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Monophyly and relationships of the tribe *Exaceae* (Gentianaceae) inferred from nuclear ribosomal and chloroplast DNA sequences

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Abstract

Both chloroplast *trn*L (UAA) intron and nuclear ribosomal ITS sequences highly confirmed the monophyly of the tribes of the Gentianaceae defined by the recent classification, and revealed the tribe *Exaceae* as a basal clade just next to the basal-most lineage, the tribe *Saccifolieae*. Within the tribe *Exaceae*, *Sebaea* (except *Sebaea madagascariensis*) appeared as the most basal clade as the sister group to the rest of the tribe. The Madagascan endemic genera *Gentianothamnus* and *Tachiadenus* were very closely related to each other, together standing as sister to a clade comprising *Sebaea madagascariensis*, *Ornichia*, and *Exacum*. The saprophytic genus *Cotylanthera* nested deeply inside *Exacum*. *Sebaea madagascariensis* was shown closer to the Madagascan endemic genus *Ornichia* than to any other sampled *Sebaea* species. *Exacum* appeared as the most derived taxon within this tribe. The topology of the phylogenetic trees conform with the Gondwana vicariance hypothesis regarding the biogeography of *Exaceae*. However, no evidence for matching the older relationships within the family to the tectonic history could be corroborated with various divergence time analyses. Divergence dating estimated a post-Gondwana diverging of the Gentianaceae about 50 million years ago (MYA), and the tribe *Exaceae* as about 40 MYA. The Mozambique Channel land-bridge could have played an important role in the biogeographic history of the tribe *Exaceae*.

1. Introduction

The Gentianaceae are a widespread family of 87 genera and about 1650 species (Struwe et al., 2002), that shows extensive morphological diversification and interesting distribution patterns. Phylogenetic studies on different infrafamilial lineages of the family using both molecular and non-molecular approaches have achieved important progress during the last decade (Adams, 1995; Albert and Struwe, 1997; Chassot et al., 2001; Chen et al., 2000a,b; Gielly and Taberlet, 1996; Gielly et al., 1996; Hagen and Kadereit, 2000, 2001; Ho and Liu, 1990; Ho et al., 1994; Ho and Pringle, 1995; Hungerer and Kadereit, 1998; Liu and Ho, 1992; Mészàros, 1994; Mészáros et al., 1996; Struwe and Albert, 1997, 1998a,b; Struwe et al., 1994, 1997, 1998, 2002; Thiv et al., 1999a,b; Yuan and Küpfer, 1995, 1997; Yuan et al., 1996). However, the only available comprehensive infrafamilial classification of the family was that of Gilg's established a century ago based mainly on pollen and gross morphology (Gilg, 1895), until the recent revision of the classification proposed by Struwe et al. (2002) based principally on the phylogeny inferred from chloroplast *trn*L (UAA) intron and *mat*K gene sequences. Our present studies were carried out to verify this new classification by using both nuclear and chloroplast DNA evidence, and further to evaluate the biogeography and phylogenetic relationships within the basal clade, the tribe *Exaceae*.

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In the new classification of Struwe et al. (2002), the Gentianaceae were divided into six tribes. The *Exaceae* represents a small tribe with six genera and 144–184 species: *Cotylanthera* Blume, *Exacum* L., *Gentianothamnus* Humbert, *Ornichia* Klack., *Sebaea* Sol. ex R. Br. and *Tachiadenus* Griseb. *Cotylanthera* has four achlorophyllous saprophytic species found from the eastern Himalayas to Southeast Asia. Gilg (1895) included this genus within his subtribe *Exacinae*. Klackenberg (2002) accepted the inclusion of the genus within tribe *Exaceae*, and further suggested a close relationship with *Exacum* or as a derived lineage inside *Exacum* based on floral morphology. The genera *Sebaea* (ca. 150 sp., Boutique, 1972) and *Exacum* (64 sp., Klackenberg, 1985; Thulin, 2001) make up the majority of species in the tribe *Exaceae*. *Sebaea* is mainly found in South Africa, although a few species are distributed in tropical Africa, Madagascar and Indo-Malaysian areas. The taxonomy of this genus is still confusing, and no updated taxonomic revision is yet available. *Exacum* has two main centres of diversity, Madagascar and the area including Southern India and Sri Lanka, with only a few species occurring on Socotra (and the Arabian Peninsula), in the Himalayas, the Southeast Asia, New Guinea, and in the extreme northern Australia. Klackenberg (1985) monographed the genus, and proposed a phylogeny based on morphological and anatomical characters. The remaining three genera of the tribe, *Gentianothamnus* (1 sp.), *Tachiadenus* (11 sp.), and *Ornichia* (3 sp.) are all endemic to Madagascar. The monotype *Gentianothamnus* was originally included in the subtribe *Chironiinae* by Humbert (1937). Klackenberg (2002) transferred it to *Exaceae* and suggested its close relationship with *Tachiadenus* based on morphological and anatomical characters. *Tachiadenus* was originally included in the subtribe *Tachiinae* by Gilg (1895). Klackenberg (1987) revised the genus and transferred it to *Exaceae*. *Ornichia* was recognized as a separate genus and placed in *Exaceae* by Klackenberg (1986) based on species previously included in *Chironia* and *Tachiadenus*.

In addition to taxonomic revisions, Klackenberg (1985, 1987) also performed manual cladistic analyses of morphological and anatomical characters on *Exacum* and *Tachiadenus*. Klackenberg (1985) further constructed an area cladogram of *Exacum* based on his species cladogram, and concluded that Africa and Madagascar were phytogeographically more closely related to each other than to any other area, and Socotra was more closely related to Madagascar than to India. He suggested that the African species might have originated from Madagascan species by dispersal, but that Indian species represented old relicts that originated from Madagascar by vicariance events. He further speculated that the Socotran species might have originated from Madagascan ancestors through vicariance events, and had no contact with Indian species. Obviously, identifying the correct species phylogeny is crucial in evaluating such considerations.

Only six species representing four genera of the tribe *Exaceae* had been subjected to a molecular phylogenetic study using *trn*L (UAA) intron sequences (Thiv et al., 1999a). Two genera, *Cotylanthera* and *Gentianothamnus*, had not yet been sampled. Although the monophyly of the tribe was supported with regard to the sampled genera, this insufficiently sampled analysis (with regards to *Exaceae*) could not offer strong support for relationships among the genera of the tribe. The inferred phylogenies, therefore, could not provide deep insights into the historical biogeography of the tribe. By sequencing both nuclear ribosomal internal transcribed spacers (ITS1 and ITS2) and the chloroplast *trn*L (UAA) intron, we conducted a more comprehensive molecular phylogenetic studies on this tribe by sampling all genera and additional species representing different clades and distribution areas. A detailed analysis on the genus *Exacum* is to be presented separately. Here we present our phylogenetic analysis on all genera of the tribe *Exaceae* together with representatives from all other clades of the family as recognized in the current classification. The following questions are to be addressed in particular: (1) Is the phylogeny based on nuclear ribosomal DNA sequences congruent with those based on chloroplast DNA sequences? (2) Is the tribe *Exaceae* as defined by current classification monophyletic? (3) What are the relationships of *Exaceae* to other tribes of the family? (4) What are the relationships among the genera of *Exaceae*? (5) What are the biogeographic implications of the molecular phylogenies?

2. Materials and methods

2.1. Ingroup sampling and outgroup choice

As Madagascar has the largest diversity of the tribe *Exaceae* at generic level, sampling of Madagascan species was maximized to cover as many species available to us as possible. Species representing all other tribes or other distribution areas were also sampled when available. The species included in this study and their voucher information are listed in Table 1. Those sequences retrieved directly from the GenBank database are indicated with asterisks following their accession numbers. A total of 60 operational taxonomic units (OTUs) were included in this study as ingroup taxa. Seven species representing all other families of the order Gentianales (APG, 1998; Backlund et al., 2000; Struwe et al., 1994) were included as outgroups. They were *Coffea arabica* L. and *Erithalis fruticosa* L. of Rubiaceae, *Labordia tinifolia* A. Gray and *Mitreola petiolata* (Walt.) Torr. and Gray of Loganiaceae, *Gelsemium sempervirens* Ait. of

Table 1
Origin of plant material, voucher information and EMBL/GenBank accession number of sequence

