

**PHYLOGENETIC RELATIONSHIPS OF THE MYCOHETEROTROPHIC
GENUS *VOYRIA* AND THE IMPLICATIONS FOR THE
BIOGEOGRAPHIC HISTORY OF GENTIANACEAE¹**

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- *Premise of the study:* The angiosperm family Gentianaceae comprises over 1700 species in 91 genera. Gentianaceae are distributed worldwide, but most species occur in temperate zones. Phylogenetic studies demonstrate that the family consists of six monophyletic tribes. However, the phylogenetic position of the mycoheterotrophic genus *Voyria*, with a remarkable trans-Atlantic distribution, remained unknown.
- *Methods:* We used nuclear ITS and 18S rDNA and mitochondrial *apt1* and *matR* data to infer the phylogenetic position of *Voyria* in Gentianaceae. In addition, with Bayesian relaxed molecular clock analyses we obtained age estimates for the diversification of *Voyria* and Gentianaceae in general and used these results to reconstruct the ancestral areas associated with the early diversification events in Gentianaceae.
- *Key results:* Our results demonstrate that *Voyria* is an early diverging lineage within Gentianaceae with no close relationships to other mycoheterotrophic Gentianaceae lineages. *Voyria* originated in the neotropics during the Early Eocene but only reached its current transoceanic distribution around the end of the Oligocene. The neotropics were an important area for the early diversification events in Gentianaceae, most of which occurred during the Eocene.
- *Conclusions:* *Voyria* is an old, phylogenetically isolated lineage within Gentianaceae, and the current distribution of the genus is indicative of the ancestral area in which the early diversification events of Gentianaceae occurred. In parallel with many other pantropical families, our results suggest that migration of tropical taxa through Laurasia during the Early Eocene has played an important role in shaping the current global distribution of Gentianaceae.

Key words: Disjunct distribution; Gentianaceae; long-distance dispersal; mycoheterotrophy; *Voyriaeae*

Gentianaceae are the third largest family of the Gentianales, with Apocynaceae and particularly Rubiaceae being considerably more species-rich. Gentianaceae include ca. 1700 species in 91 genera (Gentian Research Network, 2011). The current classification of relationships within Gentianaceae is mainly based on a family-wide phylogenetic analysis of plastid *matK* and *trnL* data by Struwe et al. (2002), who recognized six monophyletic and well-supported tribes: Saccifolieae, Exaceae, Chironieae, Gentianeae, Helieae, and Potalieae. Subsequent phylogenetic studies in Gentianaceae have mainly focused on resolving evolutionary relationships and biogeographic history within these tribes (e.g., Yuan et al., 2003, 2005; Chassot 2003; Mansion and Struwe, 2004; Chen et al., 2005; Kissling et al., 2009; Molina and Struwe, 2009; Struwe et al., 2009a, 2009b; Favre et al., 2010). The first branching lineages of all tribes of Gentianaceae with the exception of Gentianeae involve tropical

members, suggesting that the family has a tropical origin. Major diversification events have occurred in South America, Africa, and the region around the Indian Ocean, pointing toward a southern hemisphere origin of the major clades in the family (Struwe et al., 2002). Struwe et al. (2002) speculated that the early diversification events in Gentianaceae may result from Gondwanan vicariance, but this has been challenged by molecular clock analyses indicating that Gentianaceae diversification postdates the breakup of Gondwana (Bremer et al., 2004; Yuan et al., 2005).

Despite the extensive phylogenetic work carried out in Gentianaceae, the phylogenetic and taxonomic position of one enigmatic genus remained problematic: *Voyria* Aubl. *Voyria* contains 19 species of which 18 occur in tropical South America and one (*V. primuloides* Baker) in West and Central Africa (Raynal, 1967a; Maas and Ruyters, 1986). In the neotropics the distribution of *Voyria* ranges from Mexico, Cuba, Florida and the Bahamas in the north, to Bolivia, southeast Brazil and Paraguay in the south. The main center of species diversity is the Guiana Shield, where 11 of the 18 species are native (Maas and Ruyters, 1986). In Africa, *V. primuloides* is known from Cameroon, DR Congo, Gabon, Ghana, Ivory Coast, and Liberia (Raynal, 1967a; Cheek, 2006). All species of *Voyria* have a fully mycoheterotrophic mode of life, i.e., they obtain carbohydrates from surrounding photosynthetic plants through shared arbuscular mycorrhizal fungi (Merckx et al., 2010; Courty et al.,

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2011). Most species of *Voyria* rely on relatively narrow lineages of *Glomus* fungi (Bidartondo et al., 2002; Courty et al., 2011; Merckx et al., 2012). Mycoheterotrophy allows the plants to thrive in the dark understory of tropical forests, where they generally grow in moist decaying leaf mold, and only a few species prefer drier vegetation types like savannas and savanna forests (Maas and Ruyters, 1986).

In Gentianaceae, mycoheterotrophy is not restricted to *Voyria*. The South American genus *Voyriella* (tribe Saccifolieae) consists of a single fully mycoheterotrophic species (Maas and Ruyters, 1986), and *Exacum* and *Exochaenium* (tribe Exaceae) include four Southeast Asian and one African fully mycoheterotrophic species, respectively (Raynal 1967b; Klackenberg, 2006; Kissling, 2012). Although for the latter species, *Exochaenium oliganthum* (Gilg) Kissling, green individuals have been reported as well (Kissling 2012). Natural abundance ^{13}C profiles of species in *Bartonia* and *Obolaria* (tribe Gentianeae) suggest that these chlorophyllous species can gain carbon from arbuscular mycorrhizal fungi, and are thus considered partial mycoheterotrophs (Cameron and Bolin, 2010). In addition, partial mycoheterotrophy is suggested to occur in *Curtia tenuifolia* (Aubl.) Knobl. (Saccifolieae) and species of *Neurotheca* (tribe Potalieae) as well, but evidence for this is lacking (Struwe et al., 2002; Molina and Struwe, 2009).

