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SEVERAL ORIGINS OF FLORAL OIL IN THE ANGELONIEAE, A SOUTHERN HEMISPHERE DISJUNCT CLADE OF PLANTAGINACEAE[1]

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- *Premise of the study:* Over the past 75 Myr, successive groups of plants have entered the "oil bee pollination niche," meaning that they depend on oil-collecting bees for their pollination. The highly dissimilar numbers of plant species and bee species involved in these mutualisms imply evolutionary host switching, asymmetric mutual dependencies, and uncoupled diversification. Among the clades with the best field data on oil bee behavior is the Angelonieae, which we here investigate to better understand the evolutionary time frame of this pollination syndrome.
- *Methods:* We generated nuclear and plastid data matrices for 56% of the Angelonieae species (plus outgroups) and used Bayesian methods of molecular clock dating, ancestral state reconstruction, and biogeographic inference.
- *Key results:* We found that Angelonieae have two major clades, *Angelonia* (including *Monopera*) and *Basistemon*, and *Monttea*, *Melosperma*, and *Ourisia*.
- *Conclusions:* Angelonieae date back to the Uppermost Eocene, ca. 35 (26–47) Myr ago (Ma) and diversified in dry areas of southern South America; they switched from nectar to oil as a reward four or five times over the past 25 Ma. As predicted in a previous non-clock-dated study, dispersal to Australasia dates to the Miocene/Pliocene.

Key words: molecular clock; oil-offering flowers; parallel evolution; Plantaginaceae; southern hemisphere disjunction.

Some 1600 species of plants in 11 families offer oil as a reward to their pollinators. About 1000 of them belong to the Malpighiaceae in which oil is the ancestral reward system, and the remainder are in the Orchidaceae, which have oil-offering species in many genera, Calceolariaceae (~200, all in *Calceolaria*), Cucurbitaceae (~102 in several genera), Myrsinaceae (~75, all in *Lysimachia*), and just over a dozen small genera of Iridaceae, Krameriaceae, Stilbaceae, Scrophulariaceae, Solanaceae, and Plantaginaceae (Vogel, 1974, 1988; Rasmussen and Olesen, 2000; Machado, 2004; Renner and Schaefer, 2010). How often this reward system has evolved and been lost is unknown, but minimal estimates are 28 origins and 36–40 losses (Renner and Schaefer, 2010). The oil is used by females of about 365 species of bees from a few tribes in the families Apidae and Melitidae that use it to provision their offspring or to line their larval cells (Cane et al., 1983; Vogel, 1988; Alves-dos-Santos et al., 2002; Melo and Gaglianone, 2005). Field observations over the past 45 yr have shown that these plant–bee interactions are mutually obligatory at the genus or family level, not at the species level (e.g., Rasmussen and Olesen, 2000; Machado, 2004; Martins et al., 2013), and dated molecular trees have clarified, at least in broad outline, how over the past 75 Myr successive groups of plants entered the "oil bee pollination niche" (Renner and Schaefer, 2010). However, the details of the implied switching of bees to new plant species and plant species to new pollinators remain vague, and partly for lack of field observations on the bee's foraging breadth, partly for lack of dated and densely sampled phylogenies for the interacting clades.

Among the oil-offering groups with published observations on bee behavior on the flowers is the Angelonieae tribe in the Plantaginaceae (Vogel, 1974; Simpson et al., 1990; Vogel and Machado, 1991; Vogel and Cocucci, 1995; Sérsic and Cocucci, 1999; Tadey, 2012; Martins et al., 2013; Martins and Alves-dos-Santos, 2013). This small neotropical group comprises 68 species in six genera, viz. *Angelonia* (26 species; Barringer, 1981), *Basistemon* (8 spp.; Barringer, 1985), *Melosperma* (1 sp.; Rossow, 1985), *Monopera* (2 spp.; Barringer, 1983), *Monttea* (3 spp.; Rossow, 1985) and *Ourisia* with 28 species (Albach et al., 2005; Oxelman et al., 2005; Meudt, 2006). The flowers are pollinated by some 15 species of *Centris* from several subgenera and visited by many further species of Tapinotaspidini (Martins and Alves-dos-Santos, 2013 and references therein). The phylogenetic relationships of the bee species pollinating Angelonieae have recently been clarified (Martins et al., 2014). Understanding the evolutionary relationship between them and their oil sources, however, still requires a densely sampled phylogeny of the Angelonieae that is geographically explicit and molecular-clock dated. A phylogeny of the six genera could clarify whether *Melosperma* and *Ourisia*, which lack oil-producing hair patches (they instead offer nectar to their pollinators), form a clade or evolved independently. *Basistemon* is variable for oil hairs (Barringer, 1985). Depending on the precise phylogenetic relationships among the genera, oil-offering could thus have evolved several times in the Angelonieae or it could be ancestral but lost in some species.

