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A phylogenetic analysis of Apostasioideae (Orchidaceae) based on ITS, *trn*L-F and *mat*K sequences

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Abstract. The orchid subfamily Apostasioideae consists of two genera, *Apostasia* and *Neuwiedia*. To study the position of Apostasioideae within Orchidaceae and their intra- and intergeneric relationships, a molecular phylogenetic analysis has been conducted on the nuclear ITS region and the two plastid DNA regions *trn*L-F intron and *mat*K. The two genera traditionally ascribed to Apostasioideae are each monophyletic. In *Apostasia*, *A. nuda*, with two stamens and no staminode, is sister to a clade comprising three species characterised by two stamens and one staminode. Within *Neuwiedia*, maximum parsimony analyses place *N. zollingeri* as sister to the clade formed by *N. borneensis* and *N. veratrifolia*. A family-wide phylogenetic analysis of *mat*K sequences representing all proposed subfamilies of Orchidaceae produced five moderately to well-supported clades. One of these clades, Apostasioideae, is sister to the clade formed by Vanilloideae, Cypripedioideae, Orchidoideae and Epidendroideae. High transition-transversion ratio and the absence of stop codons in the individual sequences suggest that *mat*K is at the transition from a possibly functional gene to a pseudogene in Apostasioideae, contrary to what is found in some other groups of Orchidaceae.

Key words: Molecular phylogenetics, plastid DNA, nrDNA, Orchidaceae, pseudogene, *Apostasia*, *Neuwiedia*.

Introduction

The genera *Apostasia* and *Neuwiedia* form the subfamily Apostasioideae (Orchidaceae), with a total of 15 species mainly occurring in Southeast Asia. In contrast to all other orchids, they have a very simple gynostemium and three stamens, one of which may be a staminode in *Apostasia* (Kocyan and Endress 2001). Traditionally, the subfamily is placed as sister to the rest of Orchidaceae (see Stern et al. 1993, Kocyan and Endress 2001, Rudall and Bateman 2002, for recent reviews of their taxonomic history). This placement is also supported by recent molecular (*rbc*L, 18S, *nad*1 b-c; Cameron et al. 1999, Cameron and Chase 2000, Freudenstein et al. 2000) and non-molecular analyses (Freudenstein and Rasmussen 1999). However, little information has been available on the systematic structure of these two genera. De Vogel (1969) proposed two different sections in *Apostasia* based on the presence or absence of the staminode, which represents the vestige of the median abaxial stamen: sect. *Apostasia*, with a staminode, and sect. *Adactylus* without a staminode. This division was also confirmed by a recent study on floral structures and developmental pat-

terns of Apostasioideae (Kocyan and Endress 2001). The relationships among *Neuwiedia* species still remain to be resolved, because morphological results are somewhat ambiguous (de Vogel 1969, Kocyan and Endress 2001). In order to investigate inter- and intrageneric relationships within Apostasioideae, which are to date mainly based on morphological observations, we have produced sequences from three commonly used DNA regions: 1) the internal transcribed spacer (ITS) of the nuclear ribosomal DNA (e.g. Baldwin 1992, Cox et al. 1997, Conti et al. 1999, von Balthazar et al. 2000, Whitten et al. 2000, Gravendeel et al. 2001); 2) the plastid gene *mat*K (e.g. Johnson and Soltis 1994, 1995; Conti et al. 1999; Kores et al. 2000; Whitten et al. 2000; Goldman et al. 2001; Gravendeel et al. 2001; Schönenberger and Conti 2003); and 3) the non coding *trn*L-F intergenic spacer of the plastid genome (e.g. Taberlet et al. 1991, Pfosser and Speta 1999, Whitten et al. 2000). Furthermore, a *mat*K-data matrix representing all major orchid clades has been produced to test the monophyly and the phylogenetic position of Apostasioideae within Orchidaceae.