While some species of *Voyria* are relatively common, the genus was never included in a molecular phylogenetic study. Hence *Voyria* is currently classified as 'incertae sedis' within Gentianaceae (Stevens, 2001; Struwe et al., 2002). Most phylogenetic studies of Gentianaceae are based on data from the chloroplast genome, thus explaining the exclusion of *Voyria* taxa. As in most mycoheterotrophic plants, amplification of plastid genes is difficult or in some cases impossible due to the loss or high divergent nature of the photorespiratory genes in these species (Wickett et al., 2008; Delannoy et al., 2011; Logacheva et al., 2011). Indeed, Struwe et al. (2002) reported that plastid *trnL* intron data were obtained for two *Voyria* species, but that the sequences were too divergent to confidently compare with other Gentianaceae sequences.

When DNA data from the chloroplast genome cannot be obtained or is too divergent from photosynthetic relatives to be included in a chloroplast DNA dataset, nuclear and mitochondrial DNA data may offer valuable alternatives. Yet for reasons that remain unknown, substitution rates of nuclear and mitochondrial genes or intergenic regions in heterotrophic plants are often highly elevated as well (Lemaire et al., 2011). This rate heterogeneity may cause bias in phylogenetic inference. In particular maximum parsimony methods may be misled due to 'long-branch attraction' artifacts (Merckx et al., 2009). In these cases model-based reconstruction methods should be preferred. In their 2002 analysis, Struwe et al. did obtain nuclear 18S rDNA data from *Voyria* and were able to align these sequences to include in their analysis. While these data suggested that *Voyria* be placed among the basal clades of Gentianaceae (Struwe et al., 2002), the results also questioned the monophyletic status of the genus suggesting a potential for noise within the data to have influenced the results.

To infer the phylogenetic position of *Voyria* within Gentianaceae, we analyzed nuclear 18S and ITS rDNA as well as mitochondrial *matR* and *atp1* data from 11 *Voyria* species along with representatives of all six Gentianaceae tribes. Datasets were analyzed with maximum likelihood (ML) and Bayesian inference (BI) methods to reduce the risk of long-branch attraction artifacts. In addition, a family-wide ITS dataset was

assembled and analyzed with a Bayesian relaxed clock method to place the divergence of *Voyria*, as well as Gentianaceae in general, in a temporal framework. The resulting tree was then used to infer the ancestral areas associated with the early diversification events in Gentianaceae.

MATERIALS AND METHODS

Plant material and sampling—Appendix S1 (see Supplemental Data with the online version of this article) lists all taxa included in this study with voucher information and Genbank accession numbers. In total 44 species of Gentianaceae, including 11 *Voyria* species were newly sampled for this study. Three Rubiaceae species were used as part of the outgroup as recent phylogenetic investigations suggest that Rubiaceae are the first diverging lineage of Gentianales (Soltis et al., 2011). Sequences of Apocynaceae, Loganiaceae, and Gelsemiaceae were obtained from Genbank to represent the remaining Gentianales families.

DNA extraction, PCR amplification, and sequencing—DNA was extracted from silica-dried plant material with the DNeasy Plant Mini Kit (QIAGEN, Venlo, Netherlands) following the manufacturer's instructions. Amplification of the 18S rDNA region was carried out with the primers NS1, NS2, NS3, NS4, NS5, and NS8 (White et al., 1990) under the following conditions: 1 min at 94°C followed by 30 cycles of 30 s at 94°C, 30 s at 44°C and 1 min at 72°C, followed by 7 min at 72°C. The ITS region was amplified with primers ITS1 and ITS4 from White et al. (1990) with 4 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 44°C and 1.5 min at 72°C, followed by 7 min at 72°C. Mitochondrial *matR* sequences were amplified with primers *matR-26F*, *matR-1002R*, *matR-879F*, and *matR-1858R* (Davis and Wurdack, 2004) under the following conditions: 1 min at 94°C followed by 30 cycles of 30 s at 94°C, 45 s at 52°C and 1 min at 72°C, followed by 7 min at 72°C. Mitochondrial *atp1* sequences were amplified with the primers and conditions described in Eyre-Walker and Gaut (1997). Sanger sequencing was performed by the Macrogen sequencing facilities (Macrogen, Inc., Seoul, South Korea).

Sequence assembly and alignment—Sequences were assembled and edited with Geneious Pro version 5.5.6 (Drummond et al., 2011). Sequence alignments were generated with the MAFFT version 6.814b alignment tool (Katoh et al., 2002) implemented in Geneious Pro. A few short regions of the ITS dataset totaling 57 nucleotide positions were excluded as the alignment was considered too ambiguous due to high sequence divergence in these regions. The ITS1 and ITS2 regions of the sequences obtained for *Voyria tenella* Hook. (from a population in French Guiana [KC535877]; and from a population in Colombia [KC535876]) and *V. obconica* Progel (from two separate populations in Brazil: [KC535871- KC535872]) were highly divergent from other *Voyria* species and Gentianaceae in general, and therefore we did not include the ITS data for these species in the final phylogenetic analyses. A BLAST search of these divergent sequences on Genbank did not identify close matches. Because similar (but not identical) sequences were acquired from different populations, it seems unlikely that the obtained ITS sequences are the result of contamination. A ML analysis of the ITS region including *V. tenella* and *V. obconica* places both species within *Voyria*, but on extremely long branches (Appendix S2A) (see Supplemental Data with the online version of this article). This indicates that the divergence observed in these sequences is the result of extremely high substitution rates.

Phylogenetic analyses—Phylogenetic reconstructions were conducted using both ML and BI optimality criteria. First, each of the four DNA datasets was analyzed separately. The substitution model for each DNA region was selected with jModeltest version 0.1.1 (Posada, 2008) under the Akaike Information Criterion (AIC). The best-fitting model for all datasets data was GTR+I+G. Searches for the best likelihood tree were performed with RAXML version 7.2.8 (Stamatakis, 2006). Clade support was estimated by nonparametric bootstrap analyses on 200 pseudo-replicate data sets. We assessed topological congruence between the resulting trees following Seelanan et al. (1997) using a bootstrap cutoff of 75%. A ML analysis on the combined data were performed using the same settings as described in the paragraph above, with a GTR+I+G model applied to each of the four data partitions. In addition Bayesian analyses were conducted on the combined dataset with MrBayes version 3.2.1 (Ronquist et al., 2012) with a GTR+I+G model applied to each of the four data partitions.