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To resolve these questions and to shed light on the biogeographic history of the group, we sequenced one nuclear and two plastid DNA markers from 38 of the 68 species representing the genera of the Angelonieae. Previous phylogenies only sampled a few species of Angelonieae and never included all genera. They could therefore not test whether *Angelonia*, *Basistemon*, and *Monopera*, which share similar saccate corollas (Barringer, 1981, 1983, 1985) or *Melosperma* and *Monttea*, which share curved filaments, similar nectaries and similar pollen, might form distinct clades (Rossow, 1985; Meudt, 2006). *Angelonia* itself has been divided into three sections based on the length of the corolla tube and the presence of outgrowths on the inner corolla (Barringer, 1981; our Fig. 1), characters now known to relate to the size of the pollinator (Martins and Alves-dos-Santos, 2013; Martins et al., 2013), suggesting they could have evolved in parallel as adaptations to particular *Centris* bees differing in body mass and ability to manipulate large or small oil hair fields. The question of the extent of evolutionary liability in oil bee–oil flower coevolution forms the backdrop to the current study.

MATERIALS AND METHODS

Taxon sampling—A total of 52 individuals were acquired from several herbaria (Table 1) and represent the three species of *Monttea*, the type species of *Melosperma*, seven of the eight species of *Basistemon* (the missing species, *B. argutus*, is known only from the type collection), the type species of *Monopera*, which has only one other species, and roughly half of all *Angelonia*. We further included 10 of the 28 species of *Ourisia* and as outgroups species of