Taxon	Voucher	Origin	GenBank Accession No.			Reference
			ITS1	ITS2	*trn*L (UAA)	
Anthocleista amplexicaulis Baker	S. Wohlhauser PBZT	Tsimbazaza Botanical and Zoological Park, Antananarivo, Madagascar	AJ489863	AJ489863	AJ490189	Present study
Anthocleista grandiflora Gilg	M. Callmander s.n	Region of Maroantsetra, Madagascar	AJ489864	AJ489864	AJ490190	Present study
Canscora alata (Roth) Wallich	J.C. Piso, S. Wohlhauser and L. Zeltner M024	Mangindrano, Mahajanga, Madagascar	AJ489865	AJ489865	AJ490191	Present study
Canscora andrographioides Griff, ex C.B. Clarke	P. Chassot 99–234	Between Chiangdao and Chiang Mai Thailand	AJ489866	AJ489866	AJ490192	Present study
Canscora diffusa (Vahl) Roem. and Schult.	P. Chassot 99–231	Doi Inthanon, Chomtong, Thailand	AJ489867	AJ489867	AJ490193	Present study
Centaurium madrense B.L. Robinson	G. Mansion and L. Zeltner 990 124-1	23.27.15 N/105.49.91 W-Mexico	AY047742*	AY047827*	AF402166*	Mansion (to be published)
Centaurium spicatum (L.) Fritsch	L. Zeltner 1756	30.41.030 N/06.16.150 E-Morocco	AY047792*	AY402197*	AF402253*	Mansion (to be published)
Centaurium trichanthum B.L. Robinson	G. Mansion and L. Zeltner 960608-1	38.41.58 N/122.35.87 W-California, USA	AY047710*	AY047795*	AF402199*	Mansion (to be published)
Chelonanthus alatus (Aubl.) Pulle	J. Piguet C3	Near Zamora, Ecuador	AJ489868	AJ489868	AJ490194	Present study
Chelonanthus angustifolius Gilg	J. PiguetT11	Loja, Ecuador	AJ489869	AJ489869	AJ490195	Present study
Chelonanthus purpurascens (Aubl.) Struwe and V.A. Albert	J. Piguet C92	Sawaneh, Venezuela	AJ489870	AJ489870	AJ490196	Present study
Chironia baccifera L.	M. Callmander and M. Bondallaz, A005	Cape Town, Botanical Garden Kirstenbosch, Cape, South Africa	AJ489871	AJ489871	AJ490197	Present study
Chironia laxa Gilg	M. Callmander and M. Bondallaz A003	Cape Town, Botanical Garden Kirstenbosch, Cape, South Africa	AJ489872	AJ489872	AJ490198	Present study
Chironia linoides L.	M. Callmander and M. Bondallaz A004	Cape Town, Botanical Garden Kirstenbosch, Cape, South Africa	AJ489873	AJ489873	AJ490199	Present study
Cotylanthera paucisquama C.B. Clarks	Y.-M. YUAN CN2k1-31	Gongshan, Yunnan, China	AJ489874	AJ489874	NA	Present study
Curtia tenuifolia Knobl.	M.J. Jansen-Jacob 2740 (NY)	NA	AJ242613*	AJ242614*	AJ242606*	Thiv et al. (1999a)
Eustoma exaltatum (L.) Salisb. ex G.D.	S. Wohlhauser and C. Bijleveld BZ001	Sarteneja, Shipstem Nature Reserve, Orange Walk District, N18.17.94/ W88.13.05-Belize	AJ489875	AJ489875	AJ490200	Present study
Exaculum pusillum Caruel	L. Zeltner, s.n.	Italy	AJ489876	AJ489876	AJ490201	Present study
Exacum affine I.B. Balf ex Regel	Miller et al. 8238a	Nr Hadiboh, Sokotra, Yemen	AJ489877	AJ489877	AJ490202	Present study
Exacum caeruleum Balf. f.	Miller etal. 11356	Haggeher, Sokotra, Yemen	AJ489882	AJ489882	AJ490207	Present study
Exacum fruticosum Humbert	S. Wohlhauser and J.-I. Pfund M055	Marojejy R. I., Antsiranana, S14.26.17/E49.44.64-Madagascar	AJ489885	AJ489885	AJ490210	Present study
Exacum gracilipes I.B. Balf.	Miller et al. 17126	Haggeher, Sokotra, Yemen	AJ489886	AJ489886	AJ490211	Present study
Exacum hamiltonii G. Don	J.R.I. Wood 7477	Bhutan	AJ489887	AJ489887	AJ490212	Present study
Exacum marojejyense Humbert	S. Wohlhauser and J.-I. Pfund M056	Marojejy R.I., Antsiranana, S14.26.70/E49.44.17-Madagascar	AJ489893	AJ489893	AJ490218	Present study
Exacum nummularifolium Humbert	S. Wohlhauser and J.-I. Pfund MO58	Marojejy R.I., Antsiranana, S14.26.70/E49.44.17-Madagascar	AJ489896	AJ489896	AJ490221	Present study
Exacum oldenlandioides (S. Moore) Klack.	M. Reekmans 9275	Alt. 1150 m;S03.04/E29.25-Bubanza Burundi	AJ489897	AJ489897	AJ490222	Present study
Exacum quinquenervium Grisebach	S. Wohlhauser M063	Ankobahoba, Cote Est, Toamasin Madagascar	AJ489900	AJ489900	AJ490225	Present study

Exacum stenophyllum Klack.	J.C. Piso, S. Wohlhauser and L. Zeltner M049	Antsarasaotra, Fianarantsoa, Madagascar	AJ489902	AJ489902	AJ490227	Present study
Exacum tetragonum Roxb.	R. Lundin and J. Klackenberg 332	Chikmagalur distr., along road from Dattatreyapeetha to Ungadahalli, Karnataka, India	AJ489907	AJ489907	AJ490232	Present study
Exacum trinervium (L.) Drace	L. Zeltner SL002	Sri Lanka	AJ489909	AJ489909	AJ490234	Present study
Exacum wightianum Arn.	J. Klackenberg and R. Lundin 188	Tamil Nadu, India	AJ489913	AJ489913	AJ490238	Present study
Frasera speciosa Griseb.	Y.-M. Yuan 91–S2	Boulder, Colorado, USA	Z48146*	Z48124*	AJ315230*	Yuan and Küpfer (1995)
Gentiana algida Pall.	Y.-M. Yuan 91–S10	Trail Ridge, Rocky Mt., Colorado, USA	Z48142*	Z48117*	AJ490239	Yuan and Küpfer (1995); present study
Gentiana lutea L.	Y.-M. Yuan 91-S5	La Tourne, Neuchâtel, Switzerland	Z48122*	Z48119*	X75702*	Yuan and Küpfer (1995)
Gentiana pyrenaica L.	Y.-M. Yuan 93–14	Rila Mt., Borovetz, Bulgaria	Z48068*	Z48087*	X77895*	Yuan et al. (1996)
Gentianella umbellata (M. Bieb.) Holub	P. Küpfer 91–G3	Mt. Caucasus, Georgia	Z48102*	Z48132*	AJ315226*	Yuan and Küpfer (1995)
Gentianothamnus madagascariensis Humber	L. Gautier G020	Ansatrotro Manongrarivo R. S. 5 Mahajanga, Madagascar	AJ489914	AJ489914	AJ490240	Present study
Lomatogonium macranthum (Diels and Gilg) Fernald	Y.-M. Yuan 93–91	Ganzi, Sichuan, China	Z48108*	Z48135*	AJ315228	Yuan and Küpfer (1995)
Macrocarpaea macrophylla (Kunth) Gilg	Callejas and Balslev 1030	Antioquia, Colombia	AJ489915	AJ489915	AF102455*	Struwe et al. (1998) and unpublished
Megacodon stylophorus (C.B. Clarke) H. Smith	Y.-M. Yuan 93–182	Mt. Baima, Yunnan, China	Z48109*	Z48137*	AJ315200*	Yuan and Küpfer (1995)
Microrphium pubescens C.B. Clarke	P. Chassot 99–243	Khao Chang Lot, Phang Nga, Thailand	AJ489916	AJ489916	AJ490241	Present study
Ornichia madagascariensis Klack.	S. Wohlhauser M002	Ambavaniasy (Beforona), Toamasina, Madagascar	AJ489917	AJ489917	AJ490242	Present study
Ornichia trinervis (Desrousseaux) Klackenberg	M. Callmander s.n.	Near Mandeana, forest station, Southeast, Madagascar	AJ489918	AJ489918	AJ490243	Present study
Orphium frutescens E. Meyer	M. Callmander and M. Bondallaz A001	Cape Town, Botanical Garden Kirstenbosch, Cape, South Africa	AJ489919	AJ489919	AJ490244	Present study
Sabatia angularis (L.) Pursh	Lammers 4860	USA	AJ011467*	AJ011477*	AF102476*	Thiv et al. (1999b)
Saccifolium bandeirae Maguire and Pires	M. Piliackas et al., s.n.	NA	AJ242611*	AJ242612*	AJ242608*	Thiv et al. (1999a)
Sebaea brachyphylla Grisebach	J. Raynal 19414	Mt Meru, Kitoto, Arusha Nat. Park, Tsaratanaka, Madagascar	AJ489920	AJ489920	AJ490245	Present study
Sebaea exacoides L.	Snijman 1562	NA	NA	NA	AF102481*	Struwe et al. (1998)
Sebaea longicaulis Schniz	Poilecot P. 8000	Mont Ingangan, Nyanga, Zimbabwe	NA	NA	AJ490246	Present study
Sebaea cf. macrophylla Gilg	Bayliss 8765	NA	NA	NA	AF102482*	Struwe et al. (1998)
Sebaea madagascariensis Klack.	J.C. Piso, S. Wohlhauser and L. Zeltner M018	Miadana, Mahajanga, Madagascar	AJ489921	AJ489921	AJ490247	Present study
Swertia angustifolia Ham. ex D. Don	P. Chassot and Y.-M. Yuan 99–172	Binchuan, Yunnan, China	AJ318551*	AJ410330*	AJ315203*	Chassot et al. (2001)
Swertia calycina Franch.	Y.-M. Yuan and P. Küpfer 92–232	Yulong Snow Mt., Lijiang, Yunnan, China	AJ318554*	AJ410333*	AJ315206*	Chassot et al. (2001)
Swertia rosulata (Baker) Klack.	J.C. Piso, S. Wohlhauser and L. Zeltner M023	Tsaratanana, Tsaratanana R.I. Mahajanga, Madagascar	AJ489922	AJ489922	AJ490248	Present study
Swertia tetraptera Maxim.	Y.-M. Yuan and P. Küpfer 92–315	Maqu, Gansu, China	Z48115*	Z48139*	AJ315229*	Chassot et al. (2001); Yuan and Küpfer (1995)