Materials and methods

Taxon sampling. Our study comprises four (out of seven) *Apostasia* species, three (out of eight) *Neuwiedia* species, 15 representatives of other major orchid clades, as well as *Blandfordia punicea* (Blandfordiaceae) and *Curculigo capitulata* (Hypoxidaceae) as outgroups (Table 1). Blandfordiaceae and Hypoxidaceae have been identified as close relatives of Orchidaceae in broad analyses of Asparagales (Rudall et al. 1997, Chase et al. 2000, Fay et al. 2000) (Table 1). With the exception of *Apostasia nipponica* (kindly offered by T. Yukawa, Tsukuba Botanical Garden, Amakubo, Japan) the material was collected during field trips to Sabah/Malaysia and Tasmania/Australia by the first author. The sampling that represents less than 50 % of the known species diversity may be critizised. However, all Apostasioideae species are rare, none is available in cultivation, and some are known only from very few (old) herbarium collections. To increase the species sampling we also tried to extract DNA from herbarium material without success. Non-apostasioid orchids were sampled in various botanical gardens. Nomenclature of *Apostasia* and *Neuwiedia* follows that of de Vogel (1969). Vouchers are deposited in the Zürich herbarium [Z + ZT] or in the pickled collection of A. Kocyan. ITS and *mat*K sequences of various orchid taxa were obtained from Genbank (Table 1).

DNA extraction and sequencing. Leaf material was dried and preserved in silica gel. Total genomic DNA was extracted from dried material following the modified CTAB procedure of Doyle and Doyle (1987) or the SDS protocol after Eichenberger et al. (2000). *Apostasia nipponica* was extracted with the DNeasy™ Plant Mini Kit (Qiagen, Basel, Switzerland). The selected DNA regions were amplified with standard polymerase chain reactions (PCR). Newly designed primers (von Balthazar et al. 2000) were used to amplify the entire ITS1, 5.8S rDNA and ITS2 region in a single reaction (Baldwin 1992). Primers c and f of Taberlet et al. (1991) were used to amplify the *trn*L(UAA)5′ exon and *trn*F(GAA) of the chloroplast genome in a single reaction. The *mat*K gene was amplified in two overlapping parts using the -19F forward (CGTTCTGACCATATTGCACTATG; Kores et al. 2000, Molvray et al. 2000) and 834R reverse (AAAGACTCCARAAGATRTTG) primers, and the 580F (ACTAATACCCYATCCCATMC) and R1(CATTTTTCATTGCACACGRC) primers. Primer R1 anneals slightly upstream of the often used *trn*K-2R primer. Primers 580F, 834R and R1 were newly designed for this study. The PCR protocol used to amplify ITS, *trn*L-F intron and *mat*K was as follows: 35 cycles of 30 sec at 95 °C denaturation, 1 min annealing (50°C for ITS, 55 °C for *trn*L-F, 49 °C for *mat*K), 1 min 40 sec elongation at 72 °C. Three species appeared to contain multiple copies of ITS, and PCR products were cloned with the TOPO TA cloning® kit (Invitrogen). Several clones were sequenced and subjected to phylogenetic analyses. A putative ortholog was identified based on branch length, sequence characteristic, and phylogenetic placement, and was then used in final analyses. Prior to cycle sequencing, PCR products were purified with the Qiaquick™ PCR Purification Kit (Qiagen, Basel, Switzerland). For cycle sequencing BigDye Terminator Ready Reaction Kit (Applied Biosystems) was used. Additional primers were used for

Table 1. Species analysed in this study. Subfamily names according to Pridgeon et al. (1999).