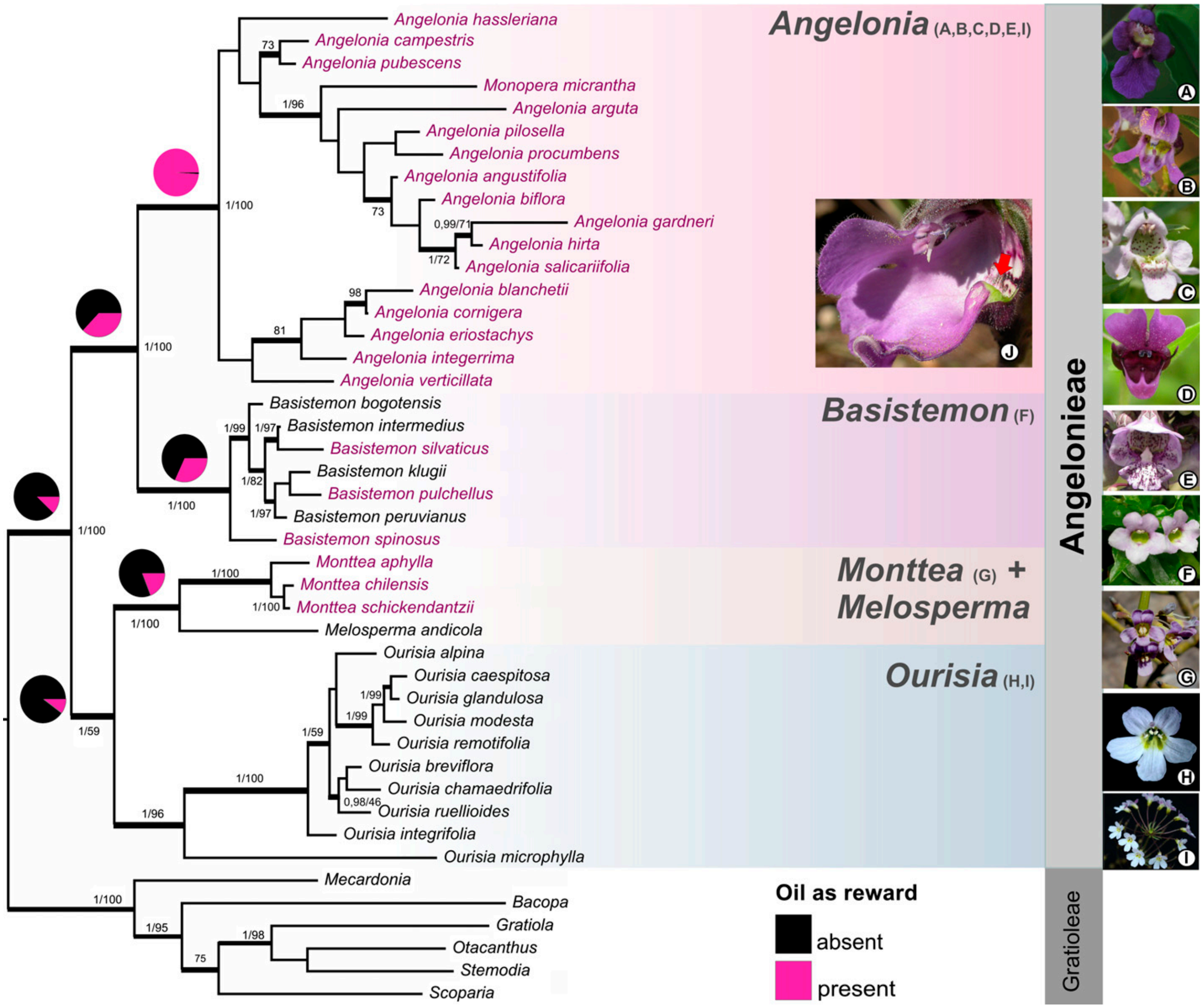

Fig. 1. Bayesian consensus tree resulting from the analysis of the combined nuclear and plastid data (44 species, 2085 aligned nucleotides), rooted between Angelonieae and Gratioleae. Numbers at nodes are Bayesian posterior probabilities and/or maximum likelihood bootstrap support values. Pie charts indicate the likelihood that floral oil hairs are absent (black) or present (purple); species names in purple indicate that the respective species has floral oil hairs. Photos on the right show the flowers of (A) *Angelonia campestris*, (B) *A. arguta*, (C) *A. salicariifolia*, (D) *A. cornigera*, (E) *A. integerrima*, (F) *Basistemon silvaticus*, (G) *Monttea aphylla*, (H, I) *Ourisia macrophylla*. (J) *Angelonia eryostachis* flower cut to show the green oil hair carpet (red arrow); corolla tube length 3 cm. *Photo credits:* (A, B) A. Aguiar, (C–F, I) A. Martins, (G) M. Taniguchi; (H) P. Garnock-Jones.

TABLE 1. Material used in this study with information on vouchers, geographic origin, and GenBank accession numbers. Numbers preceded by KM refer to newly produced sequences. Herbarium acronyms follow the Index Herbariorum (http://sweetgum.nybg.org/ih/).

