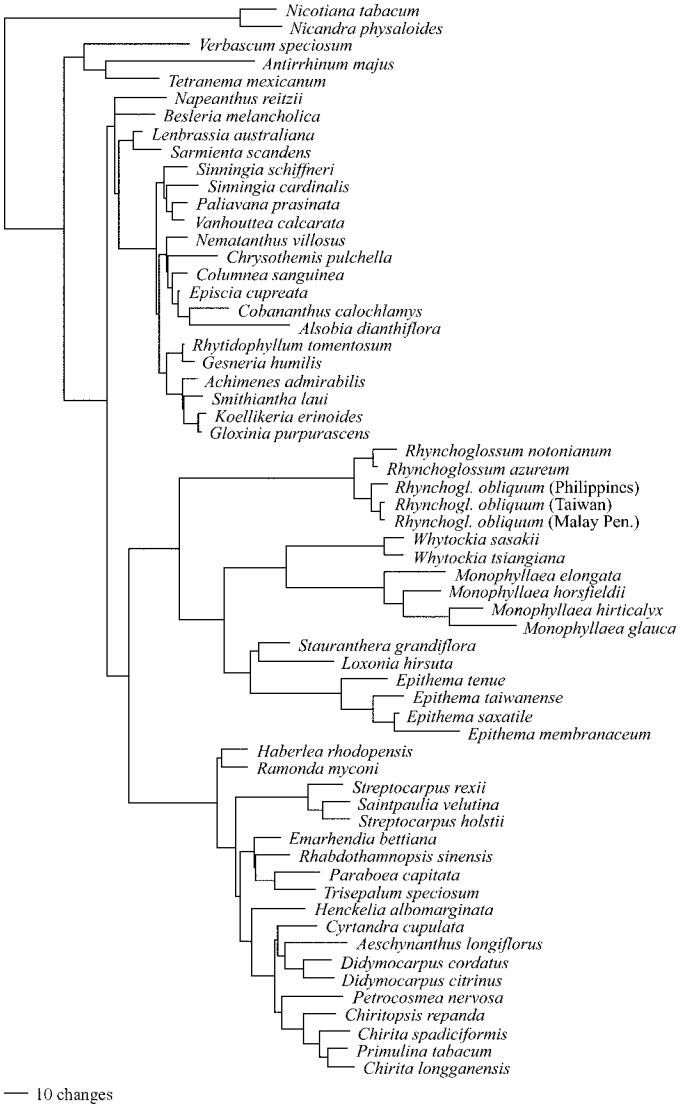

Fig. 3. Strict consensus tree of 270 most parsimonious trees based on combined *atpB-rbcL* spacer and *trnL-F* intron-spacer sequences plus alignment gap matrix (numbers above branches are decay indices, and numbers below branches are bootstrap values; jackknife values are in italic typeface). Arrows indicate conflicting results between the *atpB-rbcL* spacer and *trnL-F* intron-spacer strict consensus trees.

Monophyllaea (BS = 100%; JK = 100%; DI = 36) from the four species of *Epithema* (BS = 100%; JK = 100%; DI = 29) and *Loxonia* and *Stauranthera* (BS = 68%; JK = 64%; DI = 1), the last pair with low branch support.

Taxa belonging to subfamilies Gesnerioideae and Coronantheroideae formed a monophyletic clade albeit with low branch support (BS = 68%; JK = 64%; DI = 1) and the latter subfamily unresolved on a basal polytomy with members of the tribes Napeantheae and Beslerieae. The rest of the subfam. Cyrtandroideae (except tribe Epithemateae) formed a well-supported monophyletic group (BS = 100%; JK = 100%; DI = 33), with the European taxa basal (BS = 100%; JK = 99%; DI = 7) followed by African representatives (BS = 92%; JK = 85%; DI = 2). The Asian taxa formed several well-supported clades splitting taxa with straight and twisted capsules (*Emarhendia*, *Rhabdothamnopsis*, *Paraboea*, *Trisepalum*) (BS = 99%; JK = 97%; DI = 6).

