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Dispersing towards Madagascar: Biogeography and evolution of the Madagascan endemics of the Spermacoceae tribe (Rubiaceae) ☆

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ABSTRACT

Despite the close proximity of the African mainland, dispersal of plant lineages towards Madagascar remains intriguing. The composition of the Madagascan flora is rather mixed and shows besides African representatives, also floral elements of India, Southeast Asia, Australia, and the Neotropics. Due to its proportionally large number of Madagascan endemics, the taxonomically troublesome Spermacoceae tribe is an interesting group to investigate the origin and evolution of the herbaceous Rubiaceae endemic to Madagascar. The phylogenetic position of these endemics were inferred using four plastid gene markers. Age estimates were obtained by expanding the Spermacoceae dataset with representatives of all Rubiaceae tribes. This allowed incorporation of multiple fossil-based calibration points from the Rubiaceae fossil record. Despite the high morphological diversity of the endemic herbaceous Spermacoceae on Madagascar, only two colonization events gave rise to their current diversity. The first clade contains *Lathraeocarpa*, *Phylohydrax* and *Gomphocalyx*, whereas the second Madagascan clade includes the endemic genera *Astiella*, *Phialiphora*, *Thamnoldenlandia* and *Amphistemon*. The tribe Spermacoceae is estimated to have a Late Eocene origin, and diversified during Oligocene and Miocene. The two Madagascan clades of the tribe originated in the Oligocene and radiated in the Miocene. The origin of the Madagascan Spermacoceae cannot be explained by Gondwanan vicariance but only by means of Cenozoic long distance dispersal events. Interestingly, not only colonization from Africa occurred but also long distance dispersal from the Neotropics shaped the current diversity of the Spermacoceae tribe on Madagascar.

1. Introduction

With its unique and spectacularly rich fauna and flora, Madagascar presents itself even to the most indifferent sightseer, as a place of biological diversity. Up to 91.6% of the reptiles, 98.4% of the amphibians and 100% of the mammals (excluding bats) are endemic to the island (Garbutt, 1999; Glaw and Vences, 2000). Also for botanists Madagascar houses tremendous species richness. The island is home to more than 10,000 plant species, of which 80% occurs nowhere else in the world (Kricher, 2012). With natural vegetation varying from rainforests to spiny forests, Madagascar is one of the worlds most important biodiversity hotspots (Moat and Smith, 2007). This high diversity is even more remarkable given that certain plant lineages are poorly represented or completely absent, whereas other groups show an unrivalled diversity.

On this biodiversity hotspot, Rubiaceae is the second largest family of flowering plants with about 650 described species in 95 genera. More than 98% of the Madagascan Rubiaceae species are unique for the island, and the level of species endemism is likely to increase with every taxonomic study carried out on the region (Davis and Bridson, 2003). In general, biogeographical studies of Rubiaceae are scarce (e.g., Alejandro et al., 2005, *Mussaenda*; Malcomber, 2002, *Gaertnera*; Nepokroeff et al., 2003, Hawaiian *Psychotria*; Nie et al., 2005, *Kellogia*; Tosh et al., 2013, *Ixora*), and heretofore, not much is known on the geographic origin of the various Rubiaceae taxa in Madagascar. By generating a molecular phylogenetic hypothesis and by using dating techniques, we investigated the origin and evolution of the Madagascan members of the herbaceous Spermacoceae tribe. The use of molecular data has greatly improved our understanding of the phylogeny of this

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group (Groeninckx et al., 2009; KÍrehed et al., 2008), which is represented in Madagascar by approximately 30 species present in all terrestrial niches, from coastal dunes in the south(west) to high plateaus in the centre of the island and tropical rainforests in the north and east. Whereas some taxa are local endemics, others are more widespread with few species occurring in other tropical regions as well (e.g. *Oldenlandia corymbosa*, *O. capensis*, *O. goreensis*, *O. latifolia*, *Cordylostigma virgatum*, *C. longifolia*).

From a geohistorical point of view, Madagascar was landlocked in the centre of supercontinent Gondwana, situated between land that would eventually become Africa and India. About 165 my ago Gondwana started to split into smaller continents and Madagascar slowly moved towards its current isolated position in the Indian Ocean. At present, a vast oceanic barrier surrounds the island, with continental Africa as closest landmass approximately 400 km to the west, and India, Antarctica and Australia at a distance of 4000, 5000, and 6000 km respectively. Because of the relative short distance between Africa and Madagascar, it would be plausible that its biota has a mainly African origin. However, the composition of the Madagascan flora shows both African influences and floral elements of Asian and neotropical origin (Dejardin et al., 1973; Perrier de la Bãthie, 1936; Schatz, 1996). Because representatives of the Spermacoceae (Rubiaceae) are widespread in other tropical regions, the tribe represents an important study group to infer dispersal events towards Madagascar in evolutionary context.

It is generally accepted that the current floral composition on Madagascar is the result of an interplay between Gondwanan vicariance and Cenozoic long distance dispersal. Although Gondwanan vicariance was long considered as main evolutionary trend (Grubb, 2003; Leroy, 1978), Yoder and Nowak (2006) clearly point out that most of the present-day biota on Madagascar is the result of recent Cenozoic dispersal events, predominantly from the African continent. Moreover, in the case of Rubiaceae, no fossils are known from the Cretaceous that support a Gondwanan origin. The first fossil record of Rubiaceae is from the early Eocene (Roth and Dilcher, 1979), indicating that Rubiaceae is a relatively young family characterized by both rapid diversification and adaptive radiation.