Species	Voucher (Herbarium)	Collection site	*trnK-matK*	*trnL-F*	ITS
Angelonia angustifolia Benth.	R.L. McGregor 16461 (TEX)	Mexico: Puebla	AY492127.1	—	—
Angelonia arguta Benth.	E. Duarte & A. Castellanos 421 (M)	Brazil: Bahia, 10 km from Tucano, btw. Tucano and Euclides da Cunha, 15 Jul. 1964	KM281707	—	KM281689
Angelonia arguta Benth.	A. Aguiar 008 (UB)	Brazil: Goiás, Flores de Goiás, Margem do Rio Macaquinho, 19 Jan. 2013		KM281676	
Angelonia biflora Benth.	A. C. Martins 200 (M)	Brazil: cult. in Munich Botanical Garden (acc. 13/0882)	KM281708	KM281677	KM281690
Angelonia blanchetii Benth.	R. Harley 16787 (M)	Brazil: 8 km NW of Lagoinha, 5.5 km SW of Delfino on road to Minas do Mimoso. Caatinga/Cerrado. Ca 850 m a.s.l., 41°17′W, 10°24′S	KM281709	KM281678	—
Angelonia campestris Nees & Mart.	R. Harley 16756 (M)	Brazil: 8 km NW of Lagoinha, 5.5 km SW of Delfino on road to Minas do Mimoso. Caatinga/Cerrado.	KM281710	KM281679	—
Angelonia cornigera Hook.	R. Harley 16302 (M)	Brazil: 64 km N of Senhor do Bonfim on BA 130 highway to Juazeiro. Caatinga, ca. 400 m a.s.l., 40°15′W, 9°55′S, 25th. Feb. 1974	KM281711	KM281680	KM281691
Angelonia eriostachys Benth.	A. C. Martins 115 (ESA)	Brazil: Minas Gerais, Parque Estadual do Rio Preto, 18°12′S, 43°19′W, 1500 m a.s.l.	KM281712	KM281681	KM281692
Angelonia gardneri Hook	S. Beck 27908 (LPB)	Bolivia: Santa Cruz, 6 Jan. 2003	KM281713	KM371016	KM281693
Angelonia hassleriana Chodat	E. Zardini 33730 (MO)	Paraguay: Central, 18 Nov. 1992	KM281714	—	KM281694
Angelonia hirta Cham.	Rodrigues 30 (MO)	Brazil: Pernambuco, 4 Apr. 1995	KM281715	—	KM281695
Angelonia integerrima Spreng.	A. C. Martins 111 (ESA)	Brazil: Rio Grande do Sul, Porto Alegre, Parque Natural Morro do Osso, 30°07′S, 51°14′W, 143 m a.s.l.	KM371019	KM371014	—
Angelonia pilosella J. Kickx f.	Curtis (M)	West Indies: Near Nueva Gerona, Isla de Pinos, 26 Apr. 1904	—	KM281682	—
Angelonia pilosella J. Kickx f.	W. Greuter & Hankin 26861 (B)	Cuba: Prov. Pinar del Rio, Mun. Consolación del Sur, near Restaurant Barrigona, 3 km ESE of exit Herradura	KM281716	—	—
Angelonia procumbens Nees & Mart.	Paixão 87 (MO)	Brazil: Bahia, 18 Nov. 1998	KM281717	KM281683	KM281696
Angelonia pubescens Benth.	C. W. de Pamphilis 94/03 (PAC)	Unknown	—	AJ608618.1	—
Angelonia salicariifolia Bonpl.	A. C. Martins 113 (ESA)	Brazil: Mato Grosso do Sul, Porto Murtinho, 21°37′S, 57°49′W, 90 m	KM281718	KM371015	KM281697
Angelonia verticillata Philcox	P. von Lützelburg 176 (M)	Brazil: Bahia. Serra das Almas, 1600 m a.s.l., Carrasco, 1200 m a.s.l., 1914	KM371017	KM281684	KM281698
Bacopa monnieri (L.) Edwall	B.L. Turner & Turner 97-527 (TEX)	USA: TX, Crockett County	AY492129	AY492170	AY492095
Basistemon bogotensis Turcz.	H. van der Werff 7448 (MO)	Venezuela: Falcón	KM281719	—	KM281699
Basistemon intermedius Edwin	R. Seidel & Schulte 2184 (LPB)	Bolivia: La Paz, Sud Yungas, 700 m a.s.l.	KM281720	—	KM281700
Basistemon klugii Barringer	T. Plowman & al. 11387 (GB)	Bolivia: Mariscal Caceres, 09°09′S 076°27′W	AJ608616.1	AJ608616.1	—
Basistemon peruvianus Benth. ex B. D. Jacks.	G. Hatschbach et al., 74234 (M)	Brazil: Mato Grosso do Sul, start of road toward Mouraria do Sul (Mun. Bonito), 15 Nov. 2002	KM371018	KM281685	KM281701
Basistemon pulchellus (S. Moore) Barringer	J. Wood & Soto 25300 (LPB)	Bolivia: Beni or Pando	KM281721	KM281686	KM281702
Basistemon silvaticus (Herzog) Baehni & J. F. Macbr.	M. Nee 38032 (TEX)	Bolivia: Santa Cruz	AY492130.1	AY492171.1	AY492096
Basistemon spinosus (Chodat) Moldenke	J. Wood & Mendoza 21479 (LPB)	Bolivia: Santa Cruz, Vallegrande, 18°43′28″S 064°18′47″W, 950 m a.s.l., 25 Jan. 2005	KM281722	KM371013	—
Basistemon spinosus (Chodat) Moldenke	M. Dematteis 1972 (MBM)	Bolivia: Dep. Santa Cruz, Prov. Cordillera. 34 km N of Boyuibe, 20°15′56″S, 63°29′50″W, 896 m a.s.l., 29 Mar. 2006	—	—	KM281703
Gratiola officinalis L.	D. Albach 490 (WU)	Bulgaria: Rhodopes mountains	AY492143	—	AY492106
Gratiola pillosa Michx.	Voucher unclear	Unknown	–	AJ608591	
Mecardonia procumbens (Mill.) Small	Denny & R. Harvey 449 (TEX)	USA: TX, Bandera County	AY492152	AY492184	AY492111
Melosperma andicola Benth.	M.K. Arroyo & Humaña 206607 (TEX)	Chile: Santiago, Región Metropolitana	AY492185.1	AY492153	AY492112
Monopera micrantha (Benth.) Barringer	P. von Luetzelburg (M)	Brazil: Bahia, São Gonçalo, Apr. 1936	KM281723	—	KM281704