Taxa	Voucher number / DNA number (AK = A. Kocyan)	GenBank Accession Number		
		*mat*K	*trn*L-F	ITS
Ingroup				
Apostasioideae				
Apostasia nipponica Masam.	Yukawa 99-92 / AK92	AY557215	AY557222	AY557231
Apostasia nuda R.Br.	AK971126-3-01 / AK44	AY557214	AY557221	AY557230
Apostasia odorata Blume	AK971109-1-01 / AK15	AY557213	AY557220	AY557229
Apostasia wallichii R.Br	AK981023-1-02 / AK14	AY557212	AY557219	AY557228
Neuwiedia borneensis de Vogel	AK971114-1-01 / AK19	AY557209	AY557216	AY557225
Neuwiedia veratrifolia Blume	AK971115-1-01 / AK21	AY557211	AY557218	AY557227
Neuwiedia zollingeri Reichenb.f. var. *javanica* (J.J.Sm.) de Vogel	AK971114-1-04 / AK20	AY557210	AY557217	AY557226
Cypripedioideae				
Cypripedium calceolus L.	AK990519-1-02 / AK56	AY557208	AY557224	AY557232
Paphiopedilum glaucophyllum J. J. Smith	AK990525-1-25 / AK75	AY557205		
Phragmipedium longifolium Rolfe	AK990525-1-02 / AK71	AY557204		
Vanilloideae				
Cleistes rosea Lindl.		AJ310006*		
Pogonia ophioglossoides (L.) Jussieu		AJ310055*		
Vanilla planifolia Andr.	s.n. / AK49	AJ310079*	AY557223	U66819*
Orchidoideae				
Disa glandulosa Burch. ex Lindl.		AF263654*		
Ophrys apifera Hudson		AJ310049*		
Lower Epidendroideae				
Corymborkis veratrifolia (Reinw.) Blume	AK981020-1-01 / AK06	AY557203		
Nervilia cf. *aragoana* Gaud.		AJ310048*		
Palmorchis trilobata L.O. Williams in Woodson & Schery		PTR310052*		
Higher Epidendroideae				
Aerides multiflora Roxb.	920432 # / AP02	AY557201		
Calanthe vestita Lindl.		AF263634*		
Collabium simplex Rchb.f.	AK991017-1-04 / AK95a	AY557200		
Rhynchostylis gigantea (Lindl.) Ridl.	913013 # / AP07	AY557202		
Outgroup				
Blandfordia punicea Sweet (Blandfordiaceae)	AK981013-1-01 / AK01	AY557206		
Curculigo capitulata Kuntze (Hypoxidaceae)	AK981228-1-01 / AK05	AY557207		

* Sequence taken from GenBank
\# Voucher specimens in Leiden

sequencing: F2 and R2 for ITS (von Balthazar et al. 2000) and primers d and e for *trn*L-F (Taberlet et al. 1991). Sequences of newly designed internal *mat*K primers were as follows (5′-3′): 596R CCAGCATTGAAGGATTTG, 1251R TAGGATGACCCAATACAG, 1361R CCGCTGTGATAAYGASAAAG, 1892R TCAGTCRATYTAACCACCA, 1082F CTATTCCTTCTCTTTYMTGGG. Additionally, 731F (TCTGGAGTCTTTCTTGAGCGA; Gravendeel et al. 2001) and 1592R (TCATGAATGATCCACCAGA) were used. Purified sequencing reactions were run on an ABI Prism 377 (Applied Biosystems) automated sequencer or on an ABI Prism 3100 (Applied Biosystems). Nucleotide sequences were aligned using a combination of the software program Clustal X (Thompson et al. 1997) and MacClade 4.0 PPC (Maddison and Maddison 2000). In Clustal various gap opening and gap extension options were explored and resulting alignments manually adjusted in MacClade.

Phylogenetic analyses. In the first step, *mat*K sequences were used to perform a broader analysis on representatives of all Orchidaceae to test the monophyly of Apostasioideae and to explore their position within the family. Phylogenetic analyses were performed with PAUP* 4.0b10 (Swofford 2002) using maximum parsimony (MP) with the factory settings of the branch-and-bound search option activated. Gaps were treated as missing values. Bootstrap analysis was performed with 100 replicates under the branch-and-bound search (Felsenstein 1985). Uninformative characters were excluded from the analysis. For this analysis *Blandfordia punicea* (Blandfordiaceae) and *Curculigo capitulata* (Hypoxidaceae) were used as outgroup taxa.