History has proven that studies on oceanic islands are of considerable significance to obtain more insights in the complexity of evolution (e.g. Darwin, Wallace). Oceanic islands often represent natural evolutionary laboratories that help us to unravel general evolutionary patterns by studying the reproduction and genetics of its residents. In this study we investigate the origin and radiation of the Spermacoceae tribe on Madagascar, in order to address a number of key questions relating to the evolution and biogeography of herbaceous Rubiaceae on Madagascar, such as: What are the closest relatives of the endemic Madagascan Spermacoceae? How and when did representatives of the tribe reach the island? What mechanisms have driven the radiation of herbaceous Rubiaceae taxa on Madagascar?

2. Materials and methods

2.1. Taxon sampling

The taxon sampling for this investigation is mainly based on previous molecular phylogenetic studies of the Spermacoceae tribe (Groeninckx et al., 2009, 2010ac; KÍrehed et al., 2008; Wikstr—m et al., 2013) and is largely focussed on the relationship between the Madagascan representatives and their close relatives. The Spermacoceae which is circumscribed by Wikstr—m et al. (2013) as a tribe of c. 1000 species and 60 genera can be subdivided into three main groups: the monophyletic Spermacoceae s.s. containing 275 species (Dessein, 2003), the monophyletic lineage towards *Bouvardia* and *Manettia* consisting of ca. 130 species (Groeninckx, 2009) and the paraphyletic *Hedyotis-Oldenlandia* complex (Groeninckx et al., 2009, 2010a,b) including 500600 species (Groeninckx, 2009; Wikstr—m et al., 2013). Interestingly, it is within the latter group that the Madagascan endemics of the Spermacoceae tribe can be situated. The current dataset consists of 211 specimens representing 204 different species (*Manettia-Bouvardia* lineage: 4 species, Spermacoceae s.s: 23 species, *Hedyotis-Oldenlandia* complex: 177 species). Because of our elaborate sampling within the *Hedyotis-Oldenlandia* complex representing a broad taxonomic and geographic diversity we were able to diminish the possible effect of an incomplete taxon sampling on the biogeographic reconstruction analyses as much as possible.

Of the 177 species belonging to the paraphyletic *Hedyotis-Oldenlandia* complex, 14 species divided over 7 genera are endemic for Madagascar (*Thamnoldenlandia: T. ambovombensis*, *Amphistemon: A. humbertii and A. rakotonasolensis*, *Astiella: A. delicatula*, *A. tsaratanensis*, *A. desseinii*, *A. perrieri* and *A. latifolia*, *Lathraeocarpa: L. acicularis* and *L. decaryi*, *Gomphocalyx: G. herniarioides*, *Phylohydrax: P. madagascariensis*, and *Phialiphora: P. bevazahensis* and *P. capitulata*). Despite the difficulty of collecting all known Madagascan endemics of the Hedyotis-Oldenlandia complex, missing endemics are expected to belong to the lineage containing Astiella, Phialiphora, Thamnoldenlandia and Amphistemon (Groeninckx, 2009).

In order to provide a robust phylogenetic framework for the Spermacoceae tribe and carry out a thorough dating analysis, we expanded the dataset at family-level with 65 species to comprise representatives of all subfamilies and most tribes of Rubiaceae. This enlarged dataset consists of 22 additional Rubioideae representatives (besides the 201 already mentioned that belong to the Spermacoceae tribe), 31 representatives of the Ixoroideae subfamily, 11 representatives of the Cinchonoideae subfamily and *Luculia*. *Gentiana dinarica* (Gentianaceae), *Strychnos potatorum* (Loganiaceae) and *Ceropegia linearis* (Apocynaceae) were chosen as outgroup. Information on GenBank accessions, species names, voucher information, and references are provided in Supplementary Tables S1 and S2.

2.2. Molecular protocols and phylogenetic analyses

Total genomic DNA was isolated using a modified CTAB protocol from dried silica gel collections and fresh material from the Botanic Garden Meise. Despite several efforts, we were unable to amplify or sequence DNA from herbarium material of additional Madagascan endemics. Primers and temperature programs used for the amplification of chloroplast *rps16*, *atpB-rbcL*, *trnL-F* and *petD* follow Groeninckx et al. (2009) and KÍrehed et al. (2008). Purified amplification products were sent to Macrogen, Inc. (Seoul, South Korea) for sequencing.

Contiguous sequences were assembled using Geneious v7.0.6 (Biomatters, New Zealand). Automatic alignments were carried out with MAFFT (Katoh et al., 2002) under an E-INS-i algorithm, a 100PAM/k=2 scoring matrix, a gap open penalty of 1.3 and an offset value of 0.123. Subsequent manual finetuning of the aligned dataset was done in Geneious v7.0.6. Gaps were treated as missing data, whereas potentially informative insertions and deletions were manually coded according to the simple indel coding method of Simmons and Ochoterena (2000).

Congruency between the different datasets was inferred by a partition homogeneity test as implemented in PAUP4.0b10a (Swofford, 2003).