Table 1 (*continued*)

Taxon	Voucher	Origin	GenBank Accession No.			Reference
			ITS1	ITS2	*trn*L (UAA)	
Symphyllophyton caprifoioides Gilg	Ratter 6742	Brasil	AJ011462*	AJ011472*	AF102490*	Thiv et al. (1999b)
Tachiadenus carinatus Desrousseaux	S. Wohlhauser M059	Ambila-Lemaitso, Toamasina, Madagascar	AJ489923	AJ489923	AJ490249	Present study
Tachiadenus longiflorus Bojer ex Grisebach	S. Wohlhauser M006	Stars, Antananarivo, Madagascar	AJ489924	AJ489924	AJ490250	Present study
Voyriella parviflora (Miq.) Miq.	G. Cremers 14891	NA	AJ242615*	AJ242616*	AJ242607*	Thiv et al. (1999a); unpublished
Coffea arabica L.	NA	NA	AJ224846*	AJ224846*	AF102405*	Andreasen et al. (1999); Struwe et al. (1998)
Erithalis fruticosa L.	NA	NA	NA	NA	AF152697*	Rova et al. (2002)
Labordia tinifolia A. Gray	NA	NA	NA	NA	AF102447*	Struwe et al. (1998)
Mitreola petiolata (Walt.) Torr. and Gray	NA	NA	AF054635*	AF054635*	AF102460*	Gould (unpublished); Struwe et al. (1998)
Gelsemium sempervirens Ait.	NA	NA	NA	NA	AF102428*	Struwe et al. (1998)
Nerium oleander L.	NA	NA	NA	NA	AF214386*	Potgieter and Albert (unpublished)
Trachelospermum jasminoides Lem.	NA	NA	NA	NA	AF214439*	Potgieter and Albert (unpublished)

Sequences retrieved from GenBank are marked with an asterisk. NA, not available.

Gelsemiaceae, and *Nerium oleander* L. and *Trachelospermum jasminoides* Lem. of Apocynaceae. These outgroups were used for analyses on *trn*L (UAA) intron alone and for combined analyses, as the *trn*L (UAA) intron sequences of outgroups could be unambiguously aligned with the ingroup taxa. No outgroup was used for analyses on ITS data alone, as ITS sequences of outgroups could not be confidently aligned (see below).

2.2. DNA extraction and PCR amplification

DNA was extracted from silica gel dried leaf material (Chase and Hills, 1991), or from leaf tissue taken from herbarium sheets. Total DNA was extracted using the CTAB procedure of Doyle and Doyle (1987), or the DNeasy Plant Mini Kit (Qiagen AG, Basel). The leaf tissue was homogenized in the presence of liquid nitrogen or with a Qiagen Mixer Mill MM 300 (Qiagen AG, Basel). Both DNA fragments, ITS and *trn*L (UAA) intron, were amplified via standard PCR in 25 μl volume containing 2.5 μl 10× PCR buffer (with 1.5 mM $MgCl_2$), 0.5 μl 10 mM dNTPs, 0.5 μl of 10mM each forward and reverse primers, 0.2 μl (1U) HotStar Taq DNA polymerase (Qiagen AG, Basel), 19.8 μl H_2O, and 1 μl (ca. 10–20 ng) genomic DNA. PCRs were performed in a Biometra thermal cycler programmed for 15 min at 95 °C for the activation of the HotStar Taq DNA polymerase, followed by 35 cycles of 45 s at 94 °C, 45 s at 55 °C, 1 min at 72 °C, with a final extension period of 5 min at 72 °C. Primers ITS5 and ITS4 (White et al., 1990) were used for the amplification of ITS of the nuclear ribosomal DNA for freshly dried samples. For herbarium samples a newly designed primer, ITSPAN, by Y.-M. Yuan, was used to replace the ITS5 for better amplification. The sequence of the ITSPAN primer is 5′-TCCGGTGAAGTGTTCGGATCGC-3′. It is located about 63 bp upstream of the original ITS5, and generally gave better amplification of the ITS region when used in combination with ITS4 for the samples for which PCR amplifications failed with the primers ITS5 and ITS4. The primers "c" and "d" of Taberlet et al. (1991) were used for the amplification of the *trn*L (UAA) intron.

2.3. PCR purification and sequencing

The PCR products were checked on 0.8% agarose gels. Successfully amplified DNA fragments were then purified prior to sequencing using the QIAquick PCR purification kit (Qiagen AG, Basel) following the manufacturer's protocol. Cycle sequencing reactions were performed using the dye-terminator chemistry as implemented in the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, USA) in a Biometra thermal cycler. The same primers used for amplification were used for sequencing for both ITS and *trn*L (UAA) intron, whereas the two more internal primers ITS2 and ITS3 (White et al., 1990) were occasionally used to confirm some sequencing results for ITS. Protocols and cycling parameters suggested by the sequencing kit were followed except that the reaction volumes were scaled down to 5 μl. The cycle sequencing products were cleaned using the ethanol/sodium acetate precipitation method as suggested by the manufacturer of the sequencing kit. The purified sequencing products were resuspended in 12 μl TSR (supplied by Applied Biosystems, Foster City, USA) and then analyzed on an ABI310 automated sequencer using a 47 cm capillary and polymer POP-6™ (Applied Biosystem, Foster City, USA). Automation-generated base-calls were subsequently checked manually against the electropherograms using the software Sequence Navigator (Applied Biosystems, Foster City, USA).

2.4. Sequence alignment

The boundaries of ITS1, ITS2, and the *trn*L (UAA) intron in the studied material were determined by comparison with previous results (Gielly et al., 1996; Yuan and Küpfer, 1995). Combined sequences of ITS1, ITS2, and *trn*L (UAA) intron were preliminarily aligned with Clustal X (Thompson et al., 1997) and then manually adjusted to reflect indel events. The alignment of *trn*L (UAA) intron sequences was straightforward, and required minor manual adjustments. Potentially informative and unambiguously assessable indels in *trn*L (UAA) intron were scored as binary characters (1 for insertion, 0 for deletion) regardless of their length and were added to the data matrix (which gave 21 additional characters). The alignment of ITS sequences was problematic, so various alignment alternatives were explored to investigate the alignment consequence on phylogenetic inference. The multiple alignments were explored in two ways, taxon deletion and gap penalty alteration. Firstly, using the default penalties of Clustal X, several rounds of alignments were conducted and at each round a set of divergent taxa were removed from the data to be aligned. In this way, outgroups and too divergent ingroup taxa (tribe *Saccifolieae*, the genera *Cotylanthera, Exaculum*, *Gentiana*, *Lomatogonium*, and *Symphyllophyton*) were stepwise excluded from alignment to generate a set of reduced data matrixes. Comparison of phylogenetic analyses on this battery of alignments indicated that tree topologies started to be stabilised when outgroups and the tribe *Saccifolieae* were excluded. The reduced data set (excluding outgroups and the tribe *Saccifolieae*) was then subjected to the second round of alignment test, applying varied gap penalties for Clustal X. No significant influence on tree topologies was observed as long as the ambiguous alignment areas were excluded (data not shown). Thus the alignment of the reduced data set where outgroups and the tribe

Saccifolieae were excluded was chosen and further slightly adjusted manually, and was used for subsequent phylogenetic analyses. The indels of ITS sequences were not used as separate characters as their boundaries could not be unambiguously determined. To ensure the alignment accuracy, an alignment of the ITS and *trn*L intron sequences of the tribe *Exaceae* alone was also conducted and analyzed separately as a comparison with the broad analyses.

2.5. Phylogenetic analysis

All data sets were analyzed with heuristic searches applying the maximum parsimony optimality criterion, using PAUP* v4.0b8a or v4.0b10 (Swofford, 2000). Analyses were performed on *trn*L (UAA) intron alone, on ITS alone, and on combined ITS and *trn*L (UAA) intron. In all analyses characters were equally weighted and unordered (Fitch, 1971), with gaps treated as missing data.