A second series of analyses focused on Apostasioideae as the ingroup. To reduce problems of multiple sequence alignment in the more variable *trn*L-F and ITS regions only two taxa (*Cypripedium calceolus* and *Vanilla planifolia*) were sampled from the sister clade of Apostasioideae (also see Cameron et al. 1999, Freudenstein et al. 2000) and used for global outgroup comparison (Maddison et al. 1984). For each DNA region (ITS, *trn*L-F, *mat*K) individual analyses were performed, followed by a combined analysis of all three data sets. Due to the small number of taxa, an exhaustive search with the maximum parsimony criterion was possible, followed by a bootstrap analysis with 100 replicates under branch-and-bound search option with factory settings (Felsenstein 1985). Gaps were treated as missing values. To test homogeneity between the three data sets 1000 replicates of the incongruence length difference test (ILD; Farris et al. 1995; under partition homogeneity difference analysis in PAUP* 4.0b10) were performed under the branch-and-bound search constraints. The test was conducted by pairwise comparison of the three data sets in the following combinations: *mat*K vs. *trn*L-F, *mat*K vs. ITS, *trn*L-F vs. ITS, [*mat*K + *trn*L-F] vs. ITS, [*mat*K + ITS] vs. *trn*L-F, [*trn*L-F + ITS] vs. *mat*K. Combinability of the three data sets prior to phylogenetic analysis was also assessed by visual comparison of the trees derived from the separate data partitions.

To investigate patterns of molecular evolution and detect the presence of potential pseudogenes in our sequences, we calculated the ratio between transitions (ts) and transversions (tv) and their respective consistency (CI) and retention indices (RI). The number of transversions, their CI, and RI were obtained by using a step matrix that downweighted the transitions to zero and by reconstructing their distribution on the most parsimonious trees under ACCTRAN optimization. The corresponding values for transitions were obtained by subtracting the values obtained for transversions from the values calculated for the entire data matrices (Goldman et al. 2001, Gravendeel et al. 2001, Whitten et al. 2000).

Results

Analysis of Orchidaceae. The aligned *mat*K matrix comprised 1654 positions, of which 902 were constant, 280 uninformative, and 472 parsimony informative. The branch-and-bound search produced a single most parsimonious tree with a length of 1157 steps, a consistency index (CI) of 0.60, and a retention index (RI) of 0.73 (Table 2). Transitions (ts) were more numerous than transversions (tr), with a ts/tr ratio of 1.27 (Table 3). In the aligned sequences, most indels occurred in triplets; however, some were non-triplets ranging from 1bp to 26bp resulting in a change of the reading frame.

Five main clades were identified in the broader analysis (Fig. 1). Apostasioideae are monophyletic (100% Bootstrap Support [BS])

Table 2. Statistics from the overall Orchidaceae-*mat*K analysis and the Apostasioideae-analyses (*mat*K, *trn*L-F, ITS, combined)

	*mat*K (Orchidaceae)	*mat*K (Apostasioideae)	*trn*L-F (Apostasioideae)	ITS (Apostasioideae)	combined sequences (Apostasioideae)
Number of characters	1654	1588	1602	1033	4223
Number of constant characters	902 (54.5%)	1202 (75.7%)	1364 (85.2%)	504 (48.8%)	3070 (72.7%)
Number of uninformative characters	280 (16.9%)	218 (13.7%)	161 (10%)	206 (19.9%)	585 (13.9%)
Number of parsimony-informative characters	472 (28.6%)	168 (10.6%)	77 (4.8%)	323 (31.3%)	568 (13.4%)
Number of trees	1	1	3	2	2
Number of steps	1157	210	105	571	887
CI	0.60	0.93	0.87	0.88	0.89
RI	0.73	0.95	0.91	0.90	0.91

Table 3. Numbers of steps, CIs and RIs for the trees from transitions, transversions respectively, of the sequenced DNA regions