The best-fit nucleotide substitution model for each plastid and nuclear dataset was determined using jModelTest 2.1.4 (Posada, 2008) under the Akaike information criterion (AIC). The GTR + I + G model was found as best fit for *rps16*, *petD* and *atpB-rbcL*, whereas the GTR + G model was calculated as best substitution model for *trnL-F*. A mixed-model approach was used in which the combined dataset is partitioned in order to apply a different model of evolution on each DNA region (Ronquist and Huelsenbeck, 2003). Bayesian inference (BI) analyses were conducted with MrBayes v3.1 (Huelsenbeck and Ronquist, 2001) on three individual data partitions and a combined data matrix. Each analysis was run two times for 10 million generations. Trees were sampled every 2500 generations. Inspection of chain convergence and ESS parameters was done with TRACER v1.4 (Rambaut and Drummond, 2007).

Bayesian Inference BPP values between 0.50 and 0.95 as summarized in the 50% majority-rule consensus tree are considered to be weakly supported, whereas only BPP values above or equal 0.95 are taken into consideration (Suzuki et al., 2002; Alfaro et al., 2003).

Maximum Likelihood analyses were carried out on the CIPRES web portal using RAxML v7.2.8 (Stamatakis et al., 2008) under the GTRGAMMA model. Non-parametric ML bootstrapping analysis was calculated with 1000 bootstrap replicates.

2.3. Divergence time analysis

In order to estimate node ages within the Spermacoceae tribe, we used an expanded family-level dataset onto which several fossil calibration points were inferred. The large Rubiaceae dataset was designed to include all major Rubiaceae lineages. As no suitable fossils of Spermacoceae are known (Graham, 2009; Martinez-Millan, 2010), a large-scale Rubiaceae dating approach was indispensable as it allowed us to integrate multiple fossil calibration points and therefore minimizes the bias produced by a single calibration point. A series of well-identified and dated fossils for Rubiaceae is available from which we were able to use six in our analysis (Graham, 2009; Martinez-Millan, 2010). Following calibration points were used for age estimation: (1) the split between *Rubia* and *Galium* (=stem node for both genera) was set at 5.3 mya based on the first appearance of fossil pollen during the late Miocene (Graham, 2009), (2) fossil records of Oligocene pollen for *Coprosma* resulting in a stem node constraint at 23.8 mya (Graham, 2009), (3) the stem node of *Chiococca* constrained at 5.3 mya corresponding to the occurrence of late Miocene leaf fossils (Graham, 2009), (4) a constraint of 14.55 mya for the stem node of *Gardenia* (middle Miocene pollen fossils; Graham, 2009), (5) calibration of *Ixora* at 5.3 mya following the fossil collection of *I. casei* from the Marshall Islands (Leopold, 1969), (6) the lineage towards *Emmenopterys* was assigned a constraint of 48 mya based on the occurrence of fruits from the middle Eocene (Manchester, 1994). All fossil calibration points used in this study were modeled for BEAST under a log-normal distribution, an offset that equals the fossil calibration point, a mean of 0.5, and a standard deviation of 1. The Rubiaceae crown node was set at 68.7 mya, based on the large asterid analysis of Janssens et al. (2009) in which also Rubiaceae were incorporated and of which the results were in correspondence with the dating analysis of Bremer et al. (2004) and Bremer and Eriksson (2009). In contrast to the fossil calibration points used, this calibration point was given a normal distribution with a mean value and standard deviation of 1.0.

A chi^2 likelihood ratio test, used to assess rate heterogeneity among lineages (Felsenstein, 1988) indicated that the substitution rates in the combined dataset are not clock-like ($P < 0.001$ for all markers). A Bayesian approach using BEAST v1.8.0 was used to calculate divergence times (Drummond and Rambaut, 2007). However, in order to initiate the Bayesian dating analysis and cope with the zero likelihood issue in BEAST, we used a starting tree that was obtained by carrying out a ML analysis in RAxML v7.2.8 (Stamatakis et al., 2008) under GTRGAMMA model and using the rooted likelihood tree as input tree for a penalized likelihood (PL) analysis in r8s 1.70 (Sanderson, 2003) with all calibration points used as described above.

Because of differences in substitution model among the individual chloroplast genes, we performed a partitioned Bayesian MCMC analysis under the assumption of the Yule speciation model and a relaxed log-normal clock. Partitions were unlinked for the model of evolution. All other priors were kept as defaults. Two runs of 20 million generations were performed with sampling every 2000 generations. Convergence of the chains, as well as checking whether the ESS parameter exceeded 200 was carried out with TRACER v1.6 (Rambaut and Drummond, 2007). The two runs were combined, discarding the initial 2 million generations as burn-in, using Logcombiner v1.8.0 and a maximum clade credibility tree using a posterior probability limit of 0.5 was calculated using TreeAnnotator v1.8.0 (Drummond and Rambaut, 2007).