Four analyses, each consisting of four Markov chains starting with a random tree, were run simultaneously for five million generations, sampling trees at every 1000th generation. The first 1000 sampled trees of each analysis were regarded as 'burn in' and discarded. A majority rule consensus tree was calculated using the remaining 16000 trees.

Estimation of divergence times—To obtain age estimations of the diversification events in Gentianeaceae, we constructed an ITS data set with 328 taxa, including representatives of all Gentianeaceae tribes and three Rubiaceae outgroup taxa. See Appendix S3 for a list of included accessions. Alignment was performed with MAFFT. Despite the considerable variation in the ITS sequence data an initial unconstrained ML analysis resulted in a well-resolved phylogeny, in which all tribes are monophyletic groups. However, the relationships between the tribes slightly differ from established relationships (e.g., Struwe et al., 2002). This ML tree is shown in Appendix S4 (see Supplemental Data with the online version of this article). With this ITS alignment a Bayesian lognormal relaxed clock analysis was performed in BEAST version 1.7.1 (Drummond et al., 2012), in which the topology was constrained to reflect the current knowledge of the relationships between the Gentianeaceae tribes ("T1"): (Rubiaceae, (Saccifoliae, (Exaceae, (Voyriaceae, (Chironieae, (Potalieae, (Helieae, Gentianeae)))))); (Struwe et al., 2002; this study). In addition, the analysis was repeated using a similar constraint, but with *Voyria* as the second diverging lineage of Gentianeaceae and Exaceae as the third diverging lineage of Gentianeaceae ("T2"). For both analyses three calibration priors were specified: (1) a broad normal distribution of 79 ± 10 million years ago (Ma) was applied to the root of the tree, reflecting the age estimate of the crown node of the Gentianales obtained by Janssens et al. (2009). This estimate is also consistent with that reported by Bremer et al. (2004); (2) a normal distribution of 37 ± 1 Ma on the crown node of the Potalieae, reflecting *Lisianthus* fossil pollen data from the Late Eocene (Graham, 1984; Yuan et al., 2005; Favre et al., 2010); and (3) a normal distribution of 15 ± 1 Ma on the crown node of the subtribe Swertiinae (Gentianeae) (von Hagen and Kadereit, 2002; Yuan et al., 2005; Favre et al., 2010). All other priors were set to 'uniform'. To overcome the 'zero likelihood' error that prevented the Markov chain Monte Carlo (MCMC) run from starting, we constructed a start tree with RAXML and r8s version 1.70 (Sanderson, 2002) that was in agreement with all topological and calibration priors of the BEAST analysis. The Bayesian MCMC analysis was run for 10^8 generations, sampling every 5000th generation. Using TRACER version 1.5 (Rambaut and Drummond, 2007) the effective sampling sizes of all parameters were found to exceed 200, suggesting that they are good representations of the posterior distributions. A maximum clade credibility tree was calculated on the last 15000 sampled trees using TreeAnnotator version 1.7.1 (Drummond et al., 2012).

Ancestral area reconstructions—The BEAST mean age consensus trees of T1 and T2 were pruned so that one species of each tribe was retained. We defined the following global areas of distribution for all seven Gentianeaceae tribes: N = neotropics and temperate regions of South America, P = paleotropics and temperate regions of the paleotropics, and T = northern temperate regions (Fig. 1A). Saccifoliae and tribe Helieae only occur in the neotropics (Struwe et al., 2002). Voyriaceae were also considered to have a neotropical origin, because the sole African species clearly evolved from a neotropical ancestor (see further). Exaceae are mainly found in the paleotropics. However, several species occur in temperate South Africa, Australia and New Zealand. Africa was inferred as the ancestral area of Exaceae (Kissling, 2007). Tribe Chironieae consists of three subtribes: Chironiinae with a predominantly northern temperate distribution, Coutoubeinae with a neotropical distribution, and Canscorinae with a paleotropical distribution (Struwe et al., 2002). Therefore the Chironieae was considered present in all three regions. Potalieae have a pantropical distribution and thus were assigned to both neotropics and paleotropics (Struwe et al., 2002). Lastly, most species of the tribe Gentianeae grow in northern temperate regions and this area is considered to be the ancestral area of the tribe (Struwe et al., 2002).

For the reconstruction of ancestral areas we used Lagrange 0.1 beta 2 for Mac OS 10.6 (Ree and Smith, 2008) with trees T1 and T2. We compared the results of three different dispersal models. In model 0 (M0) dispersal probabilities between all areas were set to 1.0 (e.g., no dispersal constraints). In model 1 (M1) the dispersal probability between the neotropics and the paleotropics was set to 0.1, to simulate low probability of long-distance dispersal events. Dispersal probability was set to 0.9 for dispersal from the neotropics to the northern temperate region, and from the paleotropics to the northern temperate region. Dispersal from northern temperate region into the tropics was constrained to 0.01 to avoid unlikely exchange between the neotropics and paleotropics through temperate regions. Under model 2 (M2), we assumed that warmer climate and associated migration of tropical floras to higher latitudes