For the analyses on *trn*L (UAA) intron alone, the species *Cotylanthera paucisquama* was removed as its *trn*L (UAA) data was missing. A three-step search procedure was performed with branch collapse option set to collapse if maximum length as zero. The first step, a heuristic search to save maximum of 5000 shortest trees (Maxtree = 5000), was made to obtain an empirical minimum tree length, which generated the minimum tree length of 457 steps. The second step involved 1000 replicates of random stepwise addition of sequences, and from each replicate only one tree not longer than 457 steps was saved, giving a total of 1000 trees. Finally TBR branch swapping was performed on all these trees with MULTREES and STEEPEST DESCENT options on, and MAXTREE set to 10,000, 50,000, or 100,000, respectively. The strict consensus tree from each set of these trees was compared, and was found identical in topology. However, when branch collapse option was set to collapse if minimum length as zero, heuristic searches of 1000 replicates of random addition of sequences, with TBR branch swapping, MULTREES and ACCTRAN options on, and STEEPEST DESCENT option off, generated only six trees. Their strict consensus was identical to those obtained by analyses described above. All the seven outgroup taxa were included in all these analyses.

All heuristic searches on ITS data alone or on combined data implemented 100 replicates of random addition of sequences, with TBR branch swapping, MULTREES and ACCTRAN options on, and STEEPEST DESCENT option off. Both the 'maximum' and 'minimum' branch collapse options were compared. No difference was found in topology of the strict consensus trees.

For all the results based on separate and combined data, the relative support for individual clades was evaluated by bootstrap analyses (Felsenstein, 1985). Bootstrap values were calculated using 1000 replicates of heuristic searches, with SIMPLE ADDITION SEQUENCE, TBR branch swapping, MULTREES option on, STEEPEST DESCENT option off, and a maximum of 1000 trees saved for each replicate.

2.6. Data congruence test

In order to test the congruence of ITS and *trn*L (UAA) intron data sets, we conducted the incongruence length difference test of Farris et al. (1995). The partition ITS *vs.* *trn*L (UAA) intron homogeneity tests as implemented in PAUP were performed with 1000 replicates of heuristic searches (Maxtree = 1000, TBR branch swapping, and other default settings) on the combined data matrix.

2.7. Molecular clock test

To test the assumption of clock-like evolution of the DNA sequence under study, the likelihoods of all the equally maximum parsimonious trees (obtained with branch collapse option set to minimum) were calculated separately with and without molecular clock constraints on data sets where all gapped, ambiguity and missing sites were excluded using PAUP. *C. paucisquama* was excluded from these analyses as an accelerated evolution was apparent from the phylogram (Fig. 3). The GTR + I + G model (GTR, general time reversible; I, variable proportions of invariable sites; G, gamma distributed among-site rate variation) and parameter settings (gamma shape, base frequencies) were chosen through the Hierarchical Likelihood Ratio Tests procedure as implemented in Modeltest (Version 3.06) (Posada and Crandall, 1998) for the corresponding data sets were used. Using a χ^2 distribution with $N-2$ degrees of freedom, where N is the number of terminal taxa in the trees, a likelihood ratio test was performed based on twice the difference between the log-likelihoods for clock and no-clock analyses (Muse and Weir, 1992).

2.8. Divergence time calculations

To obtain an approximate dating of branching events, specifically the divergence of the family Gentianaceae and the tribe *Exaceae*, the times of divergence were estimated for highly supported sister groups. When a molecular clock is accepted by the likelihood ratio test, the pairwise sequence divergence values between two sister groups were determined as the average of all pairwise sequence divergence values between species from the two clades. The average sequence divergence values were calculated from the uncorrected mean pairwise distance to accommodate the divergence rate previously calibrated (Richardson et al., 2001a). The

highest previously reported divergence rate 1.30×10^{-9} substitutions per site per year (s/s/y) and the lowest divergence rate 4.87×10^{-10} s/s/y for *trn*L (UAA) intron as summarised by Richardson et al. (2001a) was used. Divergence time between two lineages was estimated as half of the average divergence value between them divided by the divergence rate.

In cases where the likelihood ratio test rejected a molecular clock, maximum parsimony trees based on *trn*L intron were subjected to non-parametric rate smoothening (NPRS) (Sanderson, 1997) using the default settings in TreeEdit v.1.0a8 (Rambaut and Charleston, 2000; Richardson et al., 2001a) to obtain homogenized rates. To calibrate the trees, the minimum age of Gentianales was estimated to be 60 million years (MY) based on pollen fossil data (Muller, 1984). As an independent test for the divergence times based on rate smoothening, the branch-specific rate dating method based on the mean branch length (MBL) as implemented in Bremer (2000) and Patterson and Givnish (2002) was used to obtain estimations of the divergence times using the same calibration point. The six equally most parsimonious trees generated by heuristic searches using 'minimum as zero' branch collapse option, were examined. Analyses without the fast evolving taxa (*Curtia*, *Saccifolium*, and *Voyriella*), where only a single most parsimonious tree was obtained using the same options, were also performed to investigate their effect on the results of rate smoothening and divergence times. Branch lengths were obtained using accelerated transformation optimisation. The delayed transformation optimisation was also tested but did not cause any significant change on final results (data not shown). In all cases, the trees were rooted at the branch connecting Rubiaceae and the other outgroup taxa. No detailed dating was attempted on ITS trees, as ITS sequences of the outgroups and the tribe Saccifolieae could not be unambiguously aligned with other ingroup taxa.

3. Results

3.1. Sequence characteristics and alignments

All newly obtained sequences have been submitted to EMBL/GenBank databases. The accession numbers of the sequences used in this study are listed in Table 1. The 164 base pairs (bp) 5.8S rDNA regions have few mutations and were removed from analyses, because this part is missing in the sequences retrieved from GenBank database.

Despite of our various efforts, we were not able to amplify the *trn*L (UAA) intron and many other cpDNA fragments, such as the *rbc*L gene, from the saprophytic species *C. paucisquama*. As this plant is completely white, it may have few or degenerate plastids and its cpDNA could have undergone dramatic variation so the available primers were not able to amplify the corresponding regions any more. This species is thus omitted from the analysis of the *trn*L (UAA) intron data set, but included for combined analyses with its *trn*L (UAA) intron simply treated as missing data.

The length of unaligned *trn*L (UAA) intron sequences of the remaining OTUs ranged from 343 to 539 bp. The aligned *trn*L (UAA) intron had 636 bp. The alignment was straightforward and unambiguous except for two simple sequence repeat (SSR) regions of multiple As (13 and 72 bp, respectively in our aligned data matrix). These ambiguously alignable SSR regions (85 bp in total) were excluded in subsequent analyses. Introduced gaps were from 1 to 111 bp in length. In total, 21 potentially informative indels were scored as binary characters regardless of their sizes and were added to the sequence data. Finally the *trn*L (UAA) intron matrix had 657 characters, of which 85 (12.9%) were excluded, 310 (47.2%) were constant, 86 (13.1%) variable but uninformative, and 176 (26.8%) informative. These data resulted in uncorrected pairwise sequence divergence ranged from 0 (*Chironia baccifera* vs. *C. linoides*, *Exacum marojejyense* vs. *Exacum fruticosum*, *Canscora alata* vs. *Canscora andrographioides*, and *Sebaea brachyphylla* vs. *S. exacoides*) to 18% (*Curtia tenuifolia* vs. *Sebaea macrophylla*) among the Gentianaceae ingroup taxa.

The length of unaligned ITS1 and ITS2 sequences varied from 212 to 237 bp and from 192 to 248 bp, respectively. The alignment of the ITS region was difficult, particularly between outgroups and a region nearby the beginning of ITS2. This region of 13–52 bp was always differently aligned among our different alignment exercises, and seems to correspond with the hypervariable terminal loop region of the arm 1 in secondary structure models as revealed in *Aeschynanthus* of Gesneriaceae (Denduangboripant and Cronk, 2001). Tests of multiple alignments through stepwise exclusion of divergent taxa (see method) indicated that ITS sequences of the outgroups and the tribe *Saccifolieae* of ingroup taxa were too divergent to be confidently aligned with other ingroup taxa. The inclusions of these sequences dramatically influence phylogenetic inference of ITS sequences, particularly regarding the relationships of the basal clades (caused by both ambiguous alignment and long-branch attraction). When these sequences were excluded from alignment, the topologies of inferred trees were stabilised and further exclusion of other suspected divergent taxa such as *Cotylanthera*, *Exaculum*, *Gentiana*, *Symphyllophyton*, etc. did not alter inferred tree topologies as long as the ambiguously aligned indel regions were excluded from analyses.

The reduced ITS data matrix contained 534 characters, of which 145 (27.2%) were excluded from phylogenetic analyses, 95 (17.8%) were constant, 78 (14.6%) variable but uninformative, and 216 (40.4%) potentially

informative. These data resulted in uncorrected pairwise sequence divergence ranged from 0 (*Exacum marojejyense* vs. *Exacum fruticosum*) to 43.4% (*C. paucisquama* vs. *Canscora diffusa*). The extraordinarily high divergence value was due to the saprophytic species *C. paucisquama*, which appears to have an accelerated rate of evolution (Fig. 3). The data matrix based on the alignment covering all OTUs can be consulted or downloaded from the web pages of the Laboratory of Evolutionary Botany of the University of Neuchâtel (http://www.unine.ch/bota/ebolab/gentianaceae/gent-main.html).