2.4. Ancestral area reconstruction

Reconstruction of ancestral distribution ranges was carried out following the dispersal-extinction-cladogenesis model of Lagrange (Ree et al., 2005; Ree and Smith, 2008). Lagrange scripts were generated using the online Lagrange configurator (www.reelab.net/lagrange/configurator/index). The phylogeny obtained was trimmed in R using the APE package (Paradis, 1998) to only contain representatives of the Spermacoceae tribe. The obtained maximum clade credibility tree from the dating analysis with BEAST v1.8.0 was used as input tree. In total, seven distribution areas were defined (Asia, Australia, Africa, Madagascar, tropical America (South and Central America), temperate America (North America and northern Mexico) and the Polynesian region). This biogeographic delimitation is chosen because the focus of this study lies on the colonization of Madagascar by representatives of the Spermacoceae tribe. In addition, ancestral distribution ranges of Spermacoceae taxa that occur on both Madagascar and another continent are investigated. Uncertain phylogenetic relationships, such as the position of the *PentodonDentella* clade, did not influence the result of the ancestral area reconstruction of the endemic Madagascan clades as they are phylogenetically too distantly positioned from our lineages of interest. Maximum range size was defined at two (only 4 out of 126 taxa investigated in the ancestral area analysis occur in more than one delineated distribution area). Dispersal rates data entry was set at symmetric. As species from the Spermacoceae are on average considered to be good dispersers, no time slices were defined.

3. Results

3.1. Molecular phylogenetics

Sequence characteristics for each chloroplast data matrix are summarized in Table 1. BI and ML analyses of the different chloro-

Table 1
Sequence characteristics.

	atpB-rbcL	*rps16*	*trnL-F*	*petD*	Combined
Nr of taxa	170	254	180	248	278
Percentage of missing data	39	8	35	11	
Total length of alignment	1881	1470	1407	2850	7608
Sequence length variation	650	497	197	841	
	911	740	526	1013	
Parsimony informative characters	307	362	276	265	1210
Variable characters	481	494	363	787	2125

plast gene markers resulted in congruent topologies that were only moderately supported by Bayesian Posterior Probabilities (BPP) and ML Bootstrap support values (ML-BS; data not shown). In case of incongruency between the different datasets, the incongruent lineages did not show any support. Despite these minor discrepancies, the partition homogeneity was not significant ($P > 0.05$) indicating that the chloroplast partitions were not in conflict.

General relationships regarding the Rubiaceae subfamilies correspond to earlier studies of Bremer (2009), Bremer and Eriksson (2009), Robbrecht and Manen (2006) and Rydin et al. (2009), with the Rubioideae subfamily sister (ML-BS = 100, BPP = 1.0) to the subfamilies Cinchonoideae and Ixoroideae (ML-BS = 100, BPP = 1.0; Fig. 1, Supplementary Figs. S1S3). The relationship of the enigmatic genus *Luculia* differs somewhat as it is positioned as sister to the three recognized subfamilies of the Rubiaceae (ML-BS = 88, BPP = 0.95; Fig. 1, Supplementary Fig. 1). This result corroborates the findings of Refulio-Rodriguez and Olmstead (2014). Within the Spermacoceae, inferred relationships correspond well to the results of Groeninckx et al. (2009) and Wikstr—m et al. (2013). One of the major phylogenetic differences with the study of Wikstr—m et al. (2013) can be found in the position of the *Arctophyllum-Kohautia* clade and the species *Pentodon pentandrus*, *Dentella repens* and *Dibrachionostylus kaessneri* (Fig. 1, Supplementary Figs. 2 and 3). In addition, a phylogenetic difference for *Hedyotis swertioides* and *H. korrorensis* was observed (Supplementary Fig. S2). However, these indiscrepencies are likely the results of differences in sampling and gene markers used (see Wikstr—m et al., 2013).

The combined data matrix supports the hypothesis that at least two colonisation events towards Madagascar gave rise to the current biodiversity of the Spermacoceae on the island (Fig. 1, Supplementary Figs. 2 and 3). The first Madagascan clade contains the endemic genus *Lathraeocarpa* (with two representatives *L. decaryi* and *L. acicularis;* ML-BS = 100, BPP = 1.0), which is sister to *Gomphocalyx* and the Afro-Madagascan genus *Phylohydrax* (ML-BS = 100, BPP = 0.99). This clade is further referred to as the LGP clade throughout the text. This clade has maximum support in both ML and BI analyses (Fig. 1, Supplementary Fig. S2) and is placed sister to the African species *Manostachya ternifolia* and *Oldenlandia rosulata* with high support (ML-BS = 98, BPP = 1.0) (Fig. 1, Supplementary Fig. S2). The second Madagascan clade includes the remaining Madagascan endemics in our sampling. Within the second Madagascan clade, two subclades can be defined that both get high support in the ML and BI tree. The first subclade is the lineage consisting of *Thamnoldenlandia ambovombensis*, *Amphistemon humbertii* and *A. rakotonasolensis* (ML-BS = 94, BPP = 0.99). Within this lineage, the genus *Amphistemon* is highly supported as monophyletic group (ML-BS = 96, BPP = 0.95). The second subclade contains the genus *Phialiphora* (including *P. bevazahensis* and *P. capitulata*; ML-BS = 100, BPP = 1.0), which is sister to the genus *Astiella* (including *A. delicatula*, *A. tsaratanensis*, *A. desseinii*, *A. perrieri* and *A. latifolia* (ML-BS = 100, BPP = 1.0). The clade with the genera *Thamnoldenlandia*, *Amphistemon*, *Phialiphora* and *Astiella* will be further referred to as the TAPA clade throughout the discussion. A sister relationship between the new species *A. latifolia* and *A. perrieri* was recovered with maximum support in the MP and BI analyses (ML-BS = 100, BPP = 1.0). Sister to the *Astiella latifolia Astiella perrieri* clade is *A. desseinii* (ML-BS = 100, BPP = 1.0). The latter clade falls as an unresolved polytomy with *Astiella delicatula* and *A. tsaratanesis* (Fig. 1, Supplementary Fig. S2).