TABLE 1. Continued.

Species	Voucher (Herbarium)	Collection site	*trnK-matK*	*trnL-F*	ITS
Monttea aphylla (Miers) Benth. & Hook. F.	AAC 4463 (CORD)	Argentina: Neuquén, Partido de Confluencia, 38°95′74″S, 69°07′42″W	—	KM281687	KM281705
Monttea chilensis Gay	M. Dillon 8132 (F)	Chile: Region de Antofagasta	AY492187	AY492155	AY492114
Monttea schickendantzii Griseb.	AAC 4516 (CORD)	Argentina: Catamarca, Barranca Larga, 26°97′13″S, 66°73′83″W	—	KM281688	KM281706
Otacanthus sp.	Voucher unclear	Brazil: cult. in Bot. Garden Bonn	AY492188	AY492156	AY492115
Ourisia alpina Poeppig & Endlicher	H. Meudt et al., 40 (TEX)	Chile: Región de la Araucanía, Sierra Nevada	AY613154	—	AY613110
Ourisia breviflora Bentham in DC	H. Meudt & López 058(TEX)	Argentina: Prov. Tierra del Fuego, Paso Garibaldi	AY613155	—	AY613111
Ourisia caespitosa J. D. Hooker	H. Meudt & López 075 (TEX)	New Zealand: North Island, Mt. Ruapehu.	AY613159	JN246141	AY613115
Ourisia chamaedrifolia Benth. in DC.	H. Meudt & López 052 (TEX)	Peru: Cusco, Prov. Paucartambo.	AY613160	—	AY613116
Ourisia glandulosa J. D. Hooker	H. Meudt & López 090 (TEX)	New Zealand: South Island, Otago, the Remarkables	AY613168	JN246146	AY613124
Ourisia integrifolia R. Brown	H. Meudt & López 066 (TEX)	Australia: Tasmania, Hartz Mountains	AY613171	—	AY613127
Ourisia microphylla Poepp. & Endl.	H. Meudt & López 036 (TEX)	Chile: Maule, Cañon del Río Maule	AY492157	AY492189	AY613136
Ourisia modesta Diels	H. Meudt & López 079 (TEX)	New Zealand: cult. seeds from South Island, Nelson Hope Range	AY613181	—	AY613137
Ourisia remotifolia Arroyo	H. Meudt & López 094 (TEX)	New Zealand: South Island, Southland, Gertrude Saddle	AY613187	JN246158	AY613143
Ourisia ruellioides Kuntze	H. Meudt & López 013 (TEX) A. Sérsic 1231 (TEX)	Chile: Región de Los Lagos, Puyehue AY613144: Argentina, Prov. Santa Cruz, Perito Moreno	AY613190	AJ608579	AY613144
Scoparia dulcis L.	E. Fischer 10254 (BONN) Carr 10834 (TEX)	FN773556 + FN794072: Gabon AY492119: USA: TX, Hardin Co.	FN773556	FN794072	AY492119
Stemodia durantifolia (L.) Sw.	Reina et al., 98-198 (TEX)	Mexico: Sonora	AY492164	—	AY492120
Stemodia glabra Oerst.	B. Nordenstam & A. Anderberg 967 (S)	Nicaragua	—	AJ608566	—

Bacopa, *Gratiola*, *Otachantus*, *Scoparia*, *Stemodia*, and *Mecardonia*, which represent the Gratioleae based on Estes and Small (2008) and Schäferhoff et al. (2010).