	*mat*K (Orchidaceae)		*mat*K (Apostasioideae)		*trn*L-F (Apostasioideae)		ITS (Apostasioideae)	
	ts	tv	ts	tv	ts	tv	ts	tv
Number of steps	648	509	125	85	57	48	280	291
CI	0.54	0.65	0.93	0.93	0.88	0.85	0.99	0.78
RI	0.79	0.75	0.95	0.95	0.91	0.90	0.99	0.82
ts:tv	1.27		1.47		1.19		0.96	

and sister to a clade comprising four clades (95% BS): Vanilloideae (97% BS) are sister to a monophylum (58% BS) of Cypripedioideae (100% BS), Orchidoideae (100% BS), and Epidendroideae (84% BS), which form a ''lower'' grade and a ''higher'' clade (95% BS). Cypripedioideae is sister to clade (97% BS) that comprises Orchidoideae and Epidendroideae.

Analysis of Apostasioideae. The *mat*K data matrix for the narrower analysis comprised 1588 aligned positions, of which 1202 were constant, 218 uninformative, and 168 parsimony-informative (Table 2). An exhaustive search produced a single most parsimonious tree of 210 steps, a CI of 0.93 and a RI of 0.95. The ts/tr ratio was 1.47 (Table 3). Individual analysis of apostasioid *mat*K sequences revealed no internal stop codons. However, when aligned these sequences showed indels that were not in multiples of three bases. 1602 positions were aligned for *trn*L-F (Table 2), with 1364 constant characters, 161 uninformative, and 77 parsimony-informative (Table 2). The non-aligned *trn*L-F sequences showed remarkable length variation, ranging from 1253 bp in *Neuwiedia borneensis* to 931 bp in *Cypripedium calceolus*. Three equally