An ML analysis on the combined data set using the parameter settings suggested by Modeltest resulted in an identical tree topology compared to the MP analysis.

Analyzing both data sets individually resulted in similar tree topologies: Epithemateae are well supported (BS = 98%, JK = 93% in *atpB-rbcL* data and BS = 98%, JK = 89% in *trnL-F*) in both trees; the position of genera within Epithemateae remain the same. On the infrageneric level differences were found in (1) *Stauranthera* and *Loxonia*, for which no resolution was obtained in the *atpB-rbcL* tree. This is reflected by low support (BS = 68%; JK = 64%) in the combined tree. (2) In *Epithema* the two Malayan species *E. membranaceum* and *E. saxatile* resulted as a sister group to an African *E. tenue* and Taiwanese *E. taiwanense* clade in the *atpB-rbcL* data, whereas in the *trnL-F* tree *E. tenue* were basal to the three Asian species. (3) For the *Monophyllaea* species no resolution could be obtained with *atpB-rbcL*, but due to the strong signals in *trnL-F*, good resolution with high support (BS = 100%; JK = 100–99%) could be found in the combined tree (Fig. 2).

Conflicting results occurred further in the position of some of the remaining Cyrtandroideae, above all *Aeschynanthus*, *Cyrtandra*, *Didymocarpus*, and *Petrocosmea*. In the *atpB-rbcL*

tree subfamilies Coronantheroideae and Gesnerioideae remained unresolved on a basal polytomy; within Gesnerioideae resolution was poor for many species in this data set.

In summary the *atpB-rbcL* tree showed a much lower resolution than the *trnL-F* tree. Combining the data sets synergistically improved tree resolution, though the areas of conflicting signals (indicated with arrows in Fig. 2) have also low support in the combined tree.

Rate heterogeneities and "pruned" trees—The likelihood ratio test indicated a significant ($P > 0.001$) variation in the rate of sequence evolution across the matrix, suggesting that cpDNA of taxa of tribe Epithemateae evolves at a significantly faster rate compared to other taxa.

Removal of Epithemateae taxa from the matrix did not significantly alter the topology of the remaining taxa compared to the full analysis (only the two taxa of subfam. Coronantheroideae were resolved as sister to other New World taxa). Conversely, analyzing Epithemateae alone gave an identical tree topology within the tribe compared to the full analysis. This suggests that the rate heterogeneity has no detrimental effect due to possible long branch attraction.

DISCUSSION

Sequence evolution of plastid DNA—The two chloroplast sequence regions chosen for this phylogenetic analysis, *atpB-rbcL* and *trnL-F*, showed similar characteristics, both in the proportion of constant, phylogenetically informative/uninformative sites, as well as in the sequence divergence levels. This is not surprising as they are both noncoding plastid DNA and have similar functions. The first is a gene spacer separating the genes *atpB* and *rbcL*; the latter is a construct of an intron in the gene *trnL*, followed by a spacer region separating *trnL*(UAA)3′ exon and the *trnF*(GAA)5′ exon. However, when calculating the proportion of the various types of sites separately for the *trnL-F* intron and spacer regions, it becomes clear that the spacer sequences contain twice as many informative sites compared to the intron (44.5% instead of 22.1%). This can be explained by the presumed post-transcriptional function of introns and thus higher selection pressure and conservation as opposed to spacer regions.