Molecular phylogenetics—Total DNA was extracted from 20 mg of leaf tissue using the Nucleospin Plant II kit (Machery-Nagel, Düren, Germany) and following the manufacturer's protocol except that after homogenization, the sample was not transferred to a new tube to minimize loss of material. Samples were lysed at 65°C for between 1 and 2 h, the crude lysate was not filtered, and a single pass of elution buffer was utilized to achieve maximum concentration of DNA in the sample. Presence and concentration of DNA in samples was assessed using a NanoDrop 1000 spectrophotometer (Nanodrop Technologies, Oxfordshire, UK), cleaned with purified water and calibrated with the elution buffer used in the extraction. For recalcitrant samples, we repeated extractions with up to 60 mg of material, using the Invisorb Spin Plant Mini Kit (Stratec Molecular, Berlin, Germany). We amplified part of the plastid *trnK* intron and *matK* gene, using the primers trnK-2R and matk8F (Steele and Vilgalys, 1994) and the *trnL* intron and spacer using the Taberlet et al. (1991) UniC, D, E, and F primers, and the nuclear ribosomal DNA internal transcribed spacers (ITS) 1 and 2, as well as the 5.8S region, using the newly designed primers 18S-15F (TCGCGACGAGAAGTCCACTGAAC), 5.8S-434R (TGGTTCACGGGATTCTGCAA), 5.8S-348F (GGCTCTCGCATCGATGAAGA), and ITS-607R (CTAGTCGTGATATCCGCCGG). The PCR conditions were 94°C for 3 min; followed by 39 cycles of 94°C denaturation for 30 s, 48–50°C annealing for 1 min, and 68°C extension for 1 min; and 10 min for final elongation. Successful DNA extractions and amplifications were purified and sequenced using BigDye and a 3130-4 sequencer (Applied Biosystems, Foster City, California, USA). Sequences were examined, edited, and constructed into contigs using Geneious 6.0 software (Biomatters, Aukland, New Zealand), and all have been deposited in GenBank under the accession numbers presented in Table 1.

The cleaned sequences were automatically aligned using MAFFT (Katoh and Standley, 2013) as implemented in Geneious v. 6.1 and taking into account ITS secondary structure under the Q-INS-I strategy (Katoh and Standley, 2013). No ambiguously aligned regions were excluded, and a few minor adjustments were made by eye. Phylogenetic analyses were conducted separately for the plastid and nuclear data matrices, and in the absence of well-supported topological conflict (defined as >70% maximum likelihood bootstrap support or >0.98 posterior probability), the matrices were concatenated. Maximum likelihood (ML) trees were obtained with the program RAxML-HPC2 7.2 (Stamatakis, 2006) as implemented on the CIPRES server in January 2014 (Miller et al., 2010) with 1000 fast bootstrap replicates under the GTR + I + Γ model unlinked for the nuclear and plastid partitions. Bayesian inference with Markov chain Monte Carlo (MCMC) was performed in the program MrBayes 3.2.2 (Ronquist et al., 2012) with the same substitution model and the default two chains, each of a length of 1 million steps and sampling every 10000th step, with a burn-in of 20%. Chain convergence was assessed in the program Tracer 1.5 (Rambaut et al., 2013).

Divergence time estimation—Dating used the same data matrix and relied on the program BEAST 1.8.0 (Drummond et al., 2012), using the uncorrelated lognormal relaxed-clock model, a Yule tree speciation model, and the GTR + I + Γ substitution model with empirical base frequencies and separated data partitions. MCMC chains were run for 100 million iterations, sampling every 10000 steps. Convergence and effective sample sizes were assessed in Tracer 1.5, and when effective sample sizes for all parameters were >300, a maximum clade credibility tree was produced in the program TreeAnnotator 1.8.0 (part of the BEAST package) with a burn-in of 25%. Trees were visualized and manipulated in the program FigTree 1.4.0 (Rambaut, 2009).