When the ITS alignment was limited to the tribe *Exaceae*, the saprophytic species, *C. paucisquama*, caused alignment ambiguity and long-branch attraction in subsequent phylogenetic inference. This by-effect could be easily corrected by combining *trn*L intron data of the corresponding species in the phylogenetic analyses (with the *trn*L intron sequence of *C. paucisquama* coded as missing).

3.2. trnL intron phylogeny

Heuristic searches on the *trn*L (UAA) intron data set resulted in over 100,000 equally most parsimonious trees when branch collapse was set to 'maximum length as zero' or only six equally most parsimonious trees when branch collapse was set to 'minimum length as zero.' All these maximum parsimonious trees had 457 steps, with a consistency index (CI) of 0.74 including autapomorphies (0.68 excluding autapomorphies), and a retention index of (RI) 0.89. The strict consensus of these two sets is identical, and is shown in Fig. 1A. Bootstrap values for the clades supports (when greater than 50%) are also shown. The *trn*L (UAA) intron consensus tree was well resolved and highly supported toward the base of the tree, while the upper branches (among closely related genera or species from the same genera) were poorly resolved or received less significant support. The monophyly of most tribes and subtribes was highly supported (72–100%), except for tribe *Gentianeae* (55%) and subtribe *Chironiineae* (57%) the bootstrap supports were slightly weak. The tribe *Saccifolieae* was resolved as the most basal clade of the family Gentianaceae with high support (100%), followed by the tribe *Exaceae* (99%) and tribe *Chironieae* (89%). The remaining three tribes formed an unresolved trichotomy. Despite the high support for the monophyly of the tribe *Exaceae*, the *trn*L (UAA) intron provided no resolution on the relationship between *Exacum* and *Ornichia* (and *Sebaea madagascariensis*), and the relationships among the species within *Exacum*. The sampled species of *Sebaea*, excluding *S. madagascariensis*, formed a highly supported basal clade (100%), sister to all the remaining genera of the tribe. *Gentianothamnus* was on a polytomy together with *Tachiadenus* (96%). Together they were sister to *Exacum*, *Ornichia*, and *S. madagascariensis*, residing unresolved in a polytomy.

3.3. ITS phylogeny

Maximum parsimony analyses on alternative alignments which include both outgroups and ingroup taxa, with outgroup taxa excluded, or with both outgroup taxa and the tribe *Saccifolieae* of ingroup excluded, resulted in varied resolutions on basal relationships among the main clades (not shown). This variation was caused by alignment ambiguity and long-branch attraction introduced by the outgroups and too divergent ingroup taxa, the tribe *Saccifolieae*. When outgroups and the tribe *Saccifolieae* were excluded, alternative alignments (obtained by further excluding other divergent taxa or changing alignment penalties) gave consistent phylogenetic resolution that was also congruent with the topologies inferred from *trn*L intron data. Heuristic search on the reduced ITS data (containing 54 taxa) generated only two trees of 1233 steps (CI = 0.45 including autapomorphies, CI = 0.40 excluding autapomorphies, RI = 0.66). The strict consensus tree and bootstrap values for clade support when higher than 50% are shown in Fig. 1B. Contrary to the trees obtained from the *trn*L (UAA) intron data set, the upper branches (among closely related genera or species from the same genera) of the ITS trees were better resolved and received significant bootstrap support, while the corresponding main clades and the relationships among them were revealed identical to that obtained from *trn*L intron data. The saprophytic *C. paucisquama*, which was missing from *trn*L tree, nested within the genus *Exacum* and grouped with two Himalayan species, *E. hamiltonii* and *E. tetragonum*. Confirming the results based on *trn*L intron data, the genus *Sebaea* was shown polyphyletic. One species, *S. brachyphylla*, was revealed as the most basal clade within the tribe. Another species *S. madagascariensis* nested with the genus *Ornichia*, the same position suggested by the *trn*L intron data. ITS trees had higher resolutions than the *trn*L trees for the tribe *Exaceae*. The Madagascar endemic genus *Tachiadenus* was revealed to be monophyletic, and the monotypic genus *Gentianothamnus* was closely related to it. Provided *C. paucisquama* is included, *Exacum* was monophyletic with two main clades, an African-Madagascan clade and a Socotra-Asiatic clade, the later included three lineages, South Indian-Sri Lankan, the Himalayan, and the Socotran.

3.4. Data sets conflict test and combined ITS and trnL (UAA) phylogeny

Trees resulted from *trn*L (UAA) and ITS data sets did not show any conflict in topology when examined visually. The partition homogeneity test on *trn*L (UAA)

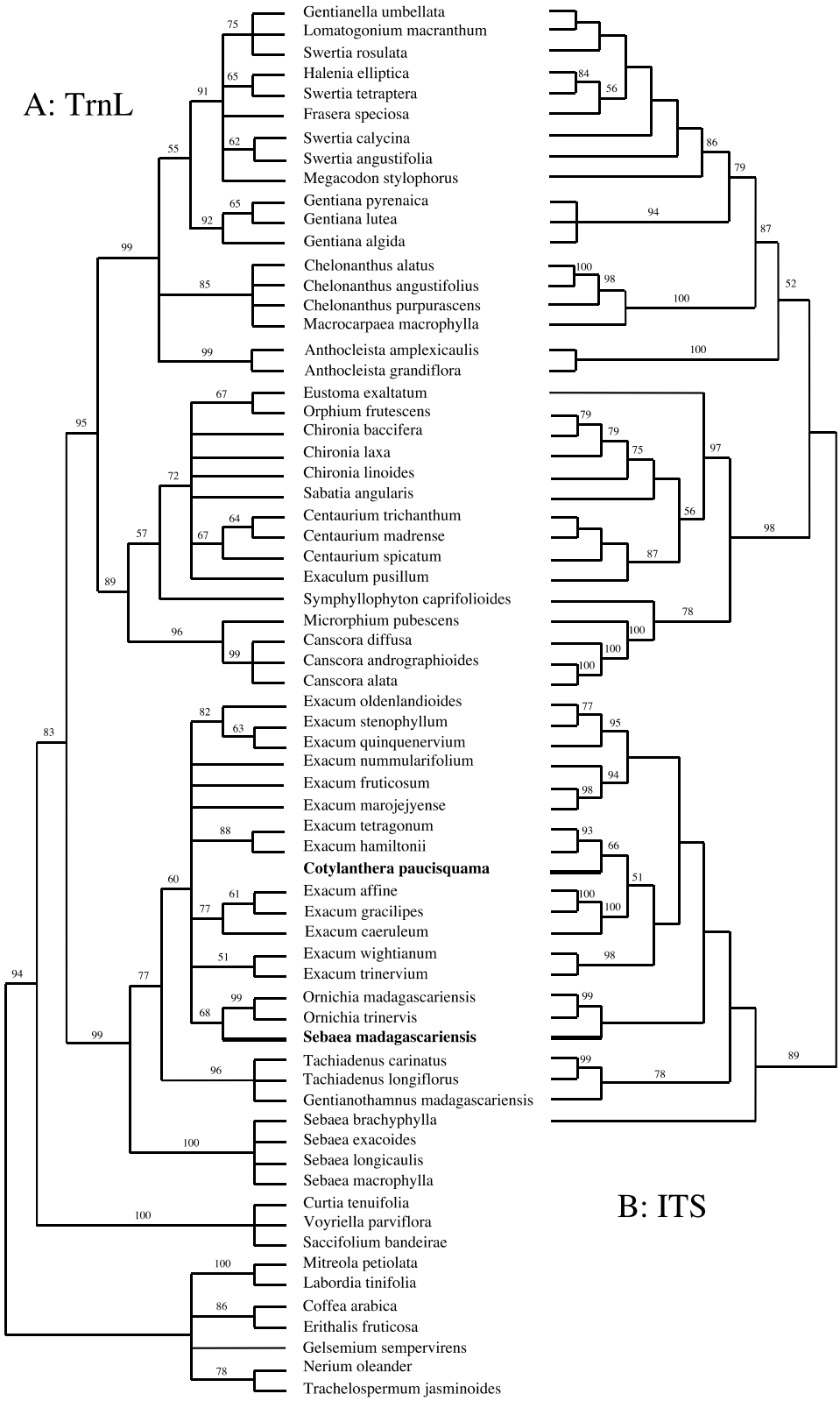

Fig. 1. Comparison of strict consensus trees obtained from separate analyses of *trn*L (UAA) intron and ITS sequence alone. Numbers above the branches are bootstrap values supporting the corresponding branch when greater than 50%. (A) The strict consensus tree of the equally parsimonious trees obtained from *trn*L intron (length = 457, CI = 0.74 including autapomorphies, CI = 0.68 excluding autapomorphies, RI = 0.89). (B) The strict consensus tree of the two equally parsimonious trees obtained from reduced ITS data matrix alone (length = 1233, CI = 0.45 including autapomorphies, CI = 0.40 excluding autapomorphies, RI = 0.66). Bold face indicates the unexpected resolutions (see text).