3.2. Divergence time estimation

Estimated divergence ages and credibility intervals are summarized in Supplementary Figs. S1 and S3. The onset of the Spermacoceae s.l. group started during the Late Eocene, whereas the Spermacoceae diverged in the Early Eocene (Fig. 1). Most lineages of the latter group emerged during the Miocene. The stem node age of the LGP clade is estimated at 22.1 mya (15.928.9, 95% highest priority density (HPD)). Its crown node age is estimated at 11.3 mya (6.317.0, 95% HPD). The split between the two species of the genus *Phylohydrax* is calculated at 0.2 mya (0.11.3, 95% HPD). For the most recent common ancestor (MRCA) of the TAPA clade, a stem node age of 27.9 mya (21.734.7, 95% HPD) and a crown node age of 25.0 mya (18.732.2, 95% HPD) was retrieved, with the divergence of the *PhialiphoraAstiella* clade estimated at 14.7 mya (9.020.8, 95% HPD) and the divergence of the *ThamnoldenlandiaAmphistemon* clade estimated at 17.0 mya (10.123.8, 95% HPD).

3.3. Ancestral area reconstruction

No unambiguous ancestral area splitting was observed for our lineages of interest involving the endemic Madagascan clades. We always observed a relative probability of at least 0.4 higher compared to the next ancestral area alternative, nor was the relative probability of our lineages of interest below 0.85. Current results clearly indicate a different biogeographic origin for the two recognized endemic Madagascan clades in the Spermacoceae (Fig. 2). The LPG clade, with *Lathraeocarpa* as first diversifying genus, has an indisputably African origin. Indeed, the LPG clade is embedded within a completely African lineage (with *Manostachya ternifolia* and *Oldenlandia rosulata* as sister to the LPG clade and the lineage towards *Amphiasma*, *Pentanopsis*, *Conostomium*, and *Oldenlandia affinis* and *O. herbacea* as sister to the LPG clade, *M. ternifolia* and *O. rosulata*). Within the LPG clade, Lagrange calculated an Afro-Madagascan origin for the ancestor of the genus *Phylohydrax*. However, due to the very recent divergence of *Phylohydrax*, a recent long distance dispersal event from Madagascar to Africa is assumed for *P. carnosa*. The interpretation of the ancestral distribution range for the second clade with Madagascan endemics (*Amphistemon*, *Astiella*, *Phialiphora*, and *Thamnoldenlandia*) is less clear, as the split between this clade and its sister (containing the genera *Houstonia*, *Arcytophyllum* and *Stenaria*) shows a division between Madagascar and tropical America, respectively. However, the MRCA of this joint clade has a well-supported ancestral distribution range situated in tropical America (Fig. 2).

In addition to the ancestral distribution range of the endemic Madagascan lineages, the ancestral area of the species with populations on Madagascar and other continent(s) was inferred. Of these, only the ancestral distribution of the Madagascan populations of *Pentodon pentandrus* and *Oldenlandia lancifolia* are unclear. All other species in the analysis with representatives on Madagascar have a clearly African origin (*Oldenlandia goreensis*, *O. affinis*, *O. capensis*, *O. herbacea*, *Mitrasacmopsis quadrivalvis*, *Agathisanthemum bojeri* and *Cordylostigma virgata*).

4. Discussion

4.1. Vicariance or dispersal?

Despite the short distance to the African mainland, the composition of the Madagascan flora is mixed containing, besides African influences, a significant percentage of Asian and neotropical elements (Yoder and Nowak, 2006). As a result of this heterogeneity, different mechanisms have been proposed that could explain the diverse Madagascan flora: (1) Gondwanan vicariance during the Cretaceous (continental drift), and (2) long-distance dispersal via wind or sea currents. Another possible dispersal mechanism explaining the floral diversity on Madagascar are the Lemurian