Plantaginaceae comprise about 90 genera with together 1900 species and have no undisputed fossil record. An angiosperm-wide dating study, calibrated with several fossils of other families, estimated the crown age of the Plantaginaceae as 46 (36–57) Ma (Bell et al., 2010). We therefore used published substitution (sub) rates to calibrate our trees and cross-validated our results by comparing the Angelonieae age found with the rate calibration to the family age found by Bell et al. (2010) with their fossil calibrations. For ITS, we used a rate of 8.34×10^{-9} subs·site^{-1}·yr^{-1}, which comes from the Primulaceae *Soldanella* (Kay et al., 2006) and is almost identical to the ITS rates of another species of small herb, the Gentianaceae *Gentianella* (8.41×10^{-9} subs·site^{-1}·yr^{-1}; Hagen and Kadereit, 2001, obtained with a fossil calibration). We used a plastid rate of 1.3×10^{-3} subs·site^{-1}·Myr^{-1}, which comes from the *trnL-F* region of *Inga* (Fabaceae; Richardson et al., 2001) and is similar to many other land plant *trnL* rates (Villarreal and Renner, 2014: table 3). The prior on each rate was a gamma distribution with an initial value of 1.0, and rates were unlinked among the nuclear and plastid partitions.

Ancestral state reconstructions for oil hairs and biogeography—We inferred the evolution of floral oil hair patches, using the states "present" or "absent." Information on the presence/absence of oil hairs came from relevant literature (Vogel, 1974; Barringer, 1981, 1983, 1985; Rossow, 1985; Simpson et al., 1990; Sérsic and Cocucci, 1999; Meudt and Simpson, 2007; Martins and Alves-dos-Santos, 2013). Species of *Angelonia*, *Monopera*, and *Monttea* as well as *Basistemon silvaticus* and *B. spinosus* were coded as "oil present", the remaining *Basistemon* species were coded as "oil absent". Barringer (1985) observed a few oil hairs in *B. pulchellus*, and as no data exist on its pollinators, we alternatively coded this species as "oil absent" or as "oil present". *Melosperma*, *Ourisia*, and the outgroups (Gratioleae) were all coded as "absent." Ancestral state reconstruction used parsimony and maximum likelihood (MK1 model; Lewis, 2001) in the program Mesquite v. 2.75 (Madison and Madison, 2011) on a maximum clade credibility chronogram.

For the biogeographic reconstruction, species occurrences were compiled from the vouchers included in this study (Table 1) and relevant literature (Barringer, 1981, 1983, 1985; Rossow, 1985; Meudt and Simpson, 2006; Souza and Giulietti, 2009). The ingroup species were assigned to one of the following regions: (A) Caatinga, Cerrado, and Chaco areas, which are seasonally dry vegetation types (Werneck, 2011); (B) northern Andes; (C) southern Andes; (D) South American Arid Diagonal, a belt of arid vegetation with <300 mm/year in rainfall that crosses South America from 2°S in the Gulf of Guayaquil to 52°S bounding the Straits of Magellan (Blisniuk et al., 2005); (E) Central America and Caribbean; (F) Australasia: Australia, Tasmania, New Zealand. The Gratioleae outgroups (*Mecardonia*, with 31 species mostly in South America; *Scoparia*, 2 species in South America; *Gratiola*, 16 species mostly in South America; *Stemodia*, 20 species mostly in South America; *Otacanthus*, ca. 10 species in South America; and *Bacopa*, with 65 species of aquatics, mostly in South America) were coded as South America (G), which in terms of the analysis comes to the same as coding them as "missing data". Reconstruction relied on Bayes-DIVA analysis using the program Reconstructed Ancestral States in Phylogenies (RASP) v. 2.1 (Yu et al., 2013), also referred to as S-DIVA (Yu et al., 2010). As input, we used 1000 trees from the Bayesian MCMC chain obtained in the BEAST analysis. Results were summarized on the majority rule consensus of the BEAST analysis, which is fully bifurcating.

RESULTS

Species and genus relationships—Our *trnL* alignment consisted of 969 sites, the *trnK-matK* alignment of 510 sites, and the ITS alignment of 606 sites. In the absence of any well-supported (>70 ML bootstrap support; *Materials and Methods*) topological conflict between the plastid and nuclear trees, the data were concatenated, yielding a matrix of 2085 sites (the matrix and trees have been submitted to TreeBASE (http://treebase.org/treebase-web/home.html, study number 16619). Bayesian and maximum likelihood trees from the combined data also showed no well-supported conflict. *Ourisia* is sister to *Melosperma* and *Monttea* (Fig. 1), and a clade of the other three genera, *Angelonia, Basistemon*, and *Monopera*, also has high support, with *M. micrantha*, the type species of the genus, deeply nested within *Angelonia*.