Phylogenetic position of Epithemateae within Gesneriaceae—There has been conflicting discussion on the systematic and phylogenetic position of Epithemateae within the family Gesneriaceae. After fusion of the tribes Klugieae and Loxonieae, the resulting Epithemateae remained at the same (tribal) rank (Burtt and Wiehler, 1995). It had, however, already become apparent that the Epithemateae were marked off from the rest of Cyrtandroideae and eventually could be regarded as a subfamily of its own (Burtt, 1977). In their molecular study of the family, using *ndhF* cpDNA sequences, Smith et al. (1997) found that the two members of Epithemateae included in their analysis (*Monophyllaea hirticalyx* and *Rhynchoglossum notonianum*) plus *Cyrtandromoea* (transferred to Scrophulariaceae by Burtt, 1965) are sister to all other Gesneriaceae. However, no branch support (given as decay index) relevant for this placement was indicated in the phylogenetic tree of Smith et al. (1997). This position would be very surprising; because of the pronounced anisocotyly Epithemateae show clear morphological affinities with other Old World Gesneriaceae. However, in a later paper combining morphology, *ndhF*, and *rbcL*, Smith (2000: Fig. 7) found that Epithemateae are sister to the remainder of Cyrtandroideae.

The present results based on combined *atpB* and *trnL-F* data confirm this result. In our analysis the Epithemateae form a sister clade to the other paleotropical Gesneriaceae, with good branch support (BS = 88; JK = 85; DI = 3; Fig. 2). As discussed later, its extant genera are probably remnants of a once much larger group, now showing—as compared to other Cyrtandroideae—severe morphological and molecular differences and discontinuities. The branches of Epithemateae are the longest in the molecular tree and, in particular, much longer than in the New World taxa, further supporting this hypothesis. Epithemateae therefore is a small and diverse group that clearly belongs to the paleotropical Gesneriaceae (subfam. Cyrtandroideae) and is well marked off from the rest of the group.

Affinities between the genera of Epithemateae—Here we briefly discuss the relationships, essentially based on the molecular data. An ample account relating to the morphological diversification and complex character evolution will be provided at a later opportunity (A. Weber, unpublished data).

Rhynchoglossum—The genus *Rhynchoglossum* comprises about ten species. The Asiatic range is from India and Sri Lanka to New Guinea. The three species described from the neotropics (*R. azureum*, *R. grandiflorum*, and *R. violaceum*) are considered to represent a single, variable species, *R. azureum* (Wiehler, 1983). The species can be roughly classified into two groups: (1) perennials with large flowers and four stamens (the former genus *Klugia*) and (2) annuals with small flowers and two stamens.

The three species/five acquisitions available for analysis, *R. notonianum* (southern India), *R. azureum* (Mexico to Peru; material from Costa Rica), and *R. obliquum* (India, southern China, Malay archipelago to New Guinea; material from the Malay peninsula, the Philippines, and Taiwan), cover both groups. These groups are reflected in the phylogram by two clades: *R. notonianum* and the neotropical *R. azureum* vs. the three acquisitions of *R. obliquum* (Fig. 3). The tree thus reflects clearly the morphology and not the geographical pattern, which can be seen as an indication that *R. azureum* is a recent introduction to the Neotropics and that it does not represent an ancient relict. The low sequence divergence of 0.7% between this species and *R. notonianum* is further evidence.

The sister position of *Rhynchoglossum* to the remaining Epithemateae is in accordance with the many and strong morphological differences (e.g., alternate, strongly asymmetrical leaves, terminal inflorescences in the form of unilateral racemes, enlarged lower lip of corolla; for details see Weber, 1978a, b, c). Nonetheless, the juxtaposition comes as a slight surprise. The genus has many special characters in common with the other Epithemateae, except the *Monophyllaea*/*Whytockia* pair. Therefore, our expectation was that the hiatus would be between *Monophyllaea*/*Whytockia* and the other Epithemateae including *Rhynchoglossum*. But according to the present data, this is not the case.

Epithema—The genus includes more than 20 species (B.L. Burtt and O. Hilliard, Edinburgh, unpublished data) and has a very wide geographical distribution: West Africa (*E. tenue*) and from northern India, southern China, and Taiwan over the Malay archipelago to New Guinea and the Solomon Islands.