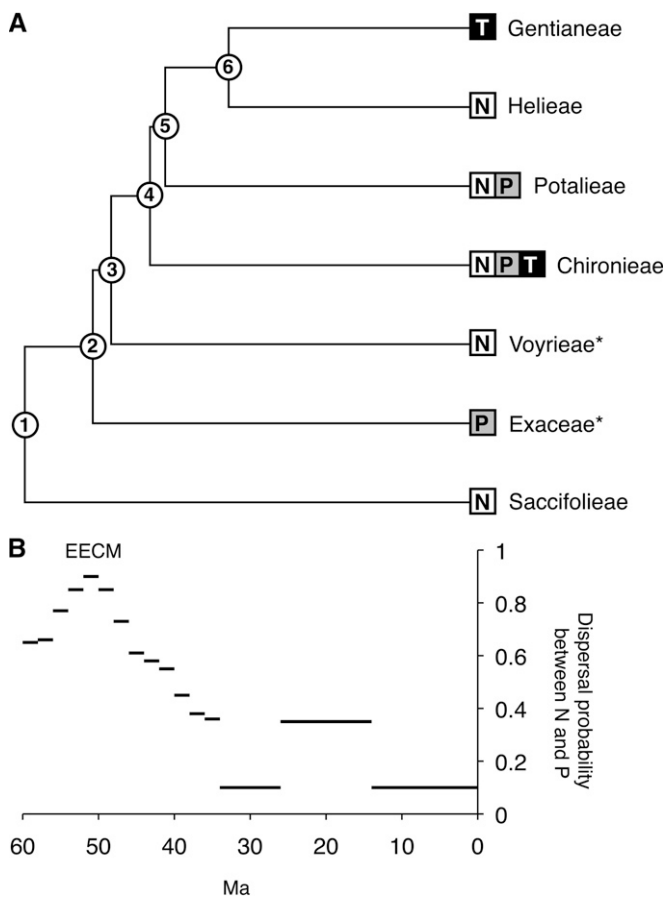


Fig. 1. (A) Topology (T1) and current distribution ranges of the tribes used for the ancestral area analyses. Node numbers correspond with Table 2. N = Neotropics, P = Paleotropics, T = Northern temperate regions. * For topology T2, the position of Voyriaceae and Exaceae was interchanged. (B) Graphical representation of the dispersal probabilities between the neotropics and the paleotropics used under model 2 (M2). EECM = Early Eocene Climatic Maximum.

has facilitated dispersal between the neotropics and the paleotropics, particularly during the warm Early Eocene (e.g., Davis et al., 2002; Renner, 2005). Therefore global temperature fluctuations were used as a proxy to model the dispersal probabilities between the neotropics and the paleotropics covering a range from 0.1 (during glacial periods) to 0.9 (during the Eocene climatic optimum): from 60 to 34 Ma dispersal probabilities were modeled in 2 Ma intervals using the deep-sea temperature reconstructions of Zachos et al. (2001); from 34 to 26 Ma dispersal probability was constrained to 0.1 representing the Oligocene glacial period (Zachos et al., 2008); from 26 to 14 Ma dispersal was constrained to 0.35 to account for the warmer temperatures during from the Late Oligocene to the Middle Miocene; from 14 Ma to the present chances of dispersal between the neotropics and the paleotropics were considered to be low and were constrained to 0.1 (see Fig. 1B for a visual representation of these dispersal probabilities). Dispersal probabilities between the tropical regions and the northern temperate region were set as described under model 1. For all models, all combinations of areas were allowed in the adjacency matrix, and baseline rates of dispersal and local extinction were estimated. In addition, for each model we compared the likelihoods when fixing the ancestral area of Gentianeaceae to either the neotropics (N), paleotropics (P), or both regions together (N+P).

RESULTS

Multigene phylogenetic analyses—The aligned sequence lengths were 649 bp (ITS), 1722 bp (18S rDNA), 1169 bp

(*atp1*), and 2054 bp (*matR*), resulting in a total length of 5595 bp. The highest likelihood tree and majority rule bootstrap consensus tree is shown for each dataset in Appendix S2 (see Supplemental Data with the online version of this article). These single-gene phylogenies do not show well-supported ($\geq 75\%$ bootstrap support [BS]) phylogenetic conflicts (except for the placement of *Voyria tenuiflora* Griseb. between the *atp1* and ITS trees) and therefore we combined the data sets for analysis. The highest-likelihood tree ($-\ln L = 32155.79$) is shown in Fig. 2 and does not show strongly supported ($\geq 75\%$ BS; $\geq 95\%$ BPP) alternative placements compared to the Bayesian analysis (not shown). In all analyses Gentianaceae are a well-supported monophyletic group (100% BS, 100% Bayesian Posterior Probability [BPP]). Saccifolieae (100% BS, 96% BPP) are sister to all other Gentianaceae with high support (100% BS, 95% BPP). The position of Exaceae (97% BS, 100% BPP) as the second diverging lineage of Gentianaceae receives weak support (63% BS, 77% BPP). Inspection of the trees sampled during bootstrap ML and Bayesian analyses reveals this is due to support obtained for an alternative topology in which *Voyria* is placed sister to all other Gentianaceae, except Saccifolieae. *Voyria* is a monophyletic group with high BS (97%) but nonsignificant BPP (89%). Gentianeae (91% BS, 100% BPP), Helieae (100% BS, 100% BPP), Potalieae (81% BS, 94% BPP), and Chironieae (98% BS, 100% BPP) form a well-supported clade (85% BS, 100% BPP). The position of Chironieae as sister group of Gentianeae, Helieae, and Potalieae receives weak support (51% BS, 87% BPP). The sister group relationship between Gentianeae and Helieae is well supported (100% BS, 100% BPP). *Voyria* consists of two well-supported clades: (1) *V. clavata* Splitg. and *V. caerulea* Aubl. (100% BS, 100% BPP); and (2) all other sampled *Voyria* species (100% BS, 95% BPP). Within the latter clade the sister group relationship between *V. tenella* and *V. obconica* is well supported (100% BS, 100% BPP). The only other node that is supported by both high BS and significant BPP is the node grouping *V. aphylla* (Jacq.) Pers., *V. aurantiaca* Splitg., and *V. tenuiflora* together.

Estimation of divergence times—The majority-rule consensus tree with mean branch lengths resulting from the BEAST relaxed molecular clock analysis under constraint T1 is shown in Fig. 3. Mean age estimations and corresponding 95% confidence intervals for the crown and stem node ages of Gentianaceae tribes for analysis T1 and T2 are listed in Table 1. The estimates date the crown node of Gentianaceae between 47.3 and 69.1 Ma (T1) or 54.6 and 78.6 Ma (T2). The divergence of *Voyria primuloides* is estimated to have occurred between 12.2 and 26.5 Ma (T1) or 12.4 and 27.7 Ma (T2).