intron vs. ITS received significant statistic support with $P = 0.99/1.0$ (without/with the taxon for which only *trn*L intron or ITS was available). Therefore both data sets were combined and were subjected to a combined analyses. The missing or excluded data, *trn*L intron of *C. paucisquama* and ITS sequences of outgroups and the tribe *Saccifoileae*, were coded as missing data. Such combined data matrix covered 67 taxa (including the seven outgroup species) and 1191 characters, of which 230 (19.3%) were excluded from analyses, 405 (34.0%) were constant, 164 (13.8%) were variable but uninformative, and 392 (32.9%) potentially informative. 150 equally most parsimonious trees with 1694 steps were generated (CI = 0.53 including autapomorphies and 0.46 excluding autapomorphies, RI = 0.74). The strict consensus of these trees is shown in Fig. 2. The consensus tree is well resolved and its topology is congruent with that generated from separated analyses on *trn*L (UAA) intron and ITS data alone. The monophyly of all currently recognized tribes and subtribes were strongly supported. The tribe *Saccifolieae* was resolved as the most basal clade within the Gentianaceae, followed by the tribe *Exaceae* as sister to the rest of the family. Tribe *Chironieae* was in turn sister to tribes *Potalieae*, *Helieae*, and *Gentianeae*. Tribe *Helieae* showed the closest relationship with tribe *Gentianeae*, being the most derived taxa of the family.

Except for the monotypic genus *Gentianothamnus* and the genera with only one species sampled for which the present phylogeny did not offer any evaluation on their monophyly, most of the genera were revealed to be monophyletic. The only exceptions were that paraphyly was revealed for *Exacum*, *Swertia*, and *Sebaea*. The paraphyly of *Swertia* has been extensively studied by Chassot et al. (2001). The Madagascan *S. rosulata* was revealed here as derived and close to *Gentianella* and *Lomatogonium* in the *Gentianella* lineage in the tribe *Gentianeae*. Within the tribe *Exaceae*, the saprophytic genus, *Cotylanthera*, was revealed as deeply nested inside the genus *Exacum*, thus the recognition of *Cotylanthera* as a distinct genus made the genus *Exacum* paraphyletic. Tests did not reveal long-branch attraction caused by *Cotylanthera* in this combined analysis. The genus *Sebaea* was also shown to be polyphyletic. While most of the sampled species grouped together as a highly supported basal clade sister to all the remaining taxa of the tribe, the Madagascan endemic species *S. madagascariensis* showed a closer relationship with the genus *Ornichia*. This similarity has been confirmed by sequences obtained from the DNAs extracted from three different individual accessions, thus the possibility of DNA sample contamination could be excluded. As far as the sampled species were concerned, *Ornichia* and *Tachiadenus* were revealed as highly supported monophyletic genera. The close relationship between the two Madagascan endemic genera *Gentianothamnus* and *Tachiadenus* was highly supported. *Ornichia* and *S. madagascariensis* together showed a close relationship with the genus *Exacum* that was revealed as the most derived taxon within the tribe *Exaceae*. *Exacum* bifurcated into two clades at the base, the Madagascar-African clade and the Asian clade. The Asian clade included three highly supported lineages as revealed by ITS data alone (Fig. 2).

Phylogenetic analyses on alignments of combined *trn*L intron and ITS sequences limited to the tribe *Exaceae* confirmed the above results. 13 equally most parsimonious trees with 629 steps were generated (CI = 0.72 including autapomorphies and 0.61 excluding autapomorphies, RI = 0.70). The topology of the strict consensus tree (not shown) was identical to that of the *Exaceae* clade of the strict consensus tree generated from the broad combined analysis including all other tribes (Fig. 2). The saprophytic *C. paucisquama* showed an accelerated evolution, as indicated by its extraordinary long branch in the phylogram of one of the 13 trees (Fig. 3). Phylogenetic analyses using only ITS data clearly showed long-branch attraction of *C. paucisquama*: tree topology changed when this species was in- or excluded. Combining the *trn*L intron data could easily correct the long-branch attraction.

3.5. Molecular clock test and estimations of divergence time

The likelihood ratio test rejected a clock-like evolutionary rate for all ITS data sets ($p < 0.05$). While a molecular clock was rejected globally for *trn*L (UAA) intron data for the whole Gentianaceae, a molecular clock could not be rejected for *trn*L (UAA) intron data of the tribe *Exaceae* alone ($p > 0.05$). We have not identified a reliable calibration for the clock of *trn*L intron of the tribe *Exaceae*, so the lowest rate 4.87×10^{-10} substitution/site/year (s/s/y) and the highest rate 1.3×10^{-9} s/s/y previously reported for *trn*L (UAA) intron (Richardson et al., 2001a) were used to estimate the divergence times of the two basal nodes of the tribe, which were supported by both ITS and *trn*L data when analyzed separately or combined. The inferred divergence times are plotted on one of the phylogenetic trees of the tribe *Exaceae* obtained from combined analyses (Fig. 3), and are also given in Table 2. According to these rates and the *trn*L intron sequence divergence, the genus *Sebaea*, the most basal clade of the tribe, has diverged from others for about 20.3–54.3 MY. The divergence between the *Gentianothamnus–Tachiadenus* lineage and the lineage including *Sebaea madagascariensis*, *Ornichia*, and *Exacum* was 11.2–29.8 MY.

The results of divergence dating on *trn*L trees based on the clock-independent NPRS and MBL methods and the calibration point of 60 MY for the minimum age of

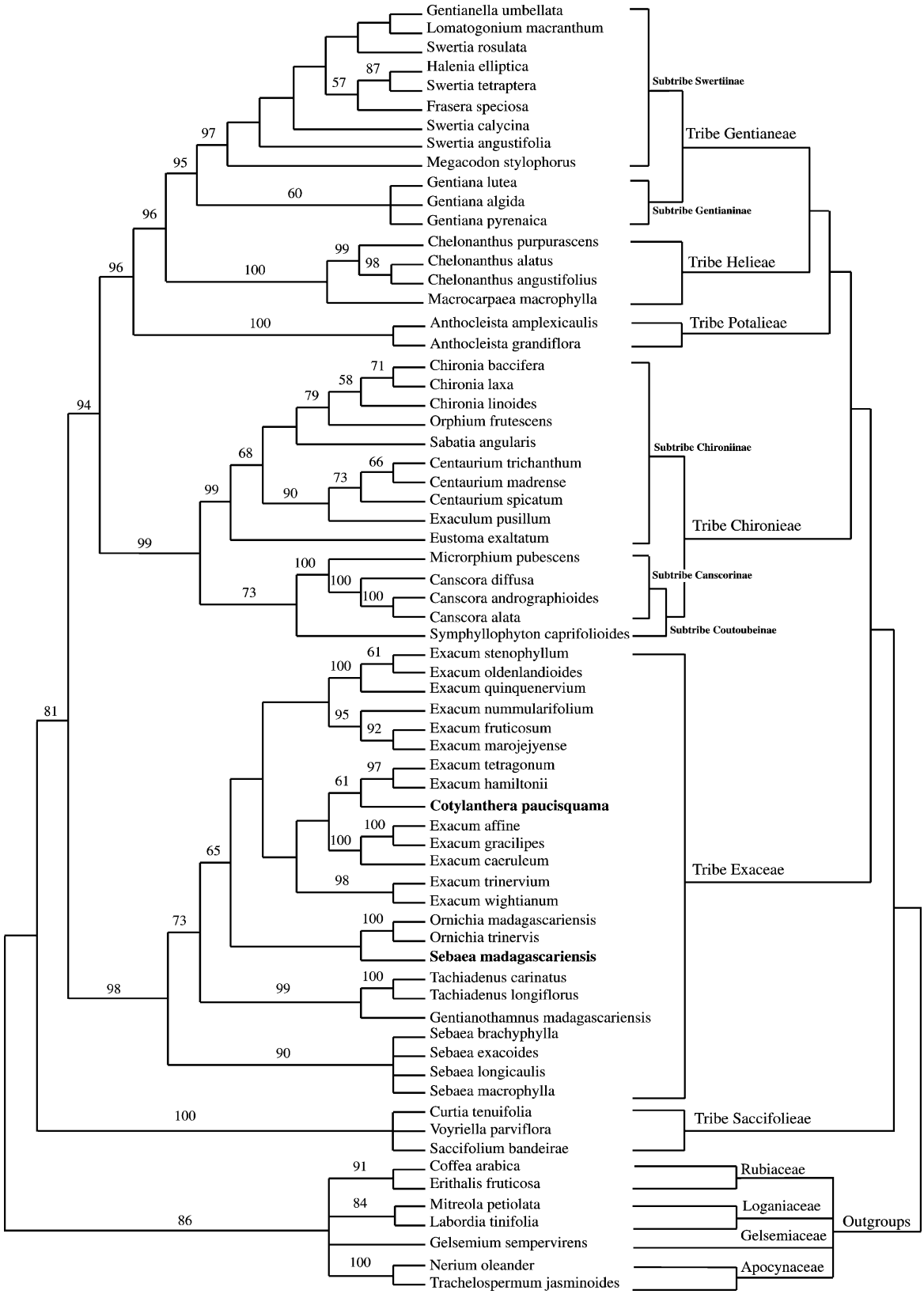

Fig. 2. Strict consensus tree of the 150 equally maximum parsimonious trees obtained from combined *trn*L (UAA) intron and ITS sequences (length = 1694, CI = 0.53 including autapomorphies, CI = 0.46 excluding autapomorphies, RI = 0.74). Numbers above the branches are bootstrap values supporting the corresponding branch when greater than 50%. Current classification is shown on the right. Bolded face indicates the unexpected resolutions (see text).

Fig. 3. Phylogram of one of the 13 equally most parsimonious trees obtained from combined analyses on ITS and *trn*L (UAA) intron sequences of the tribe *Exaceae* only (length = 629, CI = 0.72 including autapomorphies, CI = 0.61 excluding autapomorphies, RI = 0.70). Branch lengths are proportional to the substitutions supporting the branch. Note the extraordinary long branch of the saprophytic species *Cotylanthera paucisquama*, which suggested an accelerated evolution due to its saprophytism. The divergence time (million years) of the two basal nodes estimated from *trn*L intron based on the clock rate of the lowest 4.87×10^{-10} and the highest 1.3×10^{-9} as previously reported are also shown. These two nodes were supported by both ITS and *trn*L data analysed separately or combined.

the order Gentianales are summarised in Table 2. Both methods suggested very close estimations on the diverging time of the Gentianaceae (42.4–50.4 MY). For the divergence of the tribe *Exaceae* NPRS approach estimated as 34.5–49.2 MY, while MBL approach revealed a more recent divergence (19.8–25.9 MY). Similarly the divergence between genus *Sebaea* and other *Exaceae* was revealed as 22.4–28.8 and 15.6–19.1 MY respectively, both fell in the range suggested by a molecular clock. There is a clearly increased discrepancy of

Table 2
Results of divergence dating based on NPRS trees, MBL, and molecular clock applying diverse rates of gene evolution

Divergence of	*trn*L intron, six trees, NPRS[a]	*trn*L intron excl. fast taxa[b], 1 tree, NPRS	*trn*L intron, six trees, MBL[a]	*trn*L intron excl. fast taxa[b], 1 tree, MBL	Average sequence divergence of *trn*L intron of *Exaceae* only, MC rate[c]
Gentianales	60 (CP)	60 (CP)	60 (CP)	60 (CP)	n/a
Gentianaceae	42.4–47.7	50.4	43.1–48.0	42.4	n/a
Tribe *Exaceae*	34.5–39.4	49.2	19.8–21.6	25.9	n/a
Sebaea vs. other *Exaceae*	22.4–25.8	28.8	15.6–17.0	19.1	20.3–54.3
(*Tachiadenus–Gentianothamnus*) vs. (*Ornichia–Exacum* clade)	18.8–21.4	24.0	7.0–7.8	7.5	11.2–29.8
Tachiadenus vs. *Gentianothamnus*	9.7–11.6	13.2	3.2–3.5	4.1	5.5–14.7
Exacum	15.2–17.9	20.4	3.1–4.1	4.8	8.1–21.6

Divergence times are shown in million years before present (MY). NPRS, non-parametric rate smoothing (Richardson et al., 2001a; Sanderson, 1997); MBL, mean branch length (Bremer, 2000; Patterson and Givnish, 2002); MC, molecular clock; CP, calibration point based on fossil pollen date (Muller, 1984).

[a] The range shows the difference obtained from different trees for the corresponding nodes.

[b] Excluding *Curtia*, *Voyriella*, *Saccifolium*.

[c] Based on previous rates 1.30×10^{-9} and 4.87×10^{-10} s/s/y (Richardson et al., 2001a).

divergence time estimations between NPRS and MBL toward the terminals of the tree. This is probably due to the increased probability of sampling error involved in MBL method toward the tree terminals. If we consider the age of the tribe *Exaceae* as about 40 MY as suggested by NPRS, the maximum divergence rate of *trn*L intron in this tribe could be calibrated as around 8.62×10^{-10} s/s/y, which fell within the range given by Richardson et al. (2001a). Dating with ITS sequences using the previously reported ITS fast and slow rates 7.83×10^{-9} and 1.72×10^{-9} s/s/y (Richardson et al., 2001a) (despite rejection of a molecular clock) indicated a similar divergence time, 12.8–58.1 MY, for the tribe *Exaceae*, supporting the cpDNA findings (data not shown).

4. Discussions

4.1. Congruence of phylogenies based on ITS and trnL (UAA) intron

Partition homogeneity statistic test suggested that ITS and *trn*L (UAA) intron data sets were homogeneous since no character conflict was detected. When the ITS sequences of the divergent taxa, outgroups and the tribe *Saccifolieae* were excluded from aligning, the topologies of the remaining clades were also highly congruent with corresponding clades of the *trn*L trees, the monophyly of the tribes and subtribes being highly supported by both data sets. Therefore, we consider that both *trn*L intron and ITS are highly congruent for phylogenetic inference, and the majority of our discussions and conclusions were based on the combined analyses, except for *Sebaea* (based mainly on *trn*L data) and *Cotylanthera* (based on ITS data only).

4.2. Monophyly of Exaceae and its relationships with other tribes

Prior to the recent treatment by Struwe et al. (2002), the infrafamilial classification of the Gentianaceae remained incomplete or even misleading with regarding to the understanding of phylogenetic relationships among different genera. As far as the tribe *Exaceae* is concerned for an example, the genus *Gentianothamnus* had been placed in subtribe *Chironiinae* since it was described (Humbert, 1937) and *Tachiadenus* was placed in subtribe *Tachiinae* by Gilg (1895). Only the recent treatment of Struwe et al. (2002) classified them in *Exaceae*, and our present study confirmed their inclusion in *Exaceae* and showed that they are very closely related to each other. Struwe et al. (2002) based largely on phylogenetic analyses of the chloroplast *trn*L (UAA) intron sequences and partially on *mat*K sequences (Struwe et al., 1998; Thiv et al., 1999a), made the first comprehensive classification of the whole family in the context of identifying monophyletic lineages. By extended sampling of both plant taxa that were not available for previous studies (e.g., *Cotylanthera* and *Gentianothamnus*) and DNA sequences (nuclear ITS in addition to the chloroplast intron), our present phylogenetic analyses closely resembles the tribal classification of Struwe et al. (2002) with extended resolutions. Our present analyses provided strong evidence and statistical support for the monophyly of the major lineages, the tribes and subtribes of the Gentianaceae defined by the current classification. The phylogenetic hypotheses obtained from separate analyses on chloroplast *trn*L (UAA) intron (Fig. 1A) and ITS (Fig. 1B) or combined (Fig. 2) showed that the tribe *Exaceae* was a basal clade just next to the basal-most lineage, the tribe *Saccifolieae*.

4.3. Generic relationships within tribe Exaceae

The extensive taxonomic and phylogenetic studies based on morphology and anatomy done by Klackenberg (1985, 1986, 1987, 1990, 2002) have forged the shape of this tribe, and is the base for a hypothesis on relationships among the genera. The phylogenetic analyses on the Gentianaceae based on the *trn*L (UAA) intron done by Struwe et al., 1998 and Thiv et al. (1999a,b) covered six species representing four genera of the tribe. Our present molecular phylogeny corroborated Klackenberg's definition of the tribe and some relationships suggested by him for example the close relationship between *Ornichia* and *Exacum*. The genus *Sebaea* was shown basal within this tribe. Two genera, *Gentianothamnus* and *Cotylanthera*, were not available in the previous molecular studies, so their molecular phylogenetic relationships toward others were unknown. Klackenberg (2002) included *Gentianothamnus*, which was originally included within the subtribe *Chironiinae* by Humbert (1937), in the tribe *Exaceae* and suggested its close relationship with *Tachiadenus* based on morphological and anatomical characters such as long corolla tube, undulate-walled testa cells, and pollen morphology. Our present phylogeny with extended samples of species based on combined data confirmed the previous phylogeny based on *trn*L (UAA) intron sequences alone, regarding the basal position of the genus *Sebaea* (except *S. madagascariensis*) (Figs. 1A, 2, 3) and the close relationships between *Exacum* and *Ornichia* (Figs. 2, 3). Our present study further indicated that *Gentianothamnus* is closely related to *Tachiadenus*, *Sebaea* is probably polyphyletic and *Cotylanthera* nests deeply inside *Exacum*. The genus *Exacum* was resolved as monophyletic if *Cotylanthera* is included (Figs. 1B, 2, 3). However, the monophyly of *Exacum* did not receive strong bootstrap support.

4.4. Polyphyletic Sebaea

While the molecular phylogeny was mostly congruent with the current classification of the tribe *Exaceae* (Klackenberg, 1990, 2002), conflict occurred regarding the monophyly of the genus *Sebaea*. The currently defined *Sebaea* contains 150–159 species (Boutique, 1972; Paiva and Noguiera, 1990): one in New Zealand, two in Australia (Adams, 1996), one in China through India (Ho and Pringle, 1995), four in Madagascar (Klackenberg, 1990) and the rest in Africa (Klackenberg, 2002). The five species sampled in *trn*L intron phylogeny were nesting in two different clades: *S. madagascariensis* grouped with the two sampled species of *Ornichia*, while *S. brachyphylla*, *S. longicaulis*, *S. macrophylla*, and *S. exacoides* grouped together as a highly supported monophyletic, and the most basal clade of the tribe (Figs. 1A and 3). ITS data confirmed the positions of *S. madagascariensis* and *S. brachyphylla*. This result suggested that the genus *Sebaea* as it is currently defined is not monophyletic. However, the present study sampled only a fraction of the species of *Sebaea* (five out of about 150 species. Analyses including more species of *Sebaea* (and *Ornichia*) are necessary to draw conclusions on the phylogenetic status of *Sebaea*.

4.5. Cotylanthera—a saprophytic Exacum

Cotylanthera as currently recognized is a small saprophytic genus with only four species. Early taxonomic studies had placed this genus outside the Gentianaceae, but Gilg (1895) included *Cotylanthera* within his subtribe *Exacinae*. Klackenberg (2002) accepted the inclusion of the genus within tribe *Exaceae*, and further suggested that it should have a close relationship with *Exacum* or even should be a derived lineage inside *Exacum* based on floral morphology as their anthers are all opening by apical pores and all have finely perforated endothecial walls. Although *trn*L (UAA) intron was successfully amplified and sequenced from another achlorophyllous plant of the Gentianaceae, *Voyriella parviflora*, using the same set of the universal primers (Thiv et al., 1999a), we failed to generate *trn*L (UAA) intron data from *C. paucisquama* after various efforts. Studies have shown that achlorophyllous holoparasitic plants often undergone dramatic variations, usually many deletions, in their plastid DNA. For examples, the complete plastome sequence of *Epifagus virginiana* has only 70,028 bp (compared to 155,844 bp of Nicotiana tabacum, GenBank: Z00044), and the *trn*L (UAA) gene is completely deleted (Wolfe et al., 1992). In *Cuscuta cuspidata* the *trn*L (UAA) intron is reduced to 270 bp (GenBank: AF323745), and in *Cuscuta attenuata*, it is reduced to only 243 bp (GenBank: AF348404), compared to lengths between 343 and 539 bp for most the Gentianaceae. It is not clear what mechanism is involved in the cpDNA evolution of *C. paucisquama*, and it is beyond the scope of the present study. Despite that the *trn*L (UAA) intron data is not available, *C. paucisquama* nested deeply inside *Exacum* and grouped with the Himalayan species *E. hamiltonii* and *E. tetragonum* in the ITS tree. Due to an accelerated evolution in this saprophytic species as indicated by its high proportion of autapomorphic characters (112 compared to 14 in *E. tetragonum*), it showed high sequence divergence to most species of the tribe sampled here (Fig. 3). Morphologically its affinity with *Exacum* is well justified as suggested by Klackenberg (2002). However, it is not advisable at present to reduce *Cotylanthera* to *Exacum*, as the ITS data are not enough convincing, and only one species out of four was analyzed. It is preferable to further investigate this suggested affinity by studying more samples, and other genes as the accelerated evolution of its DNA sequence could cause alignment errors

and long-branch attraction. The destabilising influence on the tree topology of *Cotylanthera* is indicated when its sequence is included or excluded in the analyses of ITS data of the tribe *Exaceae* alone.

4.6. Biogeographical implications

While the Gentianaceae is a cosmopolitan family, each tribe has specific and interesting distribution patterns. Tribe *Saccifolieae* is limited to the Neotropics with a majority of species occurring on the Guayana Shield. Tribe *Exaceae* has a paleotropical, and austral African distribution with its highest diversity centred around the Indian Ocean Basin. Tribe *Chironieae* has three major lineages, each corresponding to neotropical, paleotropical, and north-temperate distribution. Tribe *Helieae* is limited to the neotropical regions. Tribe *Gentianeae* is widely distributed throughout the world with the highest diversity occurring in the Old World, and tribe *Potalieae* is disjunctively distributed in pantropical areas. The family has so far no reliable fossil record. The earliest megafossil of suggested Gentianaceae origin was the fossil flowers with *Pistillipollenites* pollen from Eocene of North America ca. 45 MY old. These fossil pollens were suggested to be associated with the relatively derived *Helieae* clade (*Macrocarpaea*) of the extant Gentianaceae (Crepet and Daghlian, 1981), but unconfirmed (Stockey and Manchester, 1986). Struwe et al. (2002) argued that this fossil might not belong to the Gentianaceae. The earliest fossil records (pollen) assigned to other families of the order Gentianales allied with the Gentianaceae were also from Eocene, and the minimum age of Gentianales was estimated to be about 60 MY (Muller, 1984) or to 53.2 MY (Magallon et al., 1999). Considering the distribution of the diversity, particularly of the basal clades, Struwe and Albert (2002) suggested an austral Gondwana origin of the family Gentianaceae.

Whether the distribution patterns of the family is linked to the breakup of the Gondwana supercontinent or from post-Gondwana dispersal/migration event (or both) still remain as open questions. The topologies of the molecular trees resemble to certain extent a Gondwana vicariance pattern. However, neither the evidence of reliable fossil records nor divergence dating results on Asterids (Magallon et al., 1999) could corroborate the Gondwana hypothesis of Gentianaceae. Using the earliest pollen fossil date suggested for Gentianales and nonparametric methods (NPRS and MBL), our present dating results suggest that the family has likely diverged for about 50 MY only (Table 2), much later than the Gondwana breakup. Obviously, fossil calibrated dating gives only a minimum age, and the rate calibration or reliable fossil identification is crucial and perhaps invokes problems. The Gentianaceae, or even the whole Gentianales, have so far not many reliable fossil records. The earliest fossil records for the basal clades of Asterids such as Cornales and Ericales are mostly from the Late Cretaceous (Turonian–Coniacian) of about 90 MY before present (Crepet, 1996; Crepet et al., 1992; Manchester, 2002; Manchester et al., 1999; Nixon and Crepet, 1993; Schonenberger and Friis, 2001; Takahashi et al., 1999). *Archaefructus*, supposed to be the earliest angiosperm fossil found so far, was 145 MY old (Sun et al., 1998). The Gentianaceae, being a relatively derived lineage among the Euasterids (APG, 1998), is unlikely diverged earlier than other Asterids. It is less likely that the Gondwana disjunctive distribution of the Gentianaceae was the direct results of a Gondwana breakup that should be at least more than 100 MY ago (McLoughlin, 2001).

Regarding the tribe *Exaceae* and particularly the genus *Exacum*, Klackenberg (1985, 2002) attributed its distribution pattern to vicariance events across the Indian Ocean Basin. He suggested a possible old vicariance between Africa (*Sebaea*) and Indian Ocean Basin (*Exacum*, *Ornichia*, and *Tachiadenus*), followed by a vicariance within *Exacum* between Madagascar and India. The initial split of the Gondwana that began in Late Jurassic ca. 165 MY (McLoughlin, 2001) or 152 MY (http://www.scotese.com/late1.htm) seems too old to explain this possible vicariance, so he hypothesized that the vicariance would have been resulted from an extended phytogeographic contact between continental Africa and Madagascar/India after the initial splitting (Klackenberg, 2002). However, our present dating obtained no evidence to corroborate the Gondwana vicariance hypothesis, as the age of the tribe *Exaceae* was estimated to be around 40 MY and the divergence of *Sebaea* from other *Exaceae* was about 30 MY, i.e., far too young to match the vicariance with the breakup of Gondwana. Post-Gondwana dispersal/migration events might have been involved in the formation of the disjunctive distribution of the tribe across the Indian Ocean Basin. A possible land-bridge, the temporal dry-out in large areas of the Mozambique Channel between 45 and 26 MY revealed by recent geological studies, has been suggested as a possible channel for mammals to colonize Madagascar (McCall, 1997). This land-bridge could serve as an important biogeographic connection between continental Africa and Madagascar for the tribe *Exaceae* as well.

The place of origin of the tribe *Exaceae* remains unresolved. Our present phylogeny suggested that the other members of *Exaceae* except for *Sebaea* likely originated in Madagascar. The genus *Sebaea* has its highest diversity in continental Africa, particularly in the Cape region, although it occurs throughout the entire paleotropical region. The phylogeny of this genus is still unknown, so the place of origin of *Sebaea* can not be suggested yet. Examples showed that the species richness in the Cape flora probably resulted from rapid

and recent (7–8 MY) diversification (Cowling and Pressey, 2001; Richardson et al., 2001b). This might also be the case for *Sebaea*. A robust phylogeny of *Sebaea* is urgently needed to allow further elaboration on the origin of the tribe *Exaceae*, and further to confirm if the vicariance between *Sebaea* and other members of *Exaceae* could be the result of a migration through the Mozambique Channel land-bridge and subsequent separation.

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