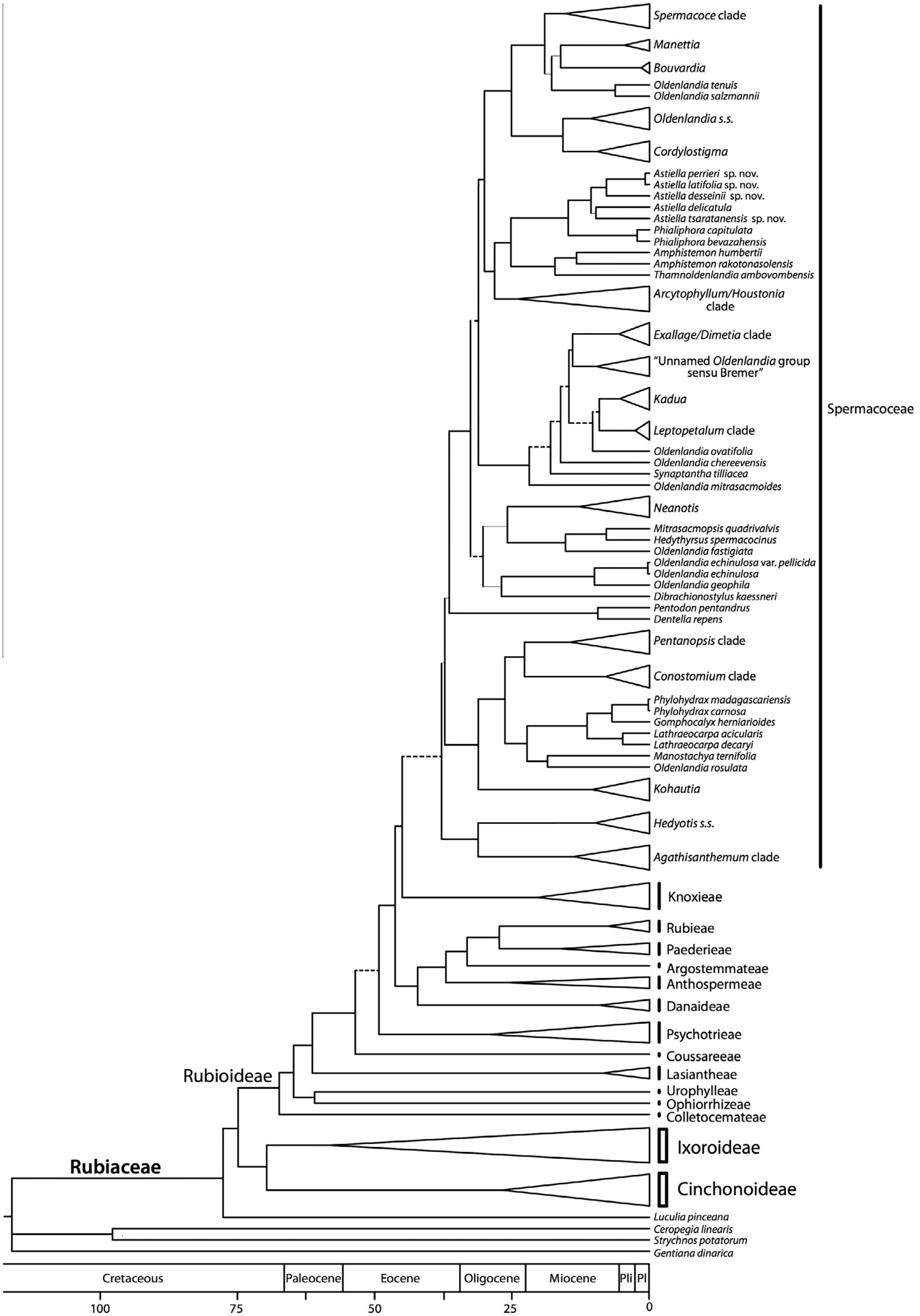

Fig. 1. Reduced BEAST chronogram of the Rubiaceae family with focus on the Spermacoceae tribe (derived from Supplementary Figs. 1 and 3). Clades that have been specified in earlier studies on the tribe or the family (e.g. Wikstr—m et al., 2013; Bremer, 2009) are shown as triangles when they did not contain Madagascan endemics. Thin branches indicate lack of support by Bayesian analysis, whereas dashed lines show low support between 0.50 and 0.95 BPP. Normal size branches indicate support above or equal to 0.95 BPP. Pli = Pliocene. Pl = Pleistocene.