Ancestral area reconstructions—The results from the ancestral area reconstructions with Lagrange are shown in Table 2. Under all models (M0, M1, M2) and topologies (T1, T2) the neotropics are inferred to be part of the ancestral area of the common ancestor of Gentianaceae, either as the sole ancestral area or together with the paleotropics. For both topologies log-likelihood scores under M1 were highest but the difference among the likelihoods of the three tested models was less than 2 log-likelihood units, and thus not statistically significant (Edwards, 1992; Ree and Smith, 2008). For T1 all models returned similar ancestral areas for the six nodes, except for node 1 for which the neotropics is the most probable ancestral area under M0 and M1, but a neotropical-paleotropical distribution is preferred under M2. Also, the ancestor of node 6 most probably occurred in the neotropics

under M0, while an ancestral neotropical-temperate ancestral range is inferred for this node under M1 and M2. A similar difference for node 6 is inferred for the models under T2. In addition, for T2, M2 preferred a neotropical-paleotropical ancestral distribution range for node 2, 3, and 4, while for M0 and M1 the ancestor of these nodes was most likely restricted to the neotropics. Under all topologies and models the neotropics were part of the ancestral area of the Gentianaceae. For the constrained analyses, all models received significantly lower log-likelihoods when the ancestral area of Gentianaceae was fixed to just the paleotropics (Table 3), except for M1 on T2.

DISCUSSION

Phylogenetic relationships of *Voyria*—Our phylogenetic results indicate that *Voyria* is a monophyletic group that is the sister lineage of all remaining Gentianaceae except for the tribes Saccifolieae and Exaceae. This position, however, is not well-supported and some of the ML bootstrap trees and trees sampled during the Bayesian MCMC run suggest that *Voyria* is the second diverging lineage of Gentianaceae. Nevertheless, both topologies indicate that *Voyria* is an early diverging lineage within Gentianaceae. The relationships obtained for the six recognized tribes of Gentianaceae are similar to previous results obtained with combinations of chloroplast and nuclear DNA data (Struwe et al., 2002; Kissling et al., 2009). Since *Voyria* is not embedded in any of these tribes, or the closest relative of a single tribe, the genus should be classified as a separate tribe: *Voyrieae* Gilg (including *Leiphaimeae* Gilg) following Albert and Struwe (1997). Our results also support the existence of two subgenera within *Voyria*, i.e., *Voyria* and *Leiphaimos*, as described by Albert and Struwe (1997) as well.

Relaxed molecular clock analysis—Our divergence age estimates of Gentianaceae are based on a sampling that consists of nearly 20% of all Gentianaceae species, but includes data from only a single DNA region. Moreover, we could use only a very limited number of calibration points. Therefore this hypothesis should be interpreted with caution. The divergence dates we obtained for nodes in Exaceae are congruent with the age estimates reported by Yuan et al. (2005) and Kissling (2007), who used different datasets and dating methods but a similar calibration strategy. However, additional fossil calibration points are needed to provide a more detailed understanding of the temporal aspects of Gentianaceae diversification.

Trans-Atlantic distribution of *Voyria*—*Voyria primuloides*, the only African species of *Voyria*, is phylogenetically embedded in *Voyria* and thus diverged from an ancestral neotropical lineage. According to our Bayesian relaxed clock analysis *V. primuloides* diverged from its neotropical ancestor during the Late Oligocene to the Middle Miocene [between 12–28 Ma]. Under the current molecular dating strategy, this age estimate should be regarded as a maximum age for this split because our incomplete sampling may lack the closest related extant relative of *V. primuloides*. This result confidently rejects the hypothesis that the trans-Atlantic distribution of *Voyria* is the result of Gondwanan vicariance; the last known land connection between South America and Africa is estimated to have disappeared at least 90 Ma (Raven and Axelrod, 1974). Also, the divergence of *V. primuloides* is estimated to have occurred