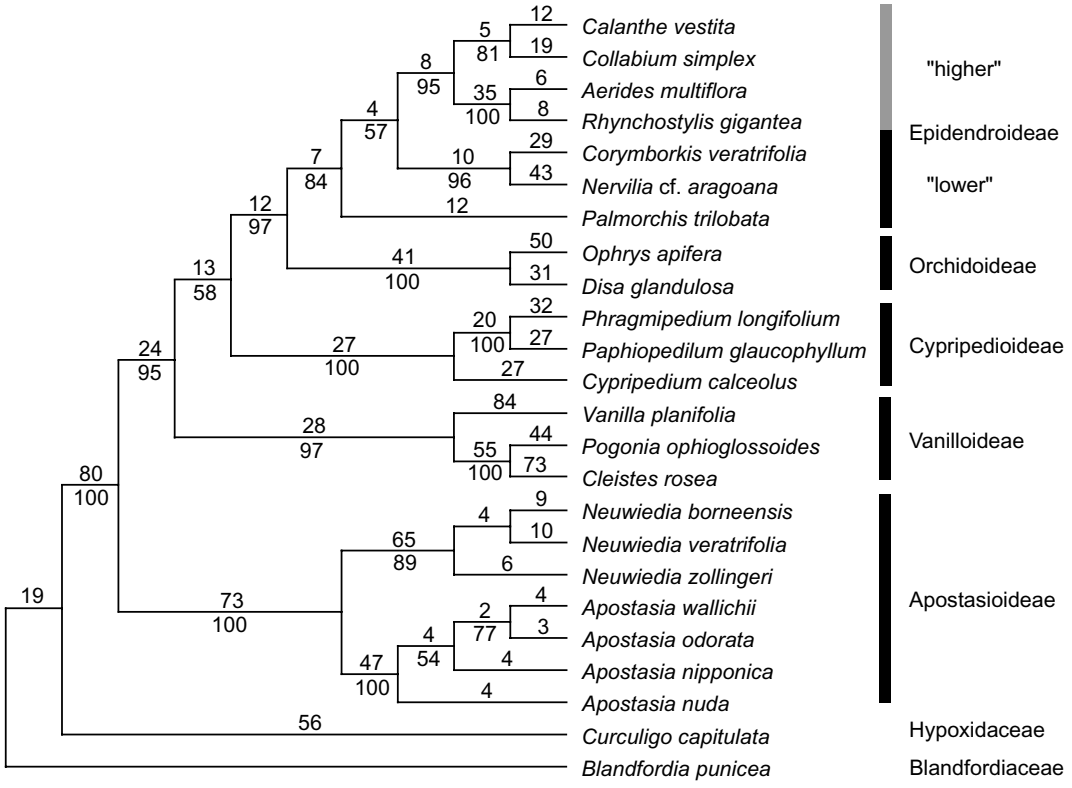

Fig. 1. The single most parsimonious tree from the *mat*K analysis: apostasioids form a monophyletic group with 100% BS support as a sister to the rest of Orchidaceae. Numbers above branches correspond to branch lengths; numbers below branches are bootstrap values; BS values below 50% are not shown

parsimonious trees were produced, with tree lengths of 105 steps, a CI of 0.87, and a RI of 0.91. The ts/tr ratio was 1.19 (Table 3). For ITS, a matrix of 1033 aligned positions was used for an exhaustive search under maximum parsimony. For *Apostasia nipponica*, only 537 bp (ITS 2), covering the 5.8S rDNA to the priming site in the 26S nrDNA region, were amplifiable. The ITS sequences yielded 504 constant, 206 uninformative, and 323 parsimony-informative characters (Table 2). ITS analysis resulted in two equally parsimonious trees with tree lengths of 571 steps, CIs of 0.88, and RIs of 0.90. The ts/tr ratio was 0.96 (Table 3).

The ILD tests indicated no homogeneity conflicts (P values ranging from 0.625 to 1); we therefore combined all three individual data sets into one data matrix. The total aligned data matrix consisted of 4223 positions, of which 3070 were constant, 585 uninformative, and 568 parsimony-informative (Table 2). The combined analysis revealed two equally most parsimonious trees of 887 steps, a CI of 0.89, and a RI of 0.91.

All data matrices, both separate and combined, produced trees with essentially the same topology (Fig. 2), except for the detailed relationships among *Apostasia nipponica*, *A. odorata* and *A. wallichii*, which collapsed to an unresolved polytomy in the *trn*L-F, ITS and combined strict consensus trees (see arrowheads in Fig. 2). In the single MP tree from *mat*K sequences, *A. nipponica* was sister to the clade formed by *A. wallichii* and *A. odorata*. *Apostasia* and *Neuwiedia* formed monophyletic sister groups with 100% bootstrap support in all trees. Within *Apostasia*, all trees supported *A. nuda* as sister to all other species representing the genus (BS ranging from 56% to 100%)