Divergence times and biogeography—The chronogram obtained from the concatenated matrix (Fig. 2) yielded a root age, i.e., the split between the Angelonieae and Gratioleae, in the Middle Eocene at 46 (34–61) Ma (95% highest posterior

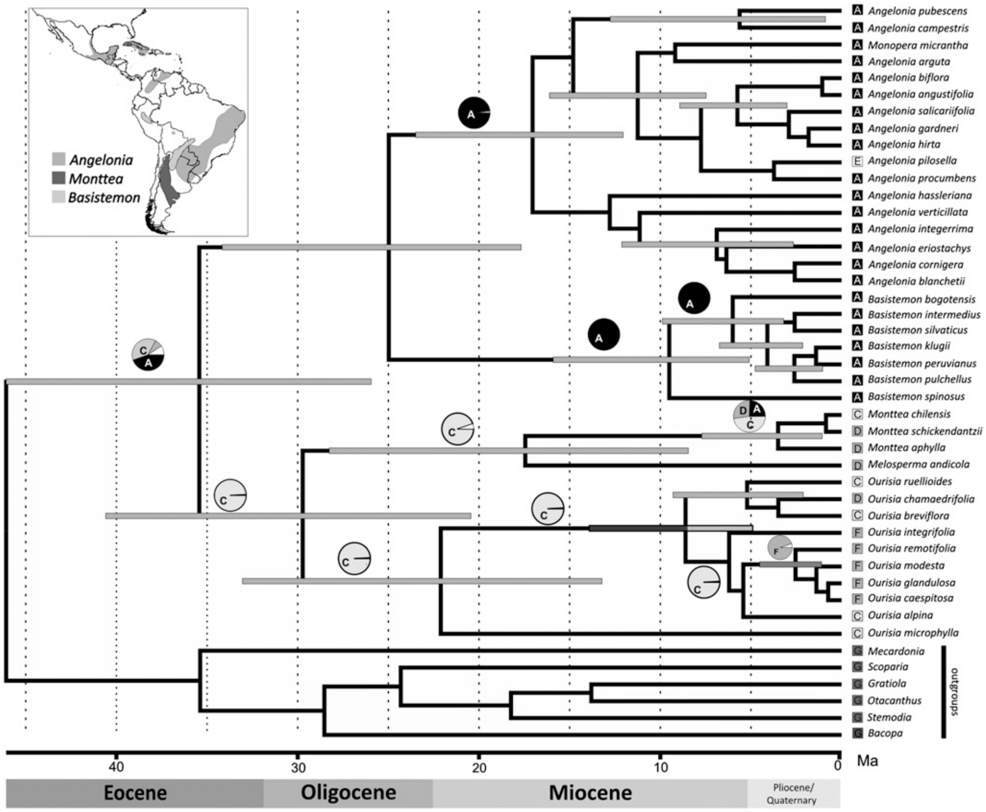

Fig. 2. Chronogram for Angelonieae obtained under a Bayesian relaxed clock model and the same data as used in Fig. 1. Bars at nodes indicate 95% confidence intervals around the respective node ages and are shown for all well-supported nodes (compare support values shown in Fig. 1). Pie charts indicate the probabilities for each alternative ancestral area derived from Bayesian ancestral reconstruction over 1000 dated trees. Squares indicate the geographic distribution of the species, with the areas being (A) South American open vegetation biomes; (B) northern Andes; (C) southern Andes; (D) South American Arid Diagonal; (E) Central America and Caribbean; (F) Australasia; (G) South America (used only for outgroups). The inset shows the geographical range of the three oil-producing clades in neotropical Plantaginaceae: *Angelonia* (including *Monopera*), *Basistemon*, and *Monttea*.

density interval), a crown age of the Angelonieae in the Uppermost Eocene at 35 (26–47) Ma, a crown age of the *Angelonia/Basistemon* clade in the Upper Oligocene at 25 (18–34) Ma, and a crown age of the *Monttea/Ourisia* clade in the Upper Oligocene at 30 (20–40) Ma. We take up the issue of cross validation of these estimates in the Discussion. The biogeographic reconstruction (Fig. 2) gave ambiguous results for the Angelonieae, which originated either in seasonally dry South American biomes or the Southern Andes. *Angelonia* appears to have initially diversified in the Caatinga, Cerrado, and