Four species (the African *E. tenue*, two Malayan species, and one Taiwanese species) were included in the analysis.

The species show remarkably little morphological variation, with a uniform and unique basic pattern: the strongly unequal pair of cotyledons is followed by a solitary leaf. Then one or two pairs of isophyllous leaves follow. The solitary and the paired leaves produce several axillary inflorescences in the form of a strongly condensed pair-flowered cyme, which is enclosed by a cucullate bract. Weber (1976a, b, 1988) showed that this unusual pattern is obviously derived from anisophylly, including the inflorescences, which are apparently reduced forms of formerly more elaborate alternicladic thyrses.

The curious and unique morphology did not allow a clear statement about the relationships with other genera. Weber (1976a, b) denied a closer relationship with *Monophyllaea* (superficial similarity in the much enlarged macrocotyledon and inflorescences) and suggested a somewhat closer relationship with *Loxonia* and *Stauranthera* (Weber, 1977b). The molecular data show that *Epithema* has a rather isolated position, but is clearly nested within Epithemateae.

Surprisingly, the African *E. tenue* is placed basal to the Asiatic species. This is suggestive of a relict status of this species in Africa from a wider distribution of the genus in the past, similar to *Saintpaulia* (Möller and Cronk, 1997).

Loxonia and Stauranthera—Loxonia is a genus of three species, whose distribution ranges from the Mentawai Islands over Sumatra to Borneo and Java (Weber, 1977a). *Stauranthera* includes about five species with a range from northeastern India and southern China southwards over the Malay archipelago to New Guinea (A. Weber, unpublished data). Only a single species of each genus could be included in the molecular analysis.

In both genera the plant architecture is very similar (strong anisophylly, sympodial structure of the flowering region, terminal inflorescences in the form of alternicladic thyrses; Weber, 1977b), but the flowers look very different. Nonetheless, Weber (1977b) suggested that the two genera *Loxonia* and *Stauranthera* are somewhat closely related. This is now corroborated by the molecular data, though the support is not very high.

Gyrogyne—Due to the lack of adequate material, the seventh genus of Epithemateae, *Gyrogyne* W.T. Wang, could not be included in the analysis (see above, Materials and methods: Ingroup taxa). Its only species, *G. subaequifolia* W.T. Wang, is known only from the type collection and is probably extinct in the wild (Y. Z. Wang, Beijing, personal communication). W. T. Wang (1981) included it in Epithemateae and placed it next to *Stauranthera* on account of the plicate calyx very similar to and characteristic of that genus. In contrast to *Stauranthera*, which is strongly anisophyllous, the leaves of *Gyrogyne* are nearly equal, which is seen as the ancestral condition by Wang (1981). No further information can be added here.

Whytockia and Monophyllaea—Whytockia is a small genus that has a more northerly and definitely extratropical distribution (southern China, Taiwan). Three species have been recognized in the revision of Weber (1982a), but recently a few more have been discovered (Wang, 1995; Wang and Li, 1997). One species, *W. tsiangiana*, was included in the analysis. *Monophyllaea* is a much larger genus and essentially confined to the ever-wet tropics from southern Thailand and the Philippines to Sumatra and Java eastwards to New Guinea. In the present analysis four species could be included: *M. horsfieldii*, *M. hirticalyx*, *M. elongata* (all Malay peninsula and belonging to subgenus [subg.] *Monophyllaea*), and *M. glauca* (Borneo, subg. *Moultonia*).

The species of *Whytockia* are uniform in gross morphology and display the simplest architecture in the present alliance: the shoots bear distinctly anisophyllous leaf pairs. Lateral shoots, in the form of pair-flowered cymes, emerge only from the axils of the plus leaves. The cymes are loosely branched and comprise a low to moderate number of (partly still large) flowers. In the simple anisophyllous shoot and inflorescence construction *Whytockia* conforms perfectly to a morphological archetype of Epithemateae, and in fact we adhere to the opinion that this genus has preserved the ancient construction of the hypothetical ancestor of that group. Weber (1976a) showed that the unifoliate architecture of *Monophyllaea* can be well derived from the pattern found in *Whytockia*.