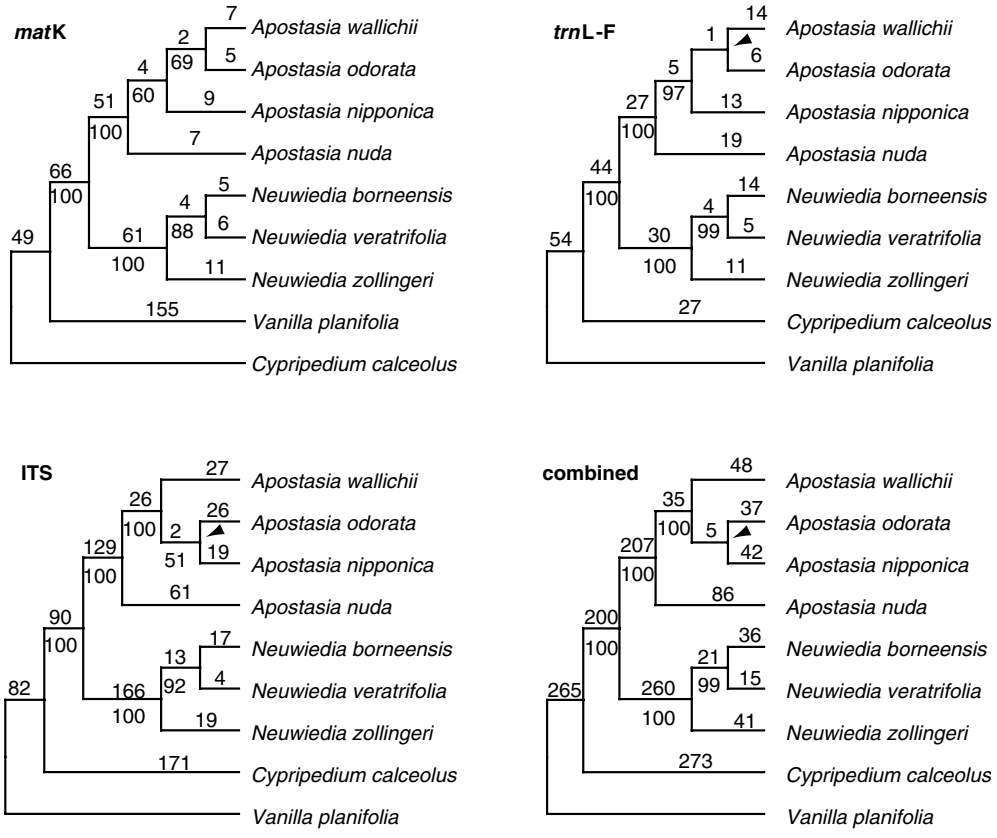

Fig. 2. One of the several most parsimonious (MP) trees of *mat*K (1 tree), *trn*L-F (3), ITS (2) and combined (2) analyses. Numbers above branches correspond to branch lengths; numbers below branches are bootstrap values. Nodes marked with an arrowhead collapse in the strict consensus trees

and within *Neuwiedia* all trees supported *N. zollingeri* as sister to *N. borneensis* and *N. veratrifolia* (BS ranging from 91% to 100%). The most parsimonious trees obtained from the *trn*L-F, ITS, and combined data sets as shown in Fig. 2 were selected to represent the highest topological similarity to the single most parsimonious *mat*K tree.

Discussion

Cladogenesis of Orchidaceae. The five main clades found in the broader analysis largely correspond to those reported in Cameron et al. (1999), Cameron and Chase (2000), and Freudenstein et al. (2000). Our cladogram is congruent with the trees published in previous studies (Neyland and Urbatsch 1995, Cameron and Chase 2000, Chase et al. 2003) in placing the vanilloids sister to the cypripedioids and the remaining monandrous orchids. However, other studies identified the cypripedioids as sister to all monandrous orchids (Cameron et al. 1999, Molvray et al. 2000). Most recently, Cameron (2003), Chase et al. (2003), and Freudenstein and Chase (2003) presented cladograms based on evidence from nine DNA regions resulting in the same tree topology supported in our study.

Cladogenesis of Apostasioideae. Our parsimony analyses strongly supported the monophyly of Apostasioideae and their sister-group relationship to the remaining orchids (also see Cameron et al. 1999, Cameron and Chase 2000, Freudenstein et al. 2000). The close relationship between *Apostasia* and *Neuwiedia* had already been suggested based on studies of early floral development in these two genera

(Kocyan and Endress 2001) and on the shared occurrence of vessels with simple perforation plates (Judd et al. 1993). Our phylogenetic results, however, contradicted the conclusion of Garay (1972), who found no close relationship between the two genera based on morphological observations. A separation into two subfamilies Neuwiedioideae and Apostasioideae as proposed by Burns-Balogh and Funk (1986) is not supported by our *mat*K analysis. In addition, a close relationship between Apostasioideae *sensu* Burns-Balogh and Funk (1986) and Cypripedioideae based on the shared occurrence of two stamens (Burns-Balogh and Funk 1986) is not supported by our *mat*K analysis. The genus *Apostasia* contains two sections (de Vogel 1969). Section *Apostasia* (*A. nipponica*, *A. odorata*, *A. wallichii*, *A. parvula*) is characterised by a staminode that is homologous to the median stamen of the outer androecial whorl of *Neuwiedia* and the only functional stamen of monandrous orchids. Section *Adactylus* (*Apostasia nuda*, *A. elliptica*, *A. latifolia*) differs from section *Apostasia* in lacking a staminode (de Vogel 1969). Our analysis supports the subdivision of the *Apostasia* into two sections *sensu* de Vogel (1969), as all sampled *Apostasia* species with a staminode (*A. nipponica*, *A. odorata*, *A. wallichii*) form a well supported clade sister to section *Adactylus* (*A. nuda*). In the light of these results and floral developmental studies (Kocyan and Endress 2001) the total loss of the staminode represents an apomorphy for section *Adactylus*; hence, the remaining staminode of section *Apostasia* is plesiomorphic. Within section *Apostasia*, only the *mat*K tree supports a clade consisting of *A. wallichii* and *A. odorata*, which is sister to *A. nipponica*; however, with relatively low bootstrap support. The *trn*L-F, ITS and combined data sets reveal no resolution among them.