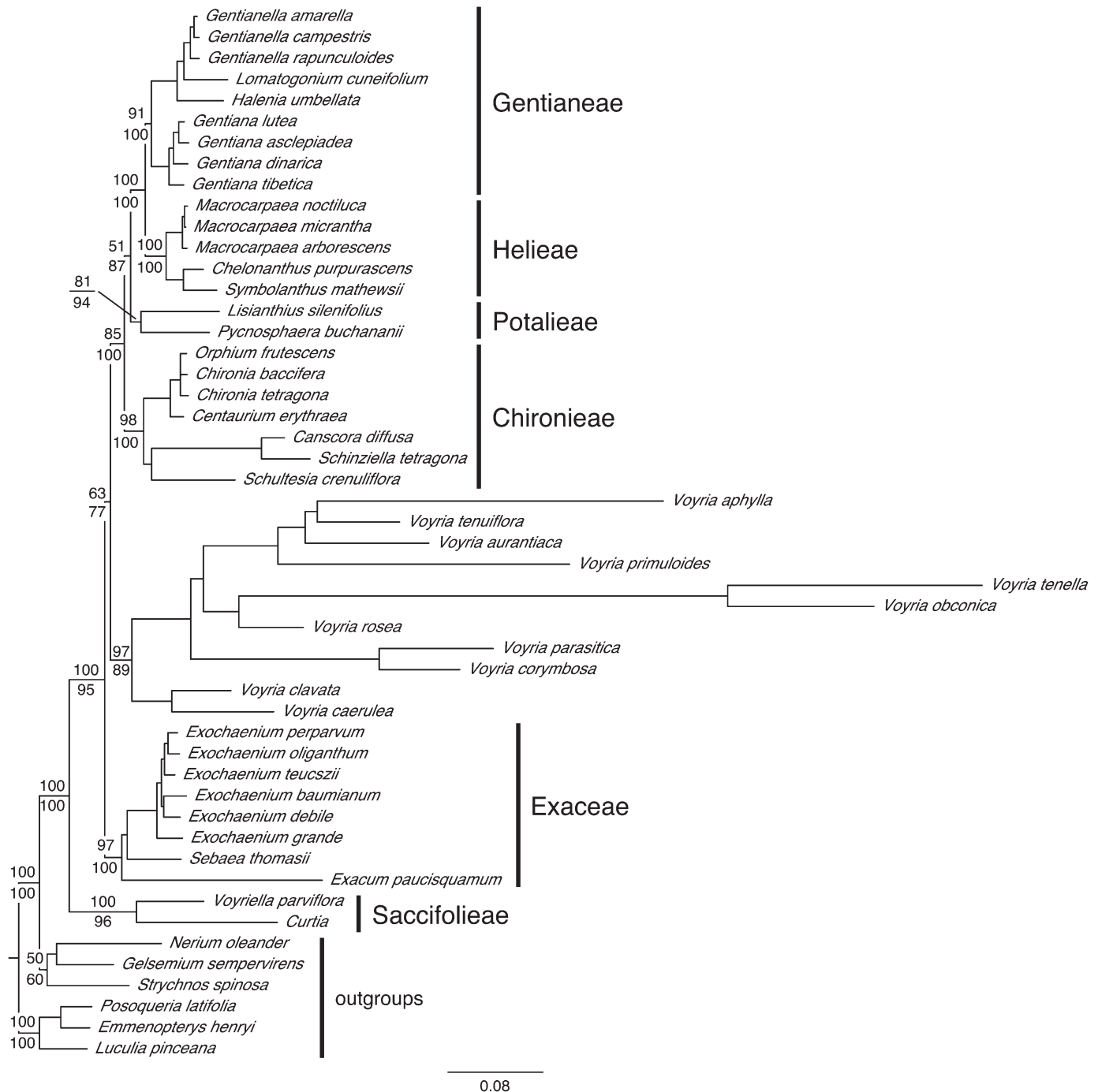


Fig. 2. The highest-likelihood tree from the 4-gene analysis. The taxon *Curtia* is represented by two species: *C. verticillaris* (Spreng.) Knobl. (18S rDNA, *matR*) and *C. tenuifolia* (Aubl.) Knobl. (ITS). Numbers above branches are bootstrap percentages. Numbers below branches are Bayesian posterior probability percentages. Names of the tribes are shown on the right and follow Struwe et al. (2002).

well after the Eocene and therefore it is unlikely that it is a relict from a boreotropical distribution of *Voyria* during the warm Early Eocene (Albert and Struwe, 1997). The Middle Miocene was characterized by warm temperatures as well, which resulted in the return of several thermophilic lineages to North America and Europe (Tiffney, 1985). Recent work has shown that this allowed for floristic interchange between North America and Europe (e.g., Tiffney, 2008; Denk et al., 2010), but it

remains unclear how important this Miocene North Atlantic Land Bridge was for interchange of tropical flora elements. Given our results, we postulate that *Voyria* reached the African tropics either by crossing the Atlantic using the Miocene North Atlantic Land Bridge, or via a long-distance dispersal event. Seeds of many species of *Voyria* are true “dust” seeds and are among the smallest known in land plants (Maas and Ruyters, 1986; Eriksson and Kainulainen, 2011). The widespread species

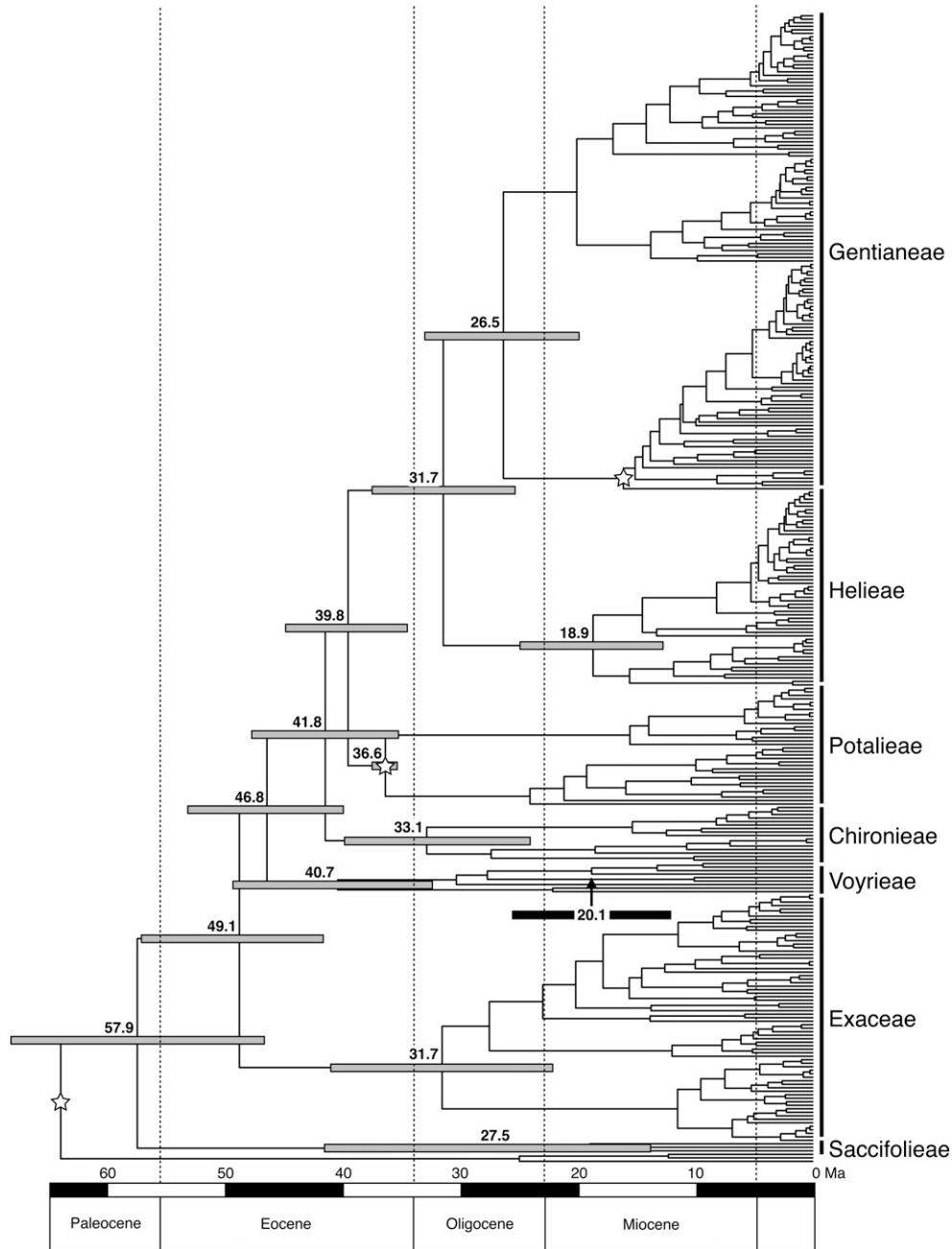


Fig. 3. Maximum clade credibility chronogram of Gentianaceae with 95% confidence intervals for the age estimates of the deep nodes shown in gray. The mean age estimates of these nodes are shown above the nodes in million years ago (Ma). Stars indicate calibration points. The divergence of *Voyria primuloides* is highlighted with an arrow, and the corresponding 95% confidence of this age estimate shown in black.