It was suggested that the relationship between these two genera must be particularly close (Weber, 1975a, b, 1976a, b). There are several uncommon floral characters (imbricate-descending aestivation of sepals; secretory canals in the sepals; chalk-secreting glands on the inner side of the sepals; descending [''antirrhinoid''] petal aestivation; bilocular ovary) that bind the two genera tightly together. The prediction of a close relationship is now considerably substantiated by the molecular data: *Whytockia* and *Monophyllaea* form a distinct clade (BS = 100%; JK = 100%; DI = 13). The phylogenetic relation of the two genera is not obvious from the molecular tree. Morphology suggests that *Whytockia* is the ancestral type. More recently, the chromosome numbers of *Whytockia* (x = 9) and *Monophyllaea* (x = 10, 11, 12) became known (Kiehn and Weber, 1998; Wang, Gu, and Hong, 1998). These numbers can be brought into a line of ascending dysploidy, in which *Whytockia* marks the starting point.

Though we have tried to analyze a greater number of *Monophyllaea* species from both subgenera (from herbarium material) we succeeded only in three species of subg. *Monophyllaea* and one from subg. *Moultonia*. The distal position of the latter is in accordance with the view that subg. *Moultonia* is more advanced than subg. *Monophyllaea*. No other statements about the relationships within *Monophyllaea* can be made at present.

Phylogenetic relationships among other alliances—The present analysis includes 56 species of Gesneriaceae representing 41 out of 147 genera. This seems adequate for evaluating the position of Epithemateae within the family and generic relationships within tribe Epithemateae, but is not enough for making far-reaching conclusions about the systematics of the family as a whole. Nonetheless, a few brief notes can be made in advance of a much broader analysis, which is currently under preparation.

1) The south-hemispherical genera *Lenbrassia* and *Sarmienta*, recently placed (together with *Depanthus*, *Coronanthera*, *Mitraria*, *Negria*, and *Rhabdothamnus*) in a separate subfamily by Wiehler (1983), form a clade that is closely associated with the neotropical Gesneriads. This has also been found by Smith and Caroll (1997) and Smith and Atkinson (1998); though the status of the alliance must be questioned.

2) The placement of the neotropical genera is in good accordance with the current systematic subdivision of neotropical Gesneriaceae (subfam. Gesnerioideae) into morphological-

ly defined sections (Wiehler, 1983) and the molecular data of Smith et al. (1997) and Zimmer et al. (2002). The suggestion of Smith et al. (1997) to add a sixth tribe (section Sinningieae) to the five tribes recognized by Wiehler (for the accomodation of *Sinningia*, *Paliavana*, and *Vanhouttea*) can be supported.

3) The paleotropical Gesneriaceae (subfam. Cyrtandroideae) consist of two blocks: the Epithemateae and the ill-resolved remaining paleotropical Gesneriaceae. In the latter, a subdivision into the three tribes as recognized by Burtt (1963: Cyrtandreae, Trichosporeae, Didymocarpeae) cannot be observed: Cyrtandreae (*Cyrtandra*) and Trichosporeae (*Agalmyla*, *Aeschynanthus*) are nested within Didymocarpeae.

4) At the present state, three alliances of different geographical distribution become apparent: (a) a European group (*Haberlea*, *Ramonda*), morphologically defined by septicidal dehiscence of the capsules, (b) an African group (*Streptocarpus*, *Saintpaulia* nested in *Streptocarpus*; for details see Möller and Cronk, 1997, 2001), and (c) a large Asiatic group. In the latter group, taxa from tropical southeastern Asia are over-represented and taxa from subtropical and temperate Asia (*Rhabdothamnopsis*, *Petrocosmea*, *Primulina*, *Chiritopsis*) are under-represented. A better representation of extra-tropical Asian taxa will provide a more differentiated and certainly more complicated picture.