In *Neuwiedia*, all trees strongly support *N. zollingeri* var. *javanica* as sister to the clade formed by *N. borneensis* and *N. veratrifolia*. This agrees in part with floral morphology. In *N. borneensis* and *N. veratrifolia* the stylar flanks are decurrent in the gynostemium, whereas this is not the case in *N. zollingeri*. However, other reproductive features unite *N. zollingeri* either with *N. veratrifolia* (relatively large flowers with the style not overtopping the stamens and enlarged filament tips vs. relatively small flowers with the style overtopping the stamens and not enlarged filament tips) or with *N. borneensis* (fleshy, berry-like fruits vs. thin-walled capsules) (de Vogel 1969, Kocyan and Endress 2001). Thus it is difficult to recognize synapomorphies. It should be emphasized that we can exclude the possibility that we have mislabeled our DNA samples, as we have extracted leaf material from different localities to test this.

Molecular evolution of *mat*K. DNA sequences with low ts/tv ratios are usually assumed to represent pseudogenes, because substitutions between purines and pyrimidines are less likely in a functional gene than substitutions within purines or pyrimidines (Graur and Li 2000). The occurrence of *mat*K pseudogenes has been reported in orchids (Kores et al. 2000, Goldman et al. 2001, Gravendeel et al. 2001, Whitten et al. 2001) and other plant families (e.g. Malpighiaceae; Cameron et al. 2001). However, the relatively high ts/tv ratio of 1.47 and the absence of frame shifts and resulting stop codons in the individual sequences suggest that *mat*K may not be a pseudogene in apostasioid orchids. The value is intermediate between those reported for *mat*K pseudogenes in orchids (1.02 for Arethuseae, Goldman et al. 2001; 0.66 for Maxillarieae, Whitten et al. 2001; 0.85 for *Coelogyne*, Gravendeel et al. 2001) and those reported for functional genes in orchids (1.89 in *rbc*L, Goldman et al. 2001) and other angiosperms (1.65 and 2.09 for *rbc*L and *atp*B, respectively, Savolainen et al. 2000). Therefore, it may be possible that *mat*K of apostasioid orchids is at the transition from a possibly functional gene to a pseudogene.

Conclusions. This study clearly shows that Apostasioideae form a clade that is sister to the remaining Orchidaceae. A similarly clear result is obtained on the relationship between the two sections of *Apostasia*: both sections are sister to each other. The loss of the staminode in

section *Adactylus* may then be an apomorphy whereas in section *Apostasia* the median stamen is the plesiomorphic state. However, within section *Apostasia* the relationship among taxa remains unresolved. Additional markers such as *psb*A-*trn*H and *atp*B-*rbc*L may give better insight in the evolution of these sections. In *Neuwiedia* all analyses yield the same topology, which contrasts with the topology one may expect observing morphological characters. Adding new taxa to the molecular dataset may be crucial for the understanding of *Neuwiedia* evolution.

The study of *mat*K gives an additional interesting result: *mat*K of Apostasioideae may be at the transition from a possibly functional gene to a pseudogene. As we have indications that *mat*K is a pseudogene in the remaining orchids, it would be interesting to know the *mat*K character of families closely related to orchids such as Asteliaceae or Hypoxidaceae.

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