V. aphylla, *V. parasitica* (Schlecht. & Cham.) Ruyters & Maas, and *V. tenella*, which also occur on many of the Caribbean islands, have winged seeds allowing them to disperse by wind over very large distances (Maas and Ruyters, 1986). In contrast a few *Voyria* species, including *V. primuloides*, bear indehiscent fruits, which have been hypothesized to disperse by water or rodents (Maas and Ruyters, 1986; Hentrich et al., 2010). When these seeds are part of muddy rainwash they may be transported for long distances on animal vectors (Albert and Struwe, 1997). A disjunct distribution between the neotropics and Africa is not uncommon in flowering plants. Renner (2004) identified 110 genera with species on both sides of the Atlantic. Within Gentianaceae,

the genera *Enicostema*, *Neurotheca* (both Potalieae), and *Schulnesia* (Chironieae) also have trans-Atlantic distribution ranges (Struwe et al., 2002). In our age estimates the crown node ages of Potalieae and Chironieae postdate the Eocene North Atlantic Land Bridge (Table 1). Thus, similar to *Voyria*, trans-Atlantic distribution patterns of these genera might be explained by either long distance dispersal or by crossing the Miocene North Atlantic Land Bridge.

Early diversification history of Gentianaceae—The majority of extant Gentianaceae species occurs in temperate regions. However, most of these temperate species belong to Gentianeae

TABLE 1. Estimates for the crown and stem node ages of the Gentianaceae tribes.

Clade	Mean node age (Ma) + 95% CI BRC topology "T1"		Mean node age (Ma) + 95% CI BRC topology "T2"	
	Area	RP	Area	RP
Gentianaceae crown (= Saccifoliae stem)	57.9 (47.3-69.1)		66.2 (54.6-78.6)	
Saccifoliae crown	27.5 (14.6-41.1)		30.8 (18.1-46.1)	
Exaceae stem	49.1 (41.5-57.4)		48.6 (41.2-57.3)	
Exaceae crown	31.7 (21.7-41.6)		32.1 (24.8-41.0)	
Voyriace stem	46.8 (40.1-54.6)		54.0 (44.8-65.2)	
Voyriace crown	40.7 (31.9-49.4)		47.1 (34.8-59.0)	
Chironieae stem	41.8 (36.7-47.7)		42.6 (37.4-49.4)	
Chironieae crown	33.1 (24.4-40.8)		34.4 (26.2-42.2)	
Potalieae stem	39.8 (35.6-44.9)		40.2 (35.7-46.0)	
Potalieae crown ¹	36.6 (34.7-38.5)		36.8 (34.8-38.7)	
Helieae stem (= Gentianeae stem)	31.7 (25.0-37.9)		32.1 (25.9-38.7)	
Helieae crown	18.9 (13.1-24.8)		20.0 (14.8-26.6)	
Gentianeae crown	26.5 (20.6-32.8)		26.9 (21.3-32.8)	

¹ A prior distribution was assigned to this node (see Materials and Methods).

(Struwe et al., 2002). The phylogenetic relationships among the Gentianaceae tribes indicate that the family has a tropical origin and that the northern temperate lineages are the result of secondary radiations during gentian evolution. Interestingly, several Gentianaceae tribes are mainly restricted to either the neotropics (e.g., Saccifoliae, Helieae, Voyriace (except *V. primuloides*)) or the paleotropics (Exaceae—except *Sebaea*). Our molecular divergence-time estimates suggest that the seven Gentianaceae tribes predominantly result from Paleocene/Eocene diversification events, and thus are not the result of Gondwanan vicariant processes.

Disjunct distributions between the neotropics and paleotropics of clades that postdate the break-up of Gondwana are often assumed to be the result of a migration to through Laurasia during the Early Eocene, when climatic conditions supported tropical vegetation at those latitudes (e.g., Davis et al., 2002; Muellner et al., 2006; Merckx et al., 2008; Antonelli et al., 2009). Such a scenario fits well with the biogeographic patterns

and divergence age estimates obtained for the early diversification events in Gentianaceae. The ancestral area reconstructions suggest that the most recent common ancestor of Gentianaceae occurred in the neotropics, or both in the neotropics and paleotropics. The latter scenario gains support when the high Early Eocene temperatures are considered to have allowed for increased dispersal probabilities (Tables 2 and 3). Under this scenario the ancestral area of the family probably showed a boreotropical distribution pattern. However, the model in which the dispersal between the neotropics and the paleotropics has a consistent low probability returned better log-likelihood scores, suggesting that a more infrequent exchange between the New and Old World tropics is a better fit for the data.

Under the latter scenario a neotropical origin of Gentianaceae is most likely (Tables 2 and 3). This neotropical origin is further supported by the distribution of Apocynaceae, the sister lineage of Gentianaceae (Soltis et al., 2011), i.e., in Apocynaceae the neotropical tribe Aspidospermeae are the sister group of the rest of the family (Simões et al., 2007), suggestive of a neotropical origin for the Apocynaceae as well. Several pantropical families have been inferred to originate in the neotropics, including Burmanniaceae (Merckx et al., 2008), Malpighiaceae (Davis et al., 2002), and Bursaceae (Weeks et al., 2005). For these families the Early Eocene North Atlantic Land Bridge was probably instrumental for their dispersal into the paleotropics. Simultaneously, several families migrated in the other direction and dispersed from their ancestral paleotropical distributions into the neotropics. Examples of the latter dispersion are Rubiaceae (Antonelli et al., 2009), Annonaceae (Couvreur et al., 2011), and Melastomataceae (Renner et al., 2001).

These examples highlight the potential importance of plant migration through Laurasia during the Early Eocene for obtaining extant distribution patterns of many tropical angiosperm families. After the Early Eocene global temperatures started to drop, resulting in a retraction of tropical flora from northern latitudes (Morley, 2000). This global cooling persisted until the Late Oligocene (Zachos et al., 2001). Our divergence time estimates suggest that the tribe Gentianeae and the subtribe Chironiinae (Chironieae), which consists predominantly of

TABLE 2. Ancestral area reconstruction obtained using different models on tree topologies T1 and T2. RP = Relative Probability, N = Neotropics, P = Paleotropics. Node numbers correspond with Fig. 1.

Tree	Model	-ln(L)	Node 1			Node 2			Node 3			Node 4			Node 5			Node 6		
			Area	RP	-ln(L)	Area	RP	-ln(L)	Area	RP	-ln(L)	Area	RP	-ln(L)	Area	RP	-ln(L)	Area	RP	-ln(L)
T1	M0	14.231	N	0.461	15.005	NP	0.500	14.924	N	0.818	14.431	N	0.770	14.492	N	0.871	14.368	N	0.500	14.924
			NP	0.378	15.203	N	0.357	15.260										NT	0.450	15.029
			NPT	0.081	16.750	NPT	0.085	16.700												
	M1	13.654	N	0.542	14.266	NP	0.629	14.119	N	0.907	13.752	N	0.872	13.791	N	0.939	13.717	NT	0.610	14.149
			NP	0.386	14.791	N	0.321	14.791										N	0.380	14.621
	M2	15.468	NP	0.525	16.112	NP	0.748	15.757	N	0.723	15.792	N	0.659	15.884	N	0.874	15.602	NT	0.620	15.946
N			0.387	16.418	N	0.149	17.370	NP	0.223	16.969	NP	0.252	16.847				N	0.368	16.467	
T2	M0	14.636	N	0.612	15.128	N	0.611	15.128	N	0.533	15.265	N	0.636	15.089	N	0.788	14.874	N	0.494	15.342
			NP	0.226	16.122	NP	0.255	16.001	NP	0.269	15.949	NP	0.164	16.443				NT	0.400	15.552
	M1	14.273	N	0.650	14.703	N	0.667	14.677	N	0.598	14.787	N	0.715	14.608	N	0.854	14.431	NT	0.484	14.998
			NP	0.227	15.756	NP	0.254	15.642	NP	0.279	15.549	NP	0.185	15.958				N	0.460	15.049
	M2	15.893	N	0.550	16.491	NP	0.528	16.533	NP	0.653	16.320	NP	0.500	16.320	N	0.794	16.123	NT	0.627	16.359
			NP	0.372	16.883	N	0.398	16.814	N	0.174	17.644	N	0.357	16.923	NP	0.148	17.805	N	0.351	16.940
								P	0.087	16.712										
								P	0.101	18.185	NPT	0.093	18.269							

TABLE 3. Inferences under different models and tree topologies with the ancestral area of the common ancestor of Gentianaceae either constricted to the neotropics (N), paleotropics (P), or both regions (N+P).

Model	Ancestral area of node 1	Tree T1			Tree T2		
		-ln(L)	Dispersal	Extinction	-ln(L)	Dispersal	Extinction
M0	N	14.990	0.009	0.005	15.126	0.009	0.005
	P	17.970	0.014	0.010	18.269	0.017	0.013
	N+P	15.043	0.007	0.002	16.035	0.007	0.003
M1	N	14.227	0.255	0.004	15.080	0.508	0.006
	P	15.573	1.048	0.011	15.320	0.890	0.011
	N+P	14.506	0.312	0.001	15.697	0.699	0.008
M2	N	16.362	0.015	0.004	16.466	0.0143	0.003
	P	19.483	0.021	0.007	20.293	0.023	0.009
	N+P	16.026	0.011	0.001	16.771	0.010	0.001

northern temperate taxa, diverged from tropical ancestors during this period.

Since our inference of the ancestral areas of the early diversification events in Gentianaceae were based on a tribe-level phylogeny we only considered very broad-scaled ancestral areas. Thus, our results do not provide information about sub-continental ancestral areas. However, the current distribution patterns of Gentianaceae lineages in South America strongly suggest that the Guiana Highlands have played a prominent role in the early diversification of Gentianaceae. Most species of tribes Saccifoliae and Voyriaceae occur here, and in the latter case the area was identified as the ancestral area for the lineage (Albert and Struwe, 1997; Struwe et al., 2002). In addition, many genera in Chironieae and Helieae are endemic to the Guiana Highlands, and molecular and morphological analyses of Helieae suggest that at least a few of these Guiana Highland genera are early diverging lineages of the tribe (Struwe et al., 2009a). Thus, we speculate that Gentianaceae arose in the Guiana Shield during the Cretaceous, similar to what is inferred for Bromeliaceae (Givnish et al., 2011) and Rapateaceae (Givnish et al., 2004; Janssen and Bremer, 2004).

Evolution of mycoheterotrophy in Gentianaceae—Our phylogenetic analyses clearly show that a fully mycoheterotrophic mode of life has evolved at least four times independently in Gentianaceae, i.e., once in both *Voyria* and *Voyriella*, once in the genus *Exochaenium*, and also at least once in *Exacum*. These results demonstrate that evolutionary shifts from autotrophy to full mycoheterotrophy in Gentianaceae have occurred in different geographic areas and in distinct geological epochs. Autotrophic ancestors of *Voyria* and *Voyriella* likely occurred in the neotropics. If we assume that mycoheterotrophy in *Voyria* evolved before the start of the diversification in the genus, then this shift dates back at least to the Early Oligocene (> 31 Ma, see Table 1). In contrast, *Exochaenium oliganthum* diverged from autotrophic ancestors in tropical Africa (Kissling et al., 2009; Kissling 2012) in the Pliocene or Late Miocene (95% CI: 2.7–7.1 Ma). Fully mycoheterotrophic *Exacum* species likely diverged from SE Asian ancestors (Yuan et al., 2003, 2005). *Exacum paucisquamum* (C.B. Clarke) Klack. is estimated to have a Miocene origin in our analyses (95% CI 9.3–16.4). Thus, mycoheterotrophy in Gentianaceae is the result of remarkable convergent evolution in different temporal and geographic contexts.

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