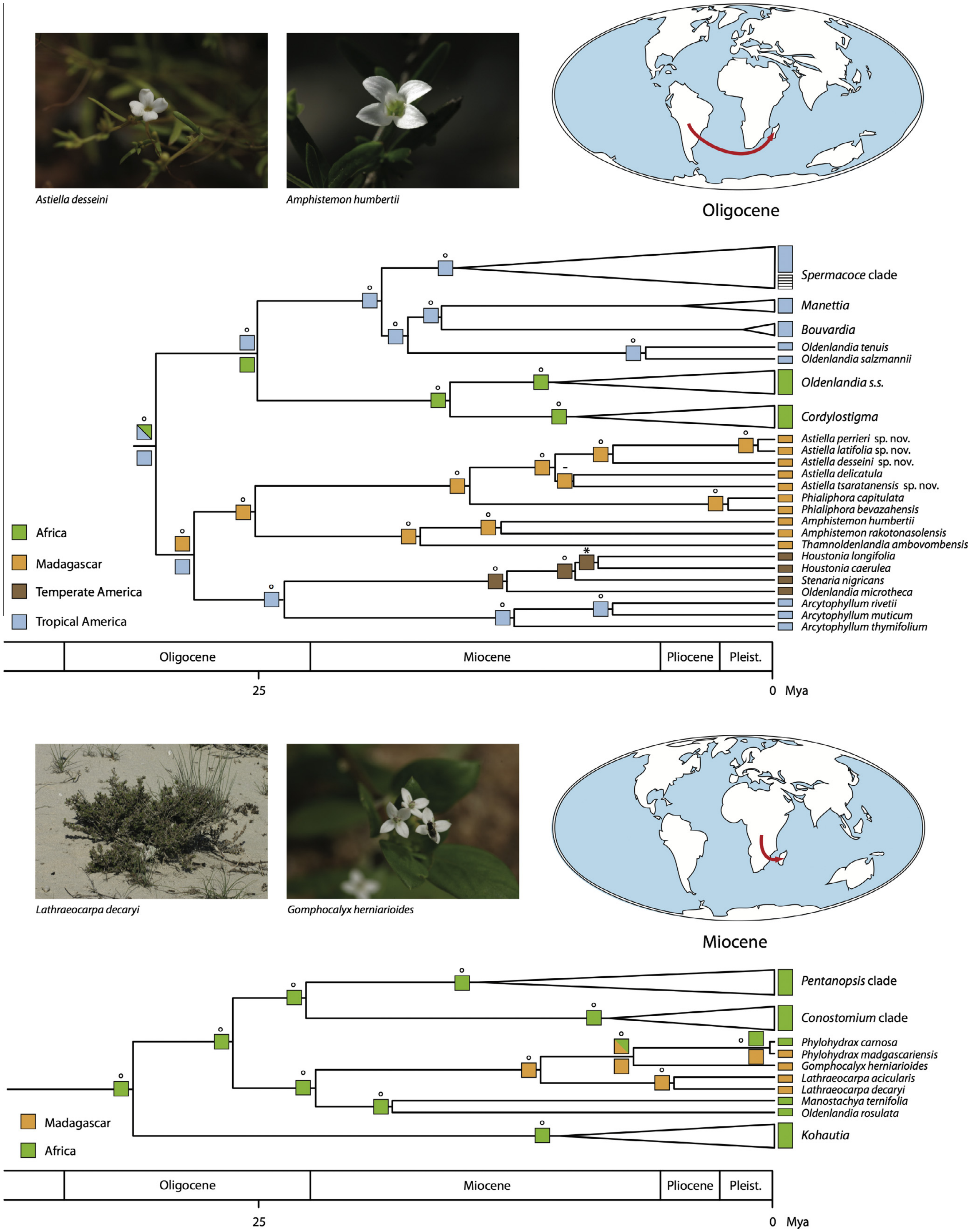

Fig. 2. Chronogram of the two lineages within the Spermacoceae tribe to which the Madagascan endemics belong, showing the result of the ancestral area reconstruction analysis using Lagrange. For the *Spermacoce* s.s. lineage, the dashed box represents a small proportion of species that occur in Australia, Africa, Asia and temperate America. The light blue box indicates the large proportion of tropical American species within that specific clade. Clades with representatives solely belonging to a certain genus receive the name of the genus. For clades containing representatives of more than one genus, informal names from Wikstr—m et al. (2013) are used. Highly supported nodes (above or equal to 0.95 BPP) are indicated with a circle above the branch, whereas nodes with low support (between 0.50 and 0.95 BPP) are highlighted with an asterisk and unsupported nodes are shown with a hyphen.

stepping stones situated in the Indian Ocean. This distribution route, however, is mainly used to explain the link between Madagascan and Asian biota. It is generally accepted that either of these mechanisms shaped the current Madagascan biodiversity, yet to what extent is still under debate (Yoder and Nowak 2006; Warren et al., 2010). Madagascars high level of biodiversity and overall endemism suggests that the evolutionary history of the different lineages could have been one of millions of years of extensive speciation and diversification. Therefore, the Gondwanan vicariance hypothesis was often considered to be a possible participator in explaining the enormous biodiversity on Madagascar (Grubb, 2003; Leroy, 1978).

Present results, however, demonstrate that the Madagascan endemics of the Spermacoceae are not the result of Gondwanan vicariance but of Cenozoic long-distance dispersal events. This result is consistent with previous studies on other flowering plants (e.g. Buerki et al., 2011; Davis et al., 2002; Janssens et al., 2006, 2009; Meve and Liede, 2002; Plana, 2003; Renner, 2004; Renner et al., 2001; Schaefer et al., 2009; Strijk et al., 2012; TrÕnel et al., 2007; Yuan et al., 2004, 2005).

A first colonization event of Madagascar by Spermacoceae lineages occurred between 32.1 and 29.0 mya and gave rise to the genera *Amphistemon*, *Thamnoldenlandia*, *Phialiphora*, and *Astiella*. The second dispersal event towards the island occurred between 25.0 and 14.4 mya and resulted in the divergence of the genera *Lathraeocarpa*, *Gomphocalyx*, and *Phylohydrax* (of which *P. carnosa* dispersed later towards eastern Africa again). Both age estimates (including their 95% confidence intervals) clearly post-date the split of India and Madagascar, ca. 87 mya (Warren et al., 2010) and that of Africa and Madagascar (together with India), ca. 145 mya (Buerki et al., 2013). Our findings corroborate the review of Yoder and Nowak (2006). They concluded that although phylogenetic analyses frequently promote an association between Madagascan and Asian biota thereby putatively implying a Gondwanan link the analyses hardly ever support a vicariant origin as divergence ages between Asian and Madagascan lineages are much more recent than the presumed age of separation between India and Madagascar. Moreover, the presence of floral elements on Madagascar from Australia, India, and Southeast Asia is often associated with the mechanism of long-distance dispersal via prevalent easterly winds and corresponding ocean currents across the Indian Ocean since the Early Paleocene (Cheke and Hume, 2008; New et al., 2005; Schatz, 1996). Additionally, in the case of Rubiaceae, no fossils are known from the Cretaceous that could support a Gondwanan origin. The earliest fossils of Rubiaceae are from the Paleocene-Eocene (Abbott, 1986; Martinez-Millan, 2010; Roth and Dilcher, 1979). Rubiaceae can be considered to be a relatively young family characterized by a species-rich and rapid radiation. Our study confirms the hypothesis that consistent dispersal played an important role in the evolution of Rubiaceae (Anderson et al., 2001; Bremer and Eriksson, 1992; Eriksson and Bremer, 1991; Malcomber, 2002; Wikstr—m et al., 2010).