5) The recent split of *Didymocarpus* (in Asia) into *Didymocarpus* sensu stricto (s.s.) and *Henckelia* (Weber and Burtt, 1998) is supported by the different position of the respective species. *Trisepalum* fits well with *Paraboea*.

Conclusions—The results obtained by the molecular analysis are in fair agreement with the morphological data and taxonomic expectations. Several predictions at the generic level are clearly supported by the molecular data: (1) *Whytockia* and *Monophyllaea*, despite their outward dissimilarity, are closely related; (2) the anisophyllous-anthocladic *Stauranthera* and *Loxonia*, despite their rather different flowers, are closely related; (3) the morphologically isolated *Epithema* (to be interpreted as derived from anisophyllous ancestors) is allied to *Loxonia* and *Stauranthera*.

Especially remarkable is the confirmation that the genera *Whytockia* and *Monophyllaea* are closely related. This supports also the view that *Monophyllaea* evolved on the Asiatic continent (Weber, 1982a, b). The great species number and the widespread occurrence of *Monophyllaea* in the humid tropics can be interpreted as an invasion from the continental subtropics and an explosive speciation in the ever-wet tropics. A south- and eastwards migration (Malay Peninsula and Sumatra: 8 species), with highest diversification in Borneo (15 species), over Celebes (1 species) and the Moluccas (2 species), and finally reaching New Guinea (3 species), appears more plausible than an eastern (New Guinea) origin and westward spread (the ''tentative'' view of Burtt, 1978). This is consistent with the view that subg. *Moultonia* evolved later than subg. *Monophyllaea*, namely when the genus had already reached Sumatra and Borneo. Both subgenera then spread to New Guinea (one species of subg. *Monophyllaea*, two of subg. *Moultonia*).

With regard to characters, some prominent floral characters of *Whytockia* and *Monophyllaea* deserve discussion: the imbricate sepal aestivation, the presence of chalk glands on the inner side, the presence of secretory canals, the antirrhinoid corolla aestivation, and the bilocular ovary. A key to the genera of Epithemateae would probably start with this character complex to separate *Whytockia*/*Monophyllaea* from the remainder of Epithemateae. In contrast, the molecular tree separates *Rhynchoglossum* first from the rest of Epithemateae. However, upon closer inspection of these floral characters, it is apparent that all represent synapomorphies (binding the two genera morphologically together), and it is well conceivable that they evolved later than the basal split.

The remaining point of discussion is the position and evolutionary state of *Rhynchoglossum*. Weber (1978a, p. 41) stated that this genus is closer allied to *Epithema*, *Loxonia*, and *Stauranthera* rather than to *Whytockia* and *Monophyllaea* and that the closest relative could be *Loxonia*. Simultaneously, however, it was expressed that *Rhynchoglossum* cannot be directly derived from *Loxonia* as the formal morphological series of inflorescence structure would suggest. The molecular tree now suggests that *Rhynchoglossum* stands apart from all other Epithemateae. This deserves interpretation. In our opinion, this does not mean that *Rhynchoglossum* is the most primitive genus of the whole alliance from which all other genera derived. Without a doubt, *Rhynchoglossum* has a very complex morphological structure and is highly derived. This side position rather seems to indicate that *Rhynchoglossum* (or more likely its ancestors) split off very early from the other taxa then existing and that there was a long period of time available for the evolution of a highly elaborate morphological pattern in isolation.

The reconstruction of the evolutionary changes in morphology of Epithemateae is a promising subject, but would go beyond the limits of the present paper. A treatment with characterization of a hypothetical ancestor of Epithemateae, reconstruction of character evolution, phytogeographical considerations, a new taxonomic circumscription, and keys to the taxa of Epithemateae will be provided in due course.

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