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Phylogeny and Biogeography of *Exacum* (Gentianaceae): A Disjunctive Distribution in the Indian Ocean Basin Resulted from Long Distance Dispersal and Extensive Radiation

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Abstract.—Disjunctive distributions across paleotropical regions in the Indian Ocean Basin (IOB) often invoke dispersal/vicariance debates. *Exacum* (Gentianaceae, tribe Exaceae) species are spread around the IOB, in Africa, Madagascar, Socotra, the Arabian peninsula, Sri Lanka, India, the Himalayas, mainland Southeast Asia including southern China and Malaysia, and northern Australia. The distribution of this genus was suggested to be a typical example of vicariance resulting from the breakup of the Gondwanan supercontinent. The molecular phylogeny of *Exacum* is in principle congruent with morphological conclusions and shows a pattern that resembles a vicariance scenario with rapid divergence among lineages, but our molecular dating analysis demonstrates that the radiation is too recent to be associated with the Gondwanan continental breakup. We used our dating analysis to test the results of DIVA and found that the program predicted impossible vicariance events. Ancestral area reconstruction suggests that *Exacum* originated in Madagascar, and divergence dating suggests its origin was not before the Eocene. The Madagascan progenitor, the most recent common ancestor of *Exacum*, colonized Sri Lanka and southern India via long-distance dispersals. This colonizer underwent an extensive range expansion and spread to Socotra-Arabia, northern India, and mainland Southeast Asia in the northern IOB when it was warm and humid in these regions. This widespread common ancestor retreated subsequently from most parts of these regions and survived in isolation in Socotra-Arabia, southern India–Sri Lanka, and perhaps mainland Southeast Asia, possibly as a consequence of drastic climatic changes, particularly the spreading drought during the Neogene. Secondary diversification from these surviving centers and Madagascar resulted in the extant main lineages of the genus. The vicariance-like pattern shown by the phylogeny appears to have resulted from long-distance dispersals followed by extensive range expansion and subsequent fragmentation. The extant African species *E. oldenlandioides* is confirmed to be recently dispersed from Madagascar. [Biogeography; DIVA; *Exacum*; Gentianaceae; ITS; phylogeny; *trn*L intron.]

The historical biogeographic connection through the paleotropical regions around the Indian Ocean Basin (IOB) area stands out as a conspicuous pattern and a key topic in the biogeography of plants and animals of the world (Raven and Axelrod, 1974; Raxworthy et al., 2002). This is primarily due to the firmly established and well-documented geologic history of this region, the sequential breakup of the Gondwana landmass, and the differential shifting history of different tectonic plates (McLoughlin, 2001). Thus, biogeographic patterns in this region provide ideal models for testing the long-lasting debate between vicariance and dispersal (Rieppel, 2002). In addition to the Gondwana vicariance and long-distance dispersal explanations, noticeably the earlier land-bridge theory (Steenis, 1962), and the more recent "Lemurian stepping-stones" hypothesis (Schatz, 1996) have been proposed to interpret this biogeographic connection. The distribution of chameleons used to be considered as a typical vicariance example related to Gondwana fragmentation, but recent studies strongly suggest that their present distribution is the result of extensive oceanic dispersals (Raxworthy et al., 2002; Rieppel, 2002). A similar situation applies to certain freshwater fish: cichlids show a typical Gondwana vicariance distribution pattern, which is congruent with the phylogenetic relationships among the lineages. Recent divergence dating, however, revealed significant discrepancy between the times of lineage divergence and the tectonic events (Vences et al., 2001). Therefore, Cenozoic dispersal was considered as a more plausible explanation than Mesozoic vicariance. A few similar debates exist in plants; for example, the oceanic dispersal versus Gondwana vicariance for the biogeography of the genus *Adansonia* (Baum et al., 1998), the circum-global dispersal versus Gondwana vicariance for the history of the family Melastomataceae (Renner and Meyer, 2001; Renner et al., 2001), migration versus vicariance in Malpighiaceae (Davis et al., 2002a, 2002b), the post-Gondwana long-distance dispersal versus Gondwana vicariance for the historical biogeography of the genus *Polyscias* (Plunkett et al., 2001), and the widespread-extinction hypothesis versus vicariance or long-distance dispersal theories for the history of the genus *Nepenthes* (Meimberg et al., 2001). Recently, Sanmartin and Ronquist (2004) also found that most of the plant patterns of suspected Gondwanan origin fit a dispersal rather than a vicariance scenario. As urged by Rieppel (2002), studies on other groups of organisms with a geographical distribution similar to that of chameleons are needed to verify whether some regularity underlies their phylogenetic and biogeographical patterns. The plant genus *Exacum* (Gentianaceae, tribe Exaceae) appears as a good model for this purpose due to its disjunctive distribution pattern in the IOB.

The genus *Exacum* consists of 64 species and shows a typical paleotropical distribution (see Fig. 1) (Klackenberg, 1985, 2002; Thulin, 2001). Our previous study has addressed its phylogenetic position within the tribe *Exaceae* (Yuan et al., 2003). Klackenberg (1985)

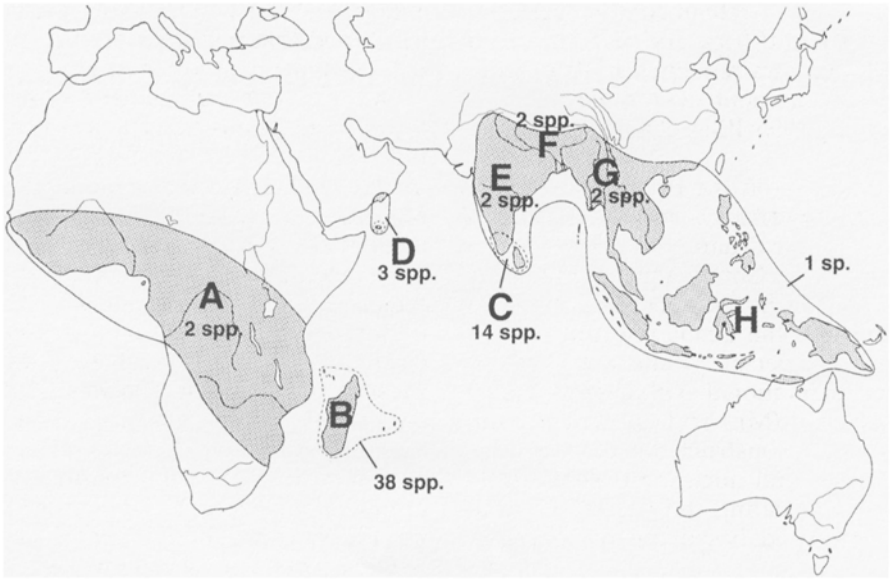

FIGURE 1. Distribution of the extant species of *Exacum* and the areas of endemism with their relevant species numbers.

monographed the genus and divided it into two sections based on phylogenetic studies of morphological and anatomical characters: sect. *Africana* consisting of African, Madagascan, Socotran and southern Arabian species, and sect. *Exacum* consisting of species of other Asian regions (Sri Lanka, India, through mainland Southeast Asia). The majority of species (38 species) occur in Madagascar. Sri Lanka and the southern tip of the Indian subcontinent (mainly the Western Ghats) is the second most species-rich area (14 species limited to this area, 3 species shared with north India, the Himalayas, and other areas). Three species are found on the southern Arabian peninsula (Dhofar of Oman and nearby Mahrah of Yemen) and the Island of Socotra (Thulin, 2001). The African continent harbors only two species: *E. oldenlandioides* widespread throughout the entire tropical Africa and *E. zombense* endemic to the Shire Highlands in Malawi (Klackenberg, 1985). Two species, *E. pedunculatum* and *E. petiolare*, are widespread in India. Two species, *E. hamiltonii* and *E. teres*, are restricted to the Himalayas, and two species, *E. pteranthum* and *E. sutaepense*, are limited to the mountainous regions between Burma and Thailand (referred to as mainland Southeast Asia herein). Only one species, *E. tetragonum*, is widespread in Indomalesia (India, the Himalayas, mainland Southeast Asia including southern China, and Malaysia) and reaches the extreme north of Australia (Arnhem Land). Based on a phylogeny reconstructed from morphological and anatomical characters, a Gondwana vicariance hypothesis has been suggested to explain the distribution pattern of *Exacum*, especially for the divergence among the species of Madagascar, India, and Socotra-Arabia (Klackenberg, 1985, 2002). Obviously, this hypothesis requires the assumption of a very old age of the genus with species divergence as early as the Gondwana breakup, in order to link the disjunctions of species distributions to the fragmentation events of the Gondwana landmass.

Paleomagnetic data and tectonic reconstruction suggested that the Gondwana breakup initiated ca. 180 million years ago (Mya) (Storey, 1995), and the Madagascar-Seychelles-India block began to separate from the Africa–South America block ca. 165 Mya, with movement ending by 121 Mya (Rabinowitz et al., 1983). Since then, Madagascar has remained in its position with respect to Africa (Coffin and Rabinowitz, 1988), whereas Australia and Antarctica separated from the Madagascar-Seychelles-India block ca. 132 Mya (Barron, 1987). India separated from Madagascar ca. 88 Mya (Storey et al., 1995) and split from the Seychelles ca. 65 Mya (Storey, 1995). Although the Seychelles block subsequently became fixed with respect to Africa, India rapidly drifted northward and collided with Laurasia during the Paleocene-Eocene. Some authors suggested the India-Asia collision occurred ca. 43 Mya (Lee and Lawver, 1995; McLoughlin, 2001), whereas the majority of authors suggest an earlier collision between 50 and 66 Mya (Besse et al., 1984; Patriat and Achache, 1984; Jaeger et al., 1989; Klootwijk et al., 1992; Beck et al., 1995; Valdiya, 2002). Sri Lanka had remained in full contact with India during all these processes until the last major sea level rise 6000 years ago, which separated Sri Lanka from India by the narrow and shallow Palk Strait (McLoughlin, 2001). After separation from Antarctica in the Cretaceous and Paleogene (96 to 35.5 Mya), the Australia–New Guinea block moved northward and collided with Southeast Asia in the late Oligocene to the beginning of the Miocene ca. 25 Mya (Lee and Lawver, 1995; Li and Powell, 2001). Socotra, a continental fragment, was located adjacent to the Dhofar region of southern Oman prior to the Gulf of Aden rifting (Jolivet and Faccenna, 2000). The Afro-Arabian plate collided with Eurasia in the eastern part around 25 Mya (Samuei et al., 1997). At about the same time the Gulf of Aden rift appeared (Richardson et al., 1995; Birse et al., 1997; Ghebreab, 1998; Fantozzi and Sgavetti,

1998), and subsequently the Arabian continent became separated from continental Africa and Socotra ca. 10 Mya (Ghebreab, 1998). Socotra remained in its position relative to the African continent ever since (Horn of Africa) (Richardson et al., 1995; Birse et al., 1997; Samuei et al., 1997).

Our recent investigations on the tribal relationships within Gentianaceae suggested a minimum age of 40 Mya for the tribe *Exaceae* (Yuan et al., 2003), which apparently argues against a Gondwanan origin of the genus *Exacum*. Alternative hypotheses to explain the historical biogeography of the genus relevant to post-Gondwana vicariance/dispersal events are therefore needed. To search for such rational explanations, we applied maximum likelihood (ML) and maximum parsimony (MP) methods to reconstruct the phylogeny of *Exacum* using chloroplast and nuclear ribosomal DNA sequences, and divergence dating and ancestral area reconstruction were conducted based on the molecular phylogeny.

Materials and Methods

Ingroup Sampling and Outgroup Choice

To obtain an accurate divergence time estimation for the tribe *Exaceae*, our previously analyzed family data matrix (Yuan et al. 2003) was expanded by the addition of two *trn*L intron sequences of *Lisianthius*, *L. longifolius* (accession AF102450), and *L. laxiflorus* (accession AF102449), retrieved from GeneBank and was reanalyzed with additional calibration points (see below).

Our *Exacum* data set included 42 sequences of the nuclear ribosomal internal transcribed spacer (ITS) regions, including the spacer 1, the 5.8S gene, and the spacer 2 and 42 chloroplast *trn*L(UAA) intron sequences (*trn*L) (Appendix 1). Sampling of *Exacum* species was maximized to represent all areas of endemism (Africa, Madagascar, Socotra-Arabia, India/Sri Lanka, and mainland Southeast Asia) and morphological diversity. About half the taxa from each geographic area were sampled. The species cover all main morphological variations such as different habits (small herb, large herb, shrub, etc.), different floral types (size of corolla, merosity, etc.), and different types of testa cells (star-shaped, isodiametric, etc.). Thirty-seven accessions representing 30 species of *Exacum* were sampled, equivalent to about 47% of the genus. Two species (out of three, all endemic to Madagascar) of *Ornichia*, the sister genus of *Exacum* (Yuan et al., 2003), were also sampled as ingroups. An additional basal clade sister to the *Exacum–Ornichia* lineage (Yuan et al., 2003) was sampled as outgroup. This additional clade, composed of the genera *Tachiadenus* (2 species sampled out of 11, all endemic to Madagascar) and *Gentianothamnus* (monotypic, endemic to Madagascar), was used as an outgroup to accommodate the dispersal-vicariance (DIVA) analyses for more reliable estimates of the ancestral areas of *Exacum* (Ronquist, 1996).

The genus *Sebaea*, the most basal clade of the tribe, was not included as an additional outgroup, as our previous analyses of the whole family including African *Sebaea* confirmed that inclusion or exclusion of this clade did not interfere with the geographic assessment for *Exacum*.

Our previous studies have shown evidence that the saprophytic genus *Cotylanthera* resided inside a monophyletic *Exacum*. It consists of four species occurring in the Himalayas, mainland Southeast Asia, and the Malaysian area. We did not include this genus in our present study because, on the one hand its sequences were incomplete (the chloroplast sequence were not obtainable); on the other hand it has been shown to have undergone an accelerated molecular evolution (cf. Yuan et al., 2003: Fig. 3), which may be problematic in the present phylogenetic analysis. However, omitting sequences of this genus had no significant effect on the tree topology (data not shown) and thus should not affect analyses on *Exacum*. Furthermore, this group showed affinities only with the more terminal branches of the phylogenetic tree of *Exacum* (*E. hamiltonii* from the Himalayas and *E. tetragonum* widespread in Indomalesia) (cf. Yuan et al., 2003: Figs. 2 and 3), and the biogeography patterns of *Exacum* across the IOB region is greatly depending on the basal branching patterns (see below).

Molecular Methods

Sequences of 18 species were previously obtained (Yuan et al., 2003). Sequences for an additional 24 accessions were newly acquired for this study. The procedures of DNA extraction, polymerase chain reaction (PCR) amplification of target fragments, purification of PCR products, and sequencing follow Yuan et al. (2003). All sequences have been deposited in GenBank (accession numbers for the *trn*L intron: AJ490202–38, 40, 42–43, 49–50; for ITS: AJ489877–914, 917–918, 923–924).

Sequence Alignment, Congruence Test, and Phylogenetic Analysis

The sequences were aligned with Clustal X (Thompson et al., 1997) followed by minor manual adjustments to improve indel events in a few tandem repeat regions. Nine base pairs near the beginning of the spacer ITS2 involved tandem repeats and could not be unambiguously aligned due to alternative alignment possibilities. They were excluded from subsequent phylogenetic analyses. The alignment does not involve severe ambiguities, therefore it was not necessary to use secondary structure for alignment. Sixteen unambiguously aligned indels (13 in ITS and 3 in *trn*L) were potentially informative. These indels were then scored as binary characters regardless of their length, and were added to the sequence data for MP analyses. The data matrix was deposited in TreeBASE and is also available from the corresponding author.

To assess the level of congruence between the *trn*L and ITS data sets, we analyzed each data set independently to see if they produced a similar topology. We also performed an incongruence length difference (ILD) test of Farris et al. (1995), implemented in PAUP* 4.0b10 (Swofford, 2000) as the partition-homogeneity

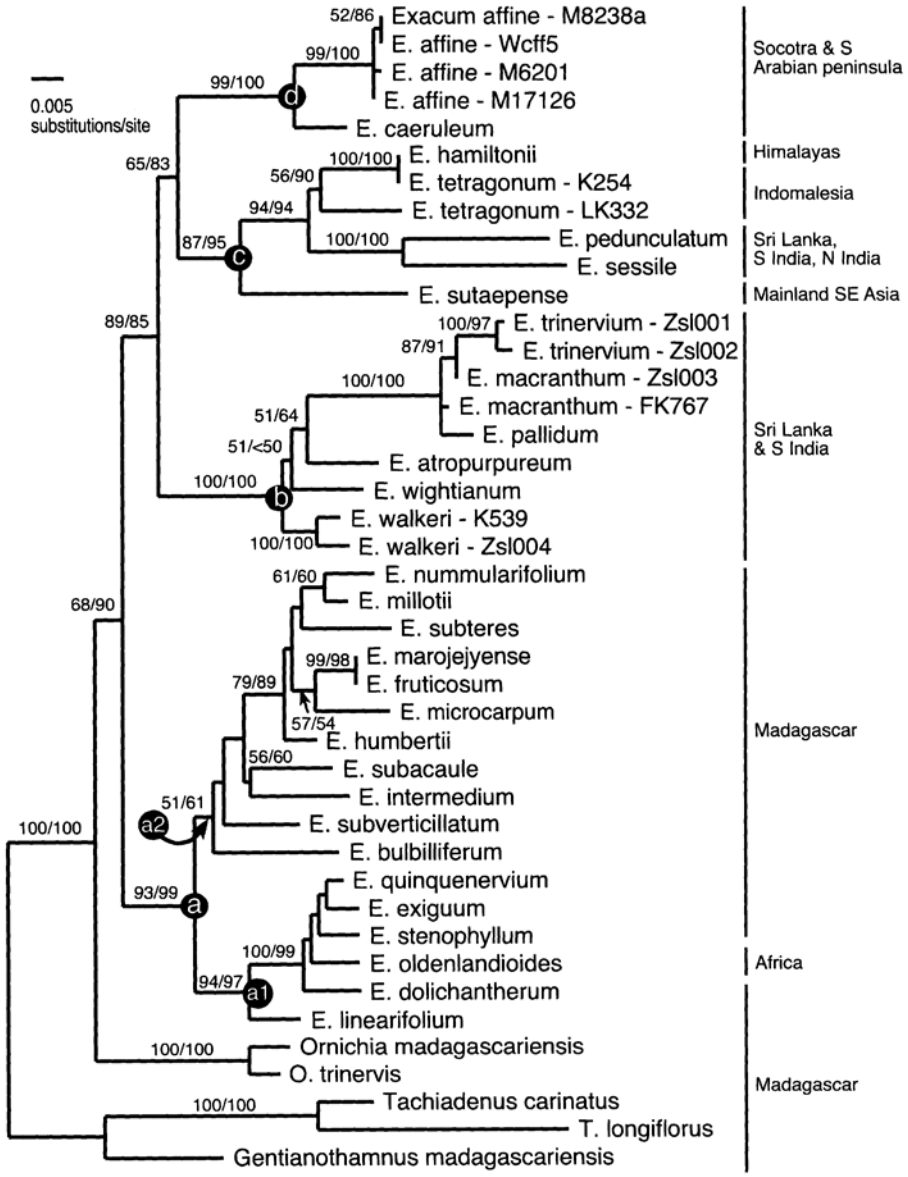

FIGURE 2. Phylogram of the single ML tree (−ln = 4350.2744), based on combined ITS and *trn*L sequences. Distributions of the terminal species are also indicated on the right. The bootstrap values of both ML and MP analyses supporting the corresponding branches are shown above the internal branches when greater than 50% (ML/MP). "a" (a1, a2), "b," "c," and "d" are the main clades discussed in the text.

test, after assuring ourselves that the properties of the data did not lend themselves to biasing this test (see results). One thousand replicates of repartitions of *trn*L intron versus ITS were conducted with heuristic searches (simple sequence addition and TBR branch-swapping). The two data sets were confirmed as congruent ($P = 0.74$) and were then combined for all further analyses.

MP analyses were conducted on separate *trn*L, ITS data sets, and on a combination of the two, applying accelerated transformation optimization (ACCTRAN) option. Heuristic searches of 1000 replicates of random sequence addition and TBR branch-swapping were performed, with MULTREES and STEEPEST DESCENT on. The relative clade support for MP analyses was also estimated by bootstrap analysis of 1000 replicates of heuristic searches with random sequence addition and TBR branch-swapping.

Phylogenetic reconstructions based on the combined data sets was also conducted using ML optimality criteria as implemented in PAUP*4.0b10 (Swofford, 2000). The TIM+I+Γ model (Posada and Crandall, 1998) and parameter settings were chosen by using the Akaike information criterion as suggested by Modeltest V3.06 (Posada and Crandall, 1998). Optimal gene trees were found via heuristic searches of 100 replicates of random sequence addition with TBR branch-swapping, MULTREES, and STEEPEST DESCENT on. The relative clade support for the ML analyses was estimated by bootstrap analysis of 100 replicates of heuristic searches

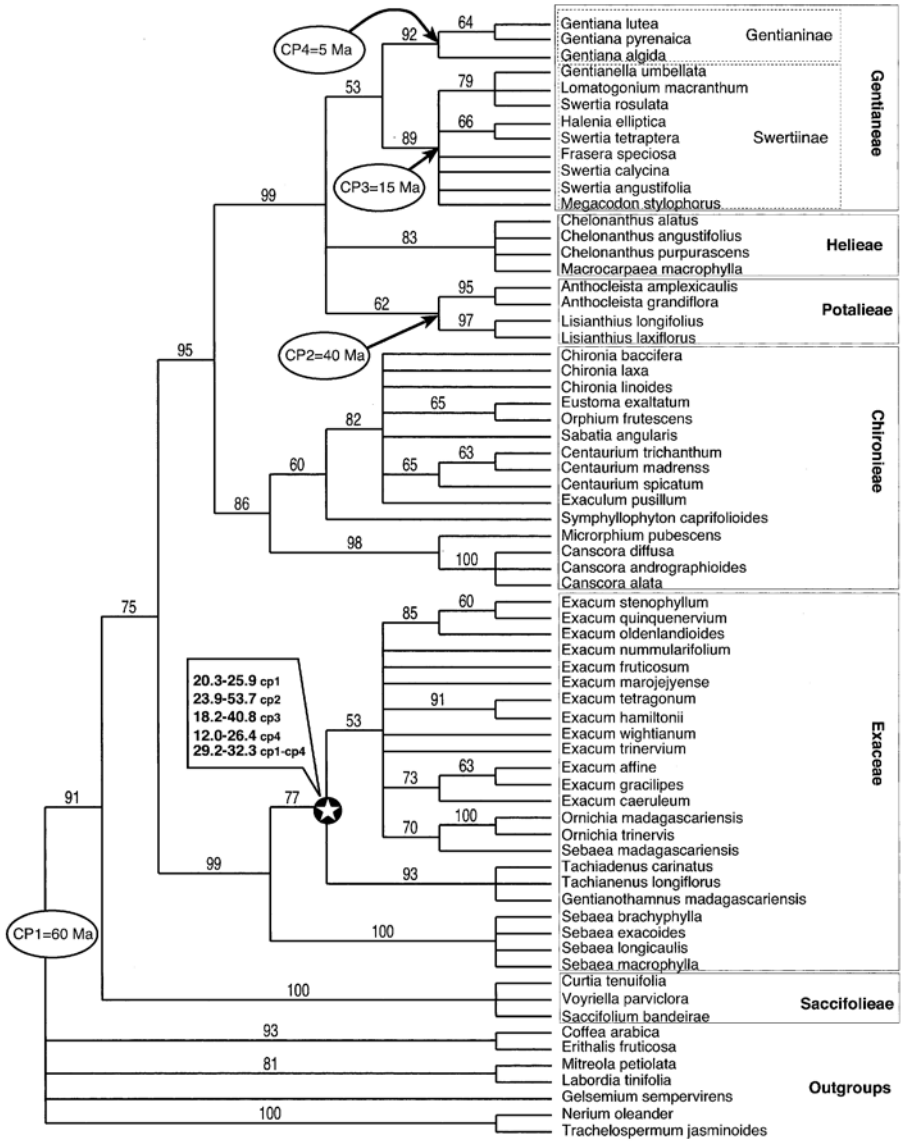

FIGURE 3. The strict consensus of six trees recovered from MP analyses (MIN collapse option) on 68 *trn*L intron sequences of Gentianaceae (length = 463, CI = 0.68 excluding autapomorphies, RI = 0.89). These trees were used to infer the divergence dates for the node marked with star, by using penalized likelihood approaches and the four calibration points CP1 through CP4 shown on the tree. The bootstrap values supporting the corresponding branches are shown above the internal branches when greater than 50%. The figures in the square are inferred divergence dates for the node indicated as a star using different calibration points. Shown on the right is the tribal classification of the family. See text for the details of the calibration points CP1 through CP4.

using the same model and parameters (Felsenstein, 1985).

Molecular Clock Test, Divergence Calibration, and Divergence Time Calculation

To obtain approximate timings of branching events within *Exacum*, it is necessary to estimate the times of divergence of the main clades of the *Exacum* tree. By comparing the likelihood ratio statistic, $-2(\ln L_{\text{clock}} - \ln L_{\text{non-clock}})$, to the χ^2 distribution with $n-2$ degrees of freedom (n = number of taxa), a molecular clock was tested (Muse and Weir, 1992). Finding that a clocklike sequence evolution had to be rejected ($P < 0.01$), the ML tree obtained in the absence of a molecular clock was then subjected to a rate smoothing applying penalized likelihood (PL) approach using the software r8s v.1.50 (Sanderson, 2002a, b). By applying appropriate calibrations, divergence times can be estimated on the smoothened tree. PL is a semiparametric smoothing method that estimates relative branching time without assuming a molecular clock. Optimal smoothing factors were chosen based on a data-driven cross-validation procedure implemented in r8s (Sanderson, 2002b).

There is no fossil record for the tribe *Exaceae* to calibrate the molecular phylogeny of *Exacum*. Our previous studies (Yuan et al., 2003) estimated a minimum age of 40 Mya for the tribe *Exaceae* calibrated on the basis of

pollen fossil of Gentianales (Muller, 1984). This minimum age of the tribe could be used directly as a calibration. However, this estimate was based on the nonparametric rate smoothing (NPRS) approach (Sanderson, 1997) with only one calibration. NPRS approaches have been proven to overfit the data, allowing too much rate variation and therefore losing predictive power (Sanderson, 2002a). As a further confirmation and cross-validation, we therefore reanalyzed our previous *trn*L intron data of the family Gentianaceae and its sister groups, with additional sequences and fossil calibrations that we recognized recently. Two *trn*L intron sequences of the genus *Lisianthius*, *L. longifolius* (accession AF102450) and *L. laxiflorus* (accession AF102449), were retrieved from GeneBank and were added to our previous data matrix to accommodate a fossil record of *Lisianthius* (Graham, 1984). This enlarged *trn*L intron matrix contained 68 taxa and 657 characters. This matrix was deposited in TreeBASE and is also available from the corresponding author. Phylogenetic reconstructions on the enlarged *trn*L intron matrix were performed using MP with 1000 replicates of heuristic searches with random sequence addition and TBR branch-swapping. Finding a molecular clock has to be rejected ($P < 0.01$) for the enlarged *trn*L intron data set, the resulting MP trees were then subjected to PL rate smoothing (Sanderson, 2002a) using the software r8s (Sanderson, 2002b). Four independent calibration points, minimum age of Gentianales (CP1 = 60 Mya) based on fossil pollen (Muller, 1984), minimum age of *Lisianthius* (CP2 = 40 Mya) based on fossil pollen (Graham, 1984), an inferred age of subtribe *Swertiinae* (CP3 = 15 Mya) (Hagen and Kadereit, 2001, 2002), and minimum age of *Gentiana* (CP4 = 5 Mya) based on fossil seeds (Mai and Walther, 1988), were used to infer the divergence dates between the outgroup *Gentianothamnus-Tachiadenus* clade and the ingroup *Exacum-Ornichia* clade. These inferred estimates of divergence dates from the global analyses were then used to calibrate the *Exacum* tree to obtain divergence estimates. Such a procedure has been successfully used by Davis et al. (2002a).

NPRS and PL may result in biased rates to the evenly weighted MP branch lengths because they are not corrected for multiple hits (Sanderson, 2002a). Ideally, the branch lengths should be corrected for multiple hits using an appropriate model of evolution (Yang, 1996). This is more critical for highly divergent sequences as the likelihood of multiple hits increases. However, in the enlarged *trn*L intron analysis, sequence divergence was moderate (maximal 18% for ingroup taxa) and our calibration dates for the *Exacum* tree inferred from PL smoothened MP trees was not affected significantly.

As an independent cross-check of the divergence-time estimates based on the smoothened ML tree, we also estimated the times of divergence of the *trn*L intron and ITS sequences employing various extreme rates measured for different plants, compiled by Richardson et al. (2001). Divergence time between a pair of species was calculated as half of divergence value divided by the rate. The highest sequence distance between a pair of species of two clades under consideration was used to ensure the divergence times were not underestimated.

Ancestral Area Reconstruction

In order to obtain the historical scenarios of the biogeography of *Exacum*, DIVA analyses were conducted to infer the ancestral areas for each internal node of the phylogeny, by using DIVA 1.1a (Ronquist, 1996, 1997), on the ML tree. DIVA reconstructs ancestral areas by minimizing dispersal and extinction events needed to explain the observed distribution pattern based on an inferred phylogeny. Vicariance is considered as the default mode of speciation. The two zero-length branches of the ML tree were arbitrarily resolved to obtain dichotomies; this had no effect on the area assignment. Based on the analyses of the distribution of *Exacum* and its relatives, we recognized eight areas of endemism: Africa, Madagascar, Sri Lanka and south India, Socotra and southern Arabian peninsula, central to northern India, the Himalayas, mainland Southeast Asia, and Malaysia plus northern Australia (Fig. 1). Terminal taxa were scored according to their distributions across the above eight areas to generate the distribution data matrix, and this data matrix was subsequently optimized onto the ML tree. Optimizations of both, unconstrained and area constrained to a maximum of two, were conducted following the reasoning of Ronquist (1996, 1997) and Donoghue et al. (2001).

RESULTS

Sequence Characteristics

The *trn*L intron sequences ranged from 487 to 495 bp in length in *Exacum*, whereas the intron was as short as 381 to 382 bp in the two species of *Ornichia* mainly due to a long gap of 106 bp. The aligned *trn*L intron matrix had 508 sequence and three indel characters. The number of variable characters was 79 (15.5%), of which 36 (7.0%) were informative. The uncorrected pairwise sequence divergence was between 0% (*E. tetragonum*—K254 versus *E. hamiltonii*; *E. macranthum*—FK767 versus *E. pallidum*; *E. marojeyense* versus *E. fruticosum*; *E. marojeyense* versus *E. humbertii*; *E. fruticosum* versus *E. humbertii*) and 3.9% (*E. sessile* versus *E. trinervium*—Zsl002) within the ingroup and maximal 4.5% (*E. sessile* versus *T. longiflorus*) overall. The ITS sequences ranged from 611 to 638 bp in length. These length variations are due to single or short indels. The ITS data matrix had 659 sequence and 13 indel characters, of which 266 (39.6%) were variable and 179 (26.6%) informative. The taxa had 0% (*E. affine*—M8238a versus *E. affine*—Wcff5; *E. tetragonum*—K254 versus *E. hamiltonii*; *E. marojeyense* versus *E. fruticosum*) to 13.0% (*E. sessile* versus *E. trinervium*—Zsl002) within ingroup and maximal 16.4% (*E. trinervium*—Zsl002 versus *T. longiflorus*) uncorrected pairwise sequence divergence across ingroup and outgroup taxa.

The two sets of sequences were revealed as congruent by the ILD test ($P = 0.74$). Recent studies and simulations suggested that the ILD test could fail to detect congruence due to different noise levels of the data sets (Dolphin et al., 2000; Yoder et al., 2001) or incongruence

(Dowton and Austin, 2002; Darlu and Lecointre, 2002) due to large difference of the sizes and evolutionary conditions of the data partitions. Our data sets had similar sizes (511 characters in *trn*L and 672 characters in ITS. ITS showed a ca. 3.5-fold higher divergence (0% to 16.4%) compared to *trn*L (0% to 4.5%), but both revealed similar tree topology when analyzed separately. Thus we consider that our two data sets were not suffering from these limitations, and, therefore, were combined. The combined data matrix had 1167 bp sequence characters plus 16 binary indel characters. It was deposited in TreeBASE and is also available on the web site http://www.unine.ch/bota/ebolab/gentianaceae/gentmain.html. Of the 1167 bp combined sequence, 9 bp (0.8%) ambiguously aligned ITS sequences were excluded from phylogenetic analyses.

Phylogenetic Analysis

MP analyses on *trn*L intron sequences alone generated 1829 equally most parsimonious trees of 90 steps (CI = 0.89 excluding autapomorphic sites, RI = 0.94). The strict consensus (not shown) is poorly resolved, and only the south India–Sri Lanka clade (b in Fig. 2) is retained. MP analyses on ITS data alone generated 2893 trees of 558 steps (CI = 0.58 excluding autopomorphic sites, RI = 0.82). The strict consensus (not shown) is highly resolved and the topology of the consensus is almost identical to that generated from the combined data. The topology of the main clades remained the same as the combined analyses, with resolution within one Madagascan clade (a2 in Fig. 2) slightly lower.

MP analyses on the combined data generated 270 most parsimonious trees of 648 steps (CI = 0.60 excluding autapomorphic sites, RI = 0.83) (results not shown). The ML analysis on the combined sequence data generated a single tree ($-\ln = 4350.2744$) (Fig. 2). The ML tree was congruent with the strict consensus of the combined MP trees and was identical to the most likely MP tree (one of the 270 MP trees that retained the highest likelihood score when measured with ML criteria).

As shown in Fig. 2, the genus *Exacum* was resolved as monophyletic, with moderate (68%, ML) to high (90%, MP) bootstrap support. The sister relationship between *Exacum* and *Ornichia* was highly supported (100%, both ML and MP). The most basal split within *Exacum* separated the highly supported Africa–Madagascar clade (93% for ML and 99% for MP; Fig. 2, clade a) from the highly supported Asia–Socotra clade (89% for ML and 85% for MP, Fig. 2). Although all the sampled Madagascan species together were monophyletic, they group into two clades: one strongly supported clade (94% for ML and 97% for MP), composed of the small-flowered species that are mainly found in open habitats (clade a1 in Fig. 2), and a weakly supported clade (51% for ML and 61% for MP), including the species with larger flowers that are found mainly in forests (clade a2 in Fig. 2). The African species, *E. oldenlandioides*, nested deeply inside the small-flowered Madagascan clade. Within the Asia–Socotra lineage, three highly supported clades were present (clades b, c, d in Fig. 2): a Sri Lanka–south India clade (100% for both ML and MP), composed of the showy species endemic to Sri Lanka and the very southern part of India (clade b); the Socotra–Arabia clade (99% for ML and 100% for MP), containing species endemic to Socotra and the southern Arabia peninsula (clade d); and the Indomalesia clade (87% for ML and 95% for MP), including the species endemic to the Himalayas, mainland Southeast Asia, the species spread in Sri Lanka and India, and the species widely spread over the entire Indomalesia area including northern Australia (clade c). The Socotra–Arabia clade showed a closer relationship to the Indomalesia clade than to any other clade (65% for ML and 83% for MP).

Divergence Calibration and Dating

In order to obtain calibrations for the *Exacum* tree, phylogenies were reconstructed for 68 representatives of the family Gentianaceae and its sister groups on *trn*L intron data. MP analyses with MIN collapse option generated six equally parsimonious trees of 463 steps (CI = 0.68 excluding autopomorphic sites, RI = 0.89). The strict consensus was well resolved and the main clades were highly supported (Fig. 3). As a molecular clock has to be rejected, these six MP trees with branch lengths optimized with ACCTRAN option were subjected to PL rate smoothing. Applying the four different calibration points to the smoothened MP trees, the divergence times between *Gentianothamnus-Tachiadenus* clade and *Exacum-Ornichia* clade (the node marked with a star in Fig. 3) are estimated as 20.3 to 25.9 Mya (CP1), 23.9 to 53.7 Mya (CP2), 18.2 to 40.8 Mya (CP3), 12.0 to 26.4 Mya (CP4), and 29.2 to 32.3 (CP1+CP2+CP3+CP4), respectively (Fig. 3).

Using these calibrations and the smoothened ML tree, the dates of the main divergence events within *Exacum* are estimated (Table 1). Dating based on maximum sequence divergences and extreme rates obtained comparable figures for corresponding nodes (Table 1). The divergence between *Exacum* and its sister group *Ornichia* was estimated to be 9.0 to 39.4 Mya, the divergence between the Africa–Madagascar clade ("a") and the Socotra–Asia clade ("b-c-d") to be about 8.2 to 35.6 Mya, the divergence between the Sri Lanka–South India clade ("b") and Socotra–Indomalesia clade ("c-d") to be about 7.4 to 31.9 Mya, and the divergence between the Socotra clade ("c") and the Indomalesia clade ("d") to be about 6.5 to 27.7 Mya. The divergence between the two Madagascan clades ("a1" and "a2") is estimated as 5.8 to 23.5 Mya, and the divergence between the African species *E. oldenlandioides* and its closest Madagascan ally is estimated to be only about 1.9 to 5.3 Mya. The divergence between the mainland Southeast Asian endemic *E. sutaepense* and its sister clade is estimated as 5.5 to 23.7 Mya. The divergence between the Socotra endemic species *E. caeruleum* and the species *E. affine*, that is common to both Socotra and the southern Arabian peninsula, is estimated to be about 2.7 to 11.6 Mya (Fig. 4). Noticeably, none of these estimates dated the divergence of *Exacum* beyond the Eocene. Meanwhile, it is conspicuous that, whatever calibration is used, the divergences among the main clades "a," "b," "c," and "d,"

TABLE 1. Estimated timing of divergence based on PL rate-smoothening approach applying different calibration points and molecular clock approach applying diverse extreme rates of gene evolution.

	Smoothened ML tree applying PL[a]					MC, *trn*L intron		MC, ITS	
Divergence of	CP1[b]	CP2[c]	CP3[d]	CP4[e]	CP1–CP4[f]	MIN[g]	MAX[h]	MIN[i]	MAX[j]
(*Gentianothamnus–Tachiadenus*) vs. (*Exacum–Ornichia*)	20.3–25.9	23.9–53.7	18.2–40.8	12.0–26.4	29.2–32.3	18.2	48.6	20.7	47.8
Exacum vs. *Ornichia*	15.2–19.3	17.9–39.4	13.6–30.1	9.0–19.7	21.7–24.0	14.3	38.2	13.4	32.3
Clade "a" vs. clade "b-c-d"	13.8–17.5	16.3–35.6	12.4–27.2	8.2–17.9	19.8–21.8	13.4	35.7	13.8	33.3
Clade "b" vs. clade "c-d"	12.4–15.7	14.6–31.9	11.2–24.5	7.4–16.1	17.8–19.6	15.0	40.0	16.0	37.9
Clade "c" vs. clade "d"	10.8–13.7	12.8–27.7	9.8–21.3	6.5–14.0	15.5–17.1	14.2	37.9	12.3	29.9
Clade "c"	9.3–11.7	10.9–23.7	6.1–18.2	5.5–12.0	13.3–14.6	11.1	29.7	8.9	26.2
Clade "d"	4.5–5.7	5.3–11.6	4.1–8.9	2.7–5.9	6.5–7.1	2.4	6.3	3.0	9.3
Clade "a1" vs. "a2"	9.7–12.1	11.5–23.5	8.8–18.3	5.8–12.4	13.8–15.0	5.5	14.8	7.9	19.8
E. oldenlandioides vs. (*E. stenophyllum–E. quinquenervium*) clade	3.0–3.4	3.5–5.3	2.8–4.5	1.9–3.6	4.0–4.1	2.4	6.3	1.8	5.7

Divergence times are shown in million years before present (Mya). PL, penalized likelihood (Sanderson, 2002a, 2002b); MC, molecular clock; CP, calibration point. The divergence of clade "a" versus "b-c-d" in bold face corresponds to the first vicariant event within *Exacum* inferred from DIVA.

[a]The range shows the difference of divergence estimates obtained from different *trn*L trees of Gentianaceae.

[b]CP1 = 60 Mya minimum age of Gentianales based on fossil pollen date (Muller, 1984).

[c]CP2 = 40 Mya minimum age of *Lisianthius* based on fossil pollen date (Graham, 1984).

[d]CP3 = 15 Mya estimated age of subtribe *Swertiinae* based on fossil pollen and geological calibration (Hagen and Kadereit, 2001, 2002).

[e]CP4 = 5 Mya minimum age of *Gentiana* based on fossil seed date (Mai and Walther, 1988).

[f]CP1–CP4 = all the four calibration points in effective at the same time.

[g]Based on the highest pairwise sequence distance and fast rate 1.30×10^{-9} substitution/site/year (s/s/y) (Richardson et al., 2001).

[h]Based on the highest pairwise sequence distance and slow rate 4.87×10^{-10} s/s/y (Richardson et al., 2001).

[i]Based on the highest Kimura 2-parameter distance and the rate 4.52×10^{-9} s/s/y calibrated for *Gentianella* (Hagen and Kadereit, 2001, 2002).

[j]Based on the highest pairwise sequence distance and slow rate 1.72×10^{-9} s/s/y (Richardson et al., 2001).

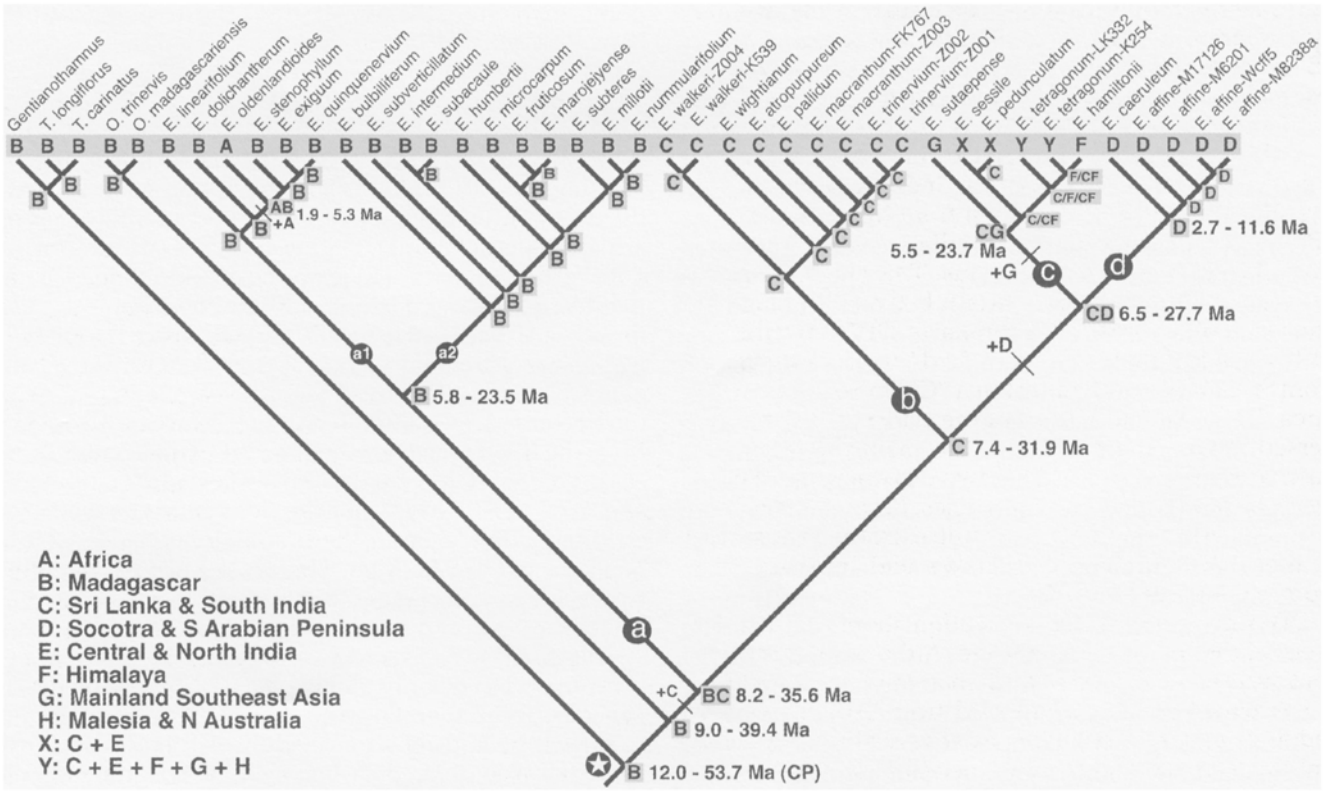

FIGURE 4. Ancestral areas reconstructed for each internal node of the phylogeny shown in Figure 2 using DIVA with two-area optimization. "A" through "H" are the areas of endemism as indicated in the Figure 1. Two coexisting ancestral areas are indicated as double letters. A bar cross a clade indicates a dispersal event to a new area leading by "+." Areas beside slants show the equally possible reconstructions for the corresponding nodes. Figures companying some nodes are the inferred divergence time for the corresponding nodes expressed in million years ago (Mya). "a" (a1, a2), "b," "c," and "d" are the main clades discussed in the text. The star marks the divergence dates inferred from the global analyses of the family Gentianaceae.

corresponding to the main areas of endemism, seem have happened within a relatively short period of time, which suggests a relatively rapid and extensive radiation of the progenitors of the clades.

Dispersal-Vicariance Analysis

As anticipated (Ronquist, 1996, 1997), unconstrained reconstructions by DIVA (not shown) suggested the three basal nodes of the tree, the most recent common ancestor (MRCA) of the outgroup (*Gentianothamnus-Tachiadenus*) and the ingroup (*Ornichia-Exacum*), the MRCA of *Exacum* and *Ornichia*, and the MRCA of *Exacum*, to be widespread in the areas "B" through "H" or "B" through "G." The area "A" (Africa) is never postulated as a possible ancestral area for these basal nodes. The MRCA of the clades "b," "c," and "d" was suggested to be spread in the areas "C" through "G" or "C" through "H." Thus, a vicariance between Madagascar (area "B") and the Asian areas plus Socotra (areas "C" through "H") is suggested for the first splitting of *Exacum*.

The optimal reconstruction of the unconstrained analyses required 13 dispersals (mainly due to widespread terminal taxa such as *E. tetragonum*). When the maximum number of areas assigned to each node is restricted to two, the optimal reconstruction of the ancestral areas required only two more dispersals than the unconstrained optimization. The results of the two-area optimization are shown in Figure 4. Madagascar (area "B") is consistently assigned to the MRCA of *Gentianothamnus-Tachiadenus* and *Ornichia-Exacum* and the MRCA of *Ornichia* and *Exacum*. The constrained optimization reveals that *Exacum* originated in Madagascar and specifies an initial dispersal (as indicated by "+C" in Fig. 4) of the MRCA of *Exacum* from Madagascar (area "B") to Sri Lanka and south India (area "C") at early diversification stage of the genus. This single dispersal resulted in the vicariance pattern between Madagascar and Sri Lanka–south India. Similarly, DIVA specifies the MRCA of the clades "b" and "c-d" to have dispersed from Sri Lanka–south India (area "C") to Socotra–Arabia (area "D"), and the MRCA of the clade "c" further dispersed from the area "C" to the mainland Southeast Asia (area "G"). Each of these two dispersals is followed by a vicariance. The African species, *E. oldenlandioides*, is also resolved as a vicariant resulted from the dispersal of the MRCA of *E. oldenlandioides* and its sister clade from Madagascar to Africa.

DIVA gives less certain resolution about the ancestral areas of the Indomalesia lineage. Although the most recent ancestor of *E. sutaepense* in mainland Southeast Asia is confirmed as being dispersed from Sri Lanka–south India, multiple possibilities exist regarding the ancestral areas of the widespread species *E. tetragonum* and its closest relatives. Either Sri Lanka–south India, or the Himalayas, or both were involved as possible ancestral areas.

DISCUSSIONS

Phylogeny of Exacum

Our present ML tree based on combined data was identical to the most likely MP tree (under ML criteria), and its topology is congruent with the *Exacum* clade structure of our previous study on the whole tribe *Exaceae* where fewer *Exacum* species were included (Yuan et al., 2003). The present phylogeny of *Exacum* based on molecular data is also highly congruent with the previous hypothesis inferred from morphological and anatomical characters (Klackenberg, 1985), with regard to the monophyly and the main infrageneric lineages resolved. However, significant difference is shown for the different position of the Socotran–Arabian clade. In our present molecular phylogeny, the Socotran–Arabian clade showed a closer relationship to the Indomalesia clade than to any other clade, whereas in Klackenberg's (1985) morphological phylogeny, they showed a closer relationship to the African–Madagascan clades rather than to any Asian clade: the Socotran–Arabian clade fell on a polytomy together with three other Madagascan–African clades. All African, Madagascan, and Socotran–Arabian clades together formed a monophyletic clade, sister to the Asian clade.

Klackenberg (1985) divided *Exacum* into two sections, sect. *Exaum* and sect. *Africana*, based on his morphological phylogeny. The relative length of the pedicel and the internode below was used as an important character to distinguish these two sections. Our present molecular phylogeny, however, suggests that his section *Africana* is polyphyletic.

Divergence Dating and Calibration

Obtaining an absolute time for a divergence/speciation offers an elegant and explicit test on whether the divergence resulted from a given historical event. Different lines of evidence have indicated that the minimum ages of the order Gentianales (53.2 Mya: Magallon et al., 1999), the family Gentianaceae (ca. 50 Mya: Yuan et al., 2003), and the tribe *Exaceae* (ca. 40 Mya: Yuan et al., 2003) are all well after the Gondwana breakup. Without an explicit dating, Klackenberg (2002) speculated that the Gentianaceae might be over 90 Mya old, based on the vicariant distribution of the basal clades of the family and an assumed correlation between this pattern and the Gondwana history. Except for our previous study where only a few *Exacum* species were sampled (Yuan et al., 2003), the absence of a temporal scale in the previous phylogeny established for *Exacum* prohibits such a direct test (Klackenberg, 1985). A temporal calibration of the phylogenetic tree of the genus *Exacum* remains necessary in order to gain insight into biogeographic consequences of post-Gondwana vicariance/dispersal events, even if a Gondwana hypothesis can be rejected based on the dating of the order Gentianales (Magallon et al., 1999), the family, or the tribe (Yuan et al., 2003). Absence of fossil data for *Exacum* and even the tribe *Exaceae* prohibits a direct calibration of the *Exacum* tree. Fossil records for the entire family Gentianaceae are scarce. Muller (1984) estimated the minimum age of the order Gentianales as 60 Mya based on fossil pollen of the other families allied with the Gentianaceae. Here we used this date as the calibration point CP1; molecular divergence dating suggested a close figure for this order (53.2 Mya: Magallon et al., 1999). The earliest megafossil of suggested

Gentianaceae origin was the fossil flowers with *Pistillipollenites* pollen from the Eocene (ca. 45 Mya) of North America. These fossil pollens were suggested to be associated with the relatively derived *Macrocarpaea* of the extant Gentianaceae (Crepet and Daghlian, 1981), but such a suggestion was unconfirmed (Stockey and Manchester, 1986). Struwe et al. (2002) doubted the Gentianaceae origin of these fossil flowers, and Crepet himself recently also upholds such a consideration (personal communication). We therefore did not use this fossil record as a calibration. Graham (1984) associated some fossil pollens isolated from lignite deposit developed from mangrove swamps of the middle to late Eocene (ca. 40 Mya) in Panama to the extant *Lisianthius*. We simply used this fossil record as our second calibration CP2. However, the taxonomic attribution of this fossil pollen needs to be further confirmed, because, as Graham (1984) admitted, the fossils differ from pollen of extant *Lisianthius* in shape, and, meanwhile, other associated fossil pollen from the same deposit were mostly mangroves such as *Rhizophora* and *Pelliceria*. The estimated age (15 Mya) of the subtribe *Swertiinae* was based on ITS sequence divergence of *Gentianella* calibrated with both fossil pollen and geological evidence (based on the ITS rate of $4.52 \pm 2.12 \times 10^{-9}$ substitutions per site per year obtained from an analysis on 44 *Gentianella* species: Hagen and Kadereit, 2001, 2002). Having similar habit and generation time, the rates of *Gentianella* may potentially applicable to *Exacum*. Thus we used both the estimated age of *Swertiinae* and the ITS rate to calibrate the *Exacum* divergence. Both gave consistent dating results (Table 1). The fossil seeds of *Gentiana* from the Upper Pliocene of Germany (ca. 5.0 Mya: Mai and Walther, 1988) seems to be a relatively recent representative. The lower bonds of the dating results we obtained are due to this "young" calibration point.

Even though our present dating on the branching events within *Exacum* was calibrated with inferred dates based on a global tree of Gentianaceae, a Gondwana vicariance scenario for the biogeography of *Exacum* can be refuted. The PL dating suggested that the divergence of *Exacum* was not beyond the Eocene. Meanwhile, our present study revealed a close correlation between PL dating and divergence rate dating on the estimates of the divergences within *Exacum*. Dating results on both *trn*L intron and ITS sequences with previously reported fast and slow rates (despite rejection of a molecular clock) are highly consistent with PL approaches in concluding that the divergence between *Exacum* and it sister group *Ornichia* was less than 40 Mya (Table 1). There is no evidence to assume that *Exacum* sequences evolve dramatically slower than other plants, and therefore it is less likely that the divergence of *Exacum* was underestimated. Fossil-calibrated NPRS and molecular clocks suggested that the divergence between the *Gentianothamnus-Tachiadenus* clade and the *Exacum-Ornichia* clade fell between 11.2 and 29.8 Mya (Yuan et al., 2003). Our present analyses applying PL approaches with additional calibrations confirmed our previous results and suggested this divergence to be 9.0 to 39.4 Mya, well after the breakup of the Gondwana continent. In fact, reconciling dates of fossil data with molecular data, Magallon et al. (1999) showed that few angiosperm lineages had evolved at that time, and the divergence of the order Gentianales (53.2 Mya) was well after Gondwana breakup. Our present PL estimate on the first divergence of *Exacum*, the divergence between the Madagascan and Indian–Sri Lankan lineages was 8.2 to 35.6 Mya, whereas geological data suggest that the most recent close contact between the Madagascar–Seychelles and Indian plates was before 65 Mya (Storey, 1995). If this time frame is correct, it is obviously impossible to interpret the history of biogeography of *Exacum* as a result of Gondwana breakup (Klackenberg, 1985, 2002).

Our *Exacum* divergence estimates further suggest that the main infrageneric lineages diverged rapidly within a relatively short period of time, regardless of which calibration point is used. The four lineages "a, b, c, d" corresponding to the main areas of endemism seemed to have emerged within ca. 8 million years (9.0–6.5 to 35.6–27.7 Mya depending on calibration point). If the Gondwana hypothesis has to be rejected, dispersals and extensive radiations have to be considered as the main causes to explain the present-day distribution pattern of *Exacum*.

Out-of-Madagascar Dispersals

Unconstrained DIVA analysis suggested the basal nodes, MRCA of *Gentianothamnus-Tachiadenus* and *Ornichia-Exacum*, the MRCA of *Exacum* and *Ornichia*, and the MRCA of *Exacum* as widespread, and the first divergence within *Exacum* as the result of a vicariance event between Madagascar and Asian areas. As mentioned above, such a vicariance without dispersal involvement is physically impossible, because at the time of this divergence (8.2 to 35.6 Mya), Madagascar (area "B") was already isolated from other areas (Storey, 1995). If we assume the coexistence of the MRCA of *Gentianothamnus-Tachiadenus* and *Ornichia-Exacum*, the MRCA of *Exacum* and *Ornichia*, and the MRCA of *Exacum*, in Madagascar, Socotra, and other Asian areas, we have to assume extinctions of *Gentianothamnus*, *Ornichia*, and *Tachiadenus* in Socotra and all other Asian areas, which is an unlikely scenario. Such a resolution is because DIVA considers wide distribution and vicariance as "default" optimization (Ronquist, 1996, 1997).

The constrained optimization of DIVA offers a more plausible estimation of the historical biogeography of *Exacum*, particularly with regard to the divergence of the basal lineages corresponding to the main areas of endemism around the IOB. As shown in Fig. 4, the constrained DIVA optimization suggested the MRCA of *Exacum* and its sister group, *Ornichia*, originated in Madagascar. The constrained DIVA optimization specified unambiguously four dispersal events within *Exacum*: an initial dispersal of the MRCA of *Exacum* from Madagascar (area "B") to Sri Lanka and south India (area "C") at early diversification stage of the genus (when there was only a single ancestral taxon in *Exacum* extant), the dispersal of the MRCA of the clades "b" and

"c-d" from Sri Lanka–South India (area "C") to Socotra–Arabia (area "D"), the dispersal of the MRCA of the clade "c" from the area "C" to the mainland Southeast Asia (area "G"), and the dispersal of the MRCA of *E. oldenlandioides* and its sister clade from Madagascar to Africa. Each of these dispersal events was followed by a vicariance. Because the present dating suggested the first divergence of *Exacum* was well after the Gondwana breakup, these unambiguous vicariance events revealed by DIVA cannot be the results of tectonic history, but instead the results of single-dispersal events. By the time of *Exacum* divergence (<35.6 Mya), the plates of mainland Africa, Madagascar, and Sri Lanka–south India had mostly attained their present-day positions (ca. 500 km between mainland Africa and Madagascar, and ca. 3600 km between Madagascar and Sri Lanka–south India), and therefore, these were necessarily long-distance dispersals.

Having originated in Madagascar, *Exacum* has experienced multiple out-of-Madagascar dispersals. The most important is the long-distance dispersal to Sri Lanka–south India, which resulted in the extensive radiation of the Socotra–Arabia and other Asian lineages in the northern IOB regions. More recent out-of-Madagascar dispersals include the dispersal to the African continent, as represented by the divergence of *E. oldenlandioides*, and to other islands around Madagascar, as indicated by the distributions of *E. stenopterum*, which occurs in both Madagascar and the Comoros, and *E. quinquenervium*, which occurs in both Madagascar and the volcanic island of Mauritius (Klackenberg, 1985).

These long-distance dispersals could be directly or via stepping-stones. The recent dispersal from Madagascar to Africa (4.7 Mya), which resulted in the divergence of *E. oldenlandioides*, could have happened directly or via the Comoros as stepping-stones (the oldest island, Mayotte, is 5.5 Mya old; Emerick and Duncan, 1982). As for the dispersal from Madagascar to the Indian subcontinent, in addition to the possibility of direct dispersal, an Eocene–Oligocene land bridge (Steenis, 1962) or the so-called "Lemurian stepping-stones" (Schatz, 1996) connecting India, Sri Lanka, the Seychelles, and Madagascar has been proposed as the main channel for plants and animals to disperse across the IOB. In fact, dispersals through this channel can be considered as stepwise long- or medium-distance dispersals because there is no geological evidence for a continuous land bridge known today (the Seychelles at present are ca. 1000 km from Madagascar and ca. 2600 km from Sri Lanka–south India) (Schatz, 1996). Further evidence is needed to confirm if such a dispersal channel has ever existed. Nevertheless, with regard to *Exacum* there is no reason to exclude the possibility of dispersals via the "Lemurian stepping-stones," although no extant *Exacum* species is found on the Seychelles (Klackenberg, 1985, 2002).

Yet the process and mechanism of these long-distance dispersals are not well understood. Without apparent epizoic or hydrozoic adaptations, the possible means of dispersal of *Exacum* are wind and birds. Birds have been proposed as the most plausible and common agents of long-distance dispersals for carrying seeds or fruits of continental plants to colonize oceanic islands. For example, it was suggested that the Hawaiian colonizer of the genus *Viola* was dispersed from the Arctic areas by migratory birds (Ballard and Sytsma, 2000). Birds might have played roles in transferring *Exacum* from Madagascar to the Sri Lanka–south India area. As soon as *Exacum* arrived in the Sri Lanka–south Indian area, it might have extensively expanded its range by means of anemochory dispersals. Although wind dispersal usually represents a slow and gradual process of range expansion, it could fulfill a relatively rapid radiation for *Exacum*: although the seeds of *Exacum* are small and light, and released from xerochastic capsules by external forces such as wind, they might be too large to be carried far by wind (like orchids). However, many extant species of *Exacum* have prominent wings along the ribs of the saclike calyx, and the calyx, particularly the wings, become conspicuously enlarged and hardened during fruit development (Klackenberg, 2002). Seeds or capsules entrapped in the calyx sac can quickly travel long distances along the ground in an open environment when caught in wind. The two most widespread species, *E. tetragonum* and *E. oldenlandioides*, indicate such a capability. The former is widespread in many open places across the entire Indomalesia region, from south India, the Himalayas, mainland Southeast Asia, to the Philippines, New Guinea, and northern Australia, whereas the latter is seen in almost the entire tropical Africa with little morphological variation, further supporting a rapid and extensive expansion in distribution.

An extensive radiation of *Exacum* in the northern IOB conforms to the paleoclimate variation in these regions (deMenocal, 1995; Cerling et al., 1997; Ramstein et al., 1997; Zachos et al., 2001; Billups and Schrag, 2002; Griffin, 2002). It is plausible to speculate that this genus originated in Madagascar when the global temperature was rising, and soon after its origin, the progenitor of *Exacum* was dispersed to the area of Sri Lanka–south India by long-distance dispersal. Following the colonization of this area, this ancestral lineage spread out in the northern IOB regions including the Socotra–Arabia area (by that time the Afro–Arabia plates had attained its connection with Asia but Socotra was still connected to Arabia) and diversified at different places as a response to the favorite warm and humid climate during the Eocene or the Miocene climatic optimum (17 to 15 Mya). At the same time, Madagascan lineages had undergone extensive diversification as well. Subsequently, following the spreading Neogene drought, particularly towards the end of the Miocene, due to a decrease of atmospheric CO_2 (Cerling et al., 1997), a global cooling linked to the reestablishment of a major ice sheet over Antarctica (Zachos e al., 2001; Billups and Schrag, 2002) and the drying up in the northern IOB regions induced mainly by the rapid uplifting of the Himalayas (deMenocal, 1995; Ramstein et al., 1997; Griffin, 2002), global vegetation experienced a drastic change (e.g., widespread increase of C4 biomass in favor of the dry climate). Consequently,

Exacum species were forced to retreat from most parts of the northern IOB regions and survived in isolation in Socotra, south India–Sri Lanka, and perhaps mainland Southeast Asia. The Madagascan lineage was probably less drastically affected. From these core areas, "secondary" diversifications and radiations occurred later on, which resulted in several relatively distantly related clusters of species—the main clades we observe today. A similar pattern has been suggested for the genus *Nepenthes* (Meimberg et al., 2001). It is thought that *Nepenthes* has originated in the north of the ancient Tethys sea or on the Indian subcontinent after it was separated from Madagascar. This progenitor is supposed to have undergone extensive spreading: on land migration to Southeast Asia, and dispersals through land bridge or stepping-stones to the Seychelles and Madagascar. This habitat expansion was followed by incisive extinctions of the widespread progenitors in the paleotropical regions (i.e., fragmentation). Subsequently, secondary diversification occurred on the Southeast Asian islands, whereas the species on the Seychelles and Madagascar remained relatively unchanged (Meimberg et al., 2001).

The collision of the Afro–Arabian plate with Asia opened another channel of migration for flora and fauna between Africa and Asia since the Miocene (Samuei et al., 1997). Furthermore, a possible land bridge between mainland Africa and Madagascar due to a temporal dryout in large areas of the Mozambique Channel between 45 and 26 Mya has been suggested as a possible channel for mammals to colonize Madagascar (McCall, 1997). Our present reconstructions of the phylogeny and ancestral areas, however, do not offer any support for a dispersal of *Exacum* from Madagascar to the Socotra–Arabian area via mainland Africa and then to India–Sri Lanka, because our phylogeny suggested that the Sri Lanka–south India lineage diverged prior to the divergence between the Socotra–Arabian and Indomalesian clades. Moreover, such a consideration has to assume a complete extinction of the first African colonizers on the entire African continent. The widespread African species *E. oldenlandioides* is firmly shown to have originated from a recent dispersal from Madagascar. The only other extant African species, *E. zombense*, endemic to the Zomba mountain of Malawi, was unavailable for our present phylogenetic assessment. However, morphologically it is similar to the recently dispersed *E. oldenlandioides* (Klackenberg, 1985) and unlikely represents the relict of the first African colonizers.

The present article has given an interesting insight into the evolution and biogeographic history of the genus *Exacum* and sheds lights on the noticeable biogeographic patterns of the paleotropical regions around the IOB. Despite some uncertainty in dating caused by the scarcity of reliable fossils, we were able to establish, to the best of our knowledge, a geographical and temporal origin of *Exacum* and older lineages therein. The evolution history of *Exacum* apparently involved a combination of long-distance dispersal events and extensive radiations.

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Appendix 1. Sources of material used for the *Exacum* molecular phylogeny. Herbaria are abbreviated as follows: E, Royal Botanic Garden Edinburgh; NEU, University of Neuchâtel; S, Swedish Museum of Natural History Stockholm. Sequences newly acquired are marked with an asterisk.

Species	Voucher specimen and herbarium[a]	Origin	GenBank accessions	
			ITS	*trn*L intron
Exacum affine I. B. Balf. ex Regel—M8238a	M & al. 8238a, E	Socotra	AJ489877	AJ490202
E. affine I. B. Balf. ex Regel—M17126 (= *E. gracilipes* I. B. Balf.)	M & al. 17126, E	Socotra	AJ489886	AJ490211
E. affine I. B. Balf. ex Regel—M6201	M & al. 6201, E	Oman	AJ489879*	AJ490204*
E. affine I. B. Balf. ex Regel—Wcff5	W cff5, NEU	Socotra	AJ489878*	AJ490203*
E. atropurpureum Bedd.	K & L 526, S	India	AJ489880*	AJ490205*
E. bulbilliferum Baker	W & C M070, NEU	Madagascar	AJ489881*	AJ490206*
E. caeruleum I. B. Balf.	M & al. 11356, E	Socotra	AJ489882	AJ490207
E. dolichantherum Klack.	W & C M064, NEU	Madagascar	AJ489883*	AJ490208*
E. exiguum Klack.	Pi, W & Z M048, NEU	Madagascar	AJ489884*	AJ490209*
E. fruticosum Humbert	W & P M055, NEU	Madagascar	AJ489885	AJ490210
E. hamiltonii G. Don	Wo 7477, E	Bhutan	AJ489887	AJ490212
E. humbertii Klack.	W & P M052, NEU	Madagascar	AJ489888*	AJ490213*
E. intermedium Klack.	W & La M060, NEU	Madagascar	AJ489889*	AJ490214*
E. linearifolium (Humbert) Klack.	M & R 6254, S	Madagascar	AJ489890*	AJ490215*
E. macranthum Arn. ex Griseb.—FK767	F & K 767, S	Sri Lanka	AJ489892*	AJ490217*
E. macranthum Arn. ex Griseb.—Zsl003	Z sl003, NEU	Sri Lanka	AJ489891*	AJ490216*
E. marojejyense Humbert	W & P M056, NEU	Madagascar	AJ489893	AJ490218
E. microcarpum Klack.	W & La M061, NEU	Madagascar	AJ489894*	AJ490219*
E. millotii Humbert	W & P M057, NEU	Madagascar	AJ489895*	AJ490220*
E. nummularifolium Humbert	W & P M058, NEU	Madagascar	AJ489896	AJ490221
E. oldenlandioides (S. Moore) Klack.	Re 9275, S	Burundi	AJ489897	AJ490222
E. pallidum (Trim.) Klack.	F & K 777, S	Sri Lanka	AJ489898*	AJ490223*
E. pedunculatum L.	B, Ke & T 4, S	Sri Lanka	AJ489899*	AJ490224*
E. quinquenervium Griseb.	W M063, NEU	Madagascar	AJ489900	AJ490225
E. sessile L.	K & L 349, S	India	AJ489901*	AJ490226*
E. stenophyllum Klack.	Pi, W & Z M049, NEU	Madagascar	AJ489902	AJ490227
E. subacaule Humbert	M 3755, S	Madagascar	AJ489903*	AJ490228*
E. subteres Klack.	W & P M053, NEU	Madagascar	AJ489904*	AJ490229*
E. subverticillatum Humbert	Madagascar, s. n., NEU	Madagascar	AJ489905*	AJ490230*
E. sutaepense Hosseus ex Craib	Ch 99 - 230, NEU	Thailand	AJ489906*	AJ490231*
E. tetragonum Roxb.—K254	Keke 254, E	Nepal	AJ489908*	AJ490233*
E. tetragonum Roxb.—LK332	L & K 332, S	India	AJ489907	AJ490232
E. trinervium (L.) Druce—Zsl001	Z sl001, NEU	Sri Lanka	AJ489910*	AJ490235*
E. trinervium (L.) Druce—Zsl002	Z sl002, NEU	Sri Lanka	AJ489909	AJ490234
E. walkeri Arn. ex Griseb.—K539	K 539, S	Sri Lanka	AJ489911*	AJ490236*
E. walkeri Arn. ex Griseb.—Zsl004	Z sl004, NEU	Sri Lanka	AJ489912*	AJ490237*
E. wightianum Arn.	K & L 188, S	India	AJ489913	AJ490238
Gentianothamnus madagascariensis Humbert	G G020, NEU	Madagascar	AJ489914	AJ490240
Ornichia madagascariensis (Baker) Klack.	W M002, NEU	Madagascar	AJ489917	AJ490242
O. trinervis (Desrousseaux) Klack.	C s. n., NEU	Madagascar	AJ489918	AJ490243
Tachiadenus carinatus (Desrousseaux) Griseb.	W M059, NEU	Madagascar	AJ489923	AJ490249
T. longiflorus Bojer ex Griseb.	W M006, NEU	Madagascar	AJ489924	AJ490250

[a] Abbreviation of the collectors: B = Bremer; C = Callmander; Ch = Chassot; F = Fagerlind; G = Gautier; K = Klackenberg; Ke = Kerr; L = Lundin; La = Laivao M = Miller; P = Pfund; Pi = Piso; R = Randrianasolo; Re = Reekmans; T = Theran; W = Wohlhauser; Wo = Wood; Z = Zeltner.