4.2. Patterns of long-distance dispersal towards Madagascar

Biogeographic studies that explain the origin of specific Rubiaceae lineages on Madagascar remain inadequate, especially in light of the large number of species in the coffee family. The few studies that have addressed this topic clearly show that the dispersal events towards Madagascar mainly occurred from Eastern Tropical Africa and Southern Africa, although colonization events from Tropical Asia are also observed (e.g. Alejandro et al., 2005; Malcomber, 2002; Maurin et al., 2007; Tosh et al., 2009, 2013; Wikstr—m et al., 2010). With an approximate distance of 400 km between mainland Africa and the west coast of Madagascar, it is clear that Africa can be considered as the most important source of flowering plant dispersal to Madagascar, especially when taking recent sea level fluctuations into account, which considerably reduced the distance between the two coastlines (Yoder and Nowak, 2006). Indeed, a first lineage of Madagascan endemics of the Spermacoceae (the LGP clade) has an indisputable African origin, a result that was also observed for all other species investigated in this study occurring in both Africa and Madagascar (e.g. *Oldenlandia affinis*, *O. herbacea*, *O. goreensis*, *O. capensis*, *Agathisanthemum bojeri*, *Cordylostigma virgata*, *Mitrasacmopsis quadrivalvis*).

Our results indicate an ancestral distribution range situated in tropical America for the second clade of Madagascan endemics, consisting of the genera *Thamnoldenlandia*, *Amphistemon*, *Phialiphora* and *Astiella* (TAPA clade). Growing evidence suggests the possibility of dispersal events from tropical America towards Madagascar via Antarctica until the Late Cretaceous (ca. 80 mya), even though there was no immediate contact between these regions (Buerki et al., 2013; Yoder and Nowak, 2006). Age estimate analyses of the Spermacoceae tribe demonstrate nonetheless that the colonization of the most recent common ancestor of the TAPA clade occurred during the Oligocene, suggesting that only long-distance dispersal can explain the link between tropical America and Madagascar for this lineage. Madagascan Spermacoceae species all have small, dry capsular fruits with one to many seeds per locule, which makes abiotic dispersal most likely for members of this tribe. For example, in *Thamnoldenlandia ambovombensis*, selection for wind dispersal has resulted in the development of winged seeds (Groeninckx et al., 2010a). However, despite the fact that seeds of native Madagascan representatives of the Spermacoceae lack adaptations for biotic dispersal, it is not unlikely for animals to act as secondarily dispersers.

Until now, only eight examples of a putative tropical American sister group relationship with a Madagascan endemic plant lineage have been detected (Buerki et al., 2013). Of these, only the endemic Madagascan genera *Haematodendron* (Myristicaceae) and *Tsoala* (Solanaceae) are embedded within a tropical American branch and could thus provide evidence that these Madagascan lineages are derived from a tropical American ancestor. The other Madagascan/tropical American sister group relationships are either clustered with representatives from several other tropical regions (e.g. *Xerosicyos* and *Siolmatra* *Zanonia*; Kocyan et al., 2007; Schaefer et al., 2009), or show little phylogenetic support meaning that other ancestral scenarios are equally possible (e.g. *Talinella* and *Talinum*; Applequist and Wallace, 2001; *Cymbidiella* and *Cyrtopodium*, van den Berg et al., 2002). Regarding the young age of both the Solanaceae subfamily Goetzeoideae to which the genus *Tsoala* belongs (<10 mya; Sðrkinen et al., 2013) and the Myristicaceae family of which *Haematodendron* is a representative (<25 mya; Sauquet et al., 2003), we assume that the distribution of these endemic Madagascan genera could be the result of a long-distance dispersal event from tropical America towards Madagascar, similar to our results on the MRCA of the endemic Rubiaceae genera *Thamnoldenlandia*, *Amphistemon*, *Phialiphora*, and *Astiella*. The presence of Spermacoceae endemics on Madagascar with a tropical American background is even more surprising as most species of this tribe are characterized by a heterostylous breeding system, which is known to complicate efficient long-distance dispersal. Many heterostylous species are self-incompatible, and one plant of each morph i.e. pin (longistyl) and thrum (brevistyl) is required to establish a population after the long-distance dispersal event. The fact that only legitimate pollination between morphs causes fruit set is a major constraint for distylous species in the colonization of oceanic islands (Baker, 1955; Barrett and Shore, 1987; Malcomber, 2002; Pailler et al., 1998).

It is therefore not surprising that heterostyly is often absent on oceanic islands. Self-compatible species are more likely to establish populations after long-distance dispersal due to their ability to reproduce in the absence of pollinators (Pailler et al., 1998). In several distylous species, however, a breakdown of the self-incompatibility system has been reported permitting self-pollination. As some of the Madagascan species of the Spermacoceae (e.g. *Astiella delicatula*) are characterized by incomplete heterostyly as possible mechanism to promote self-pollination, this feature could increase the possibility of a long-distance dispersal event from tropical America to Madagascar without the need of two morphs being dispersed at once.

Last but not least, one could always postulate that this tropical American-Madagascan connection is the result of a putative loss of an African or Asian ancestor, thereby obscuring the ancestral area analysis. However, because of the proportionally short branches for this clade and its most closely related lineages, the probability of such an extinction event is low, yet this hypothesis should not be completely ruled out.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.10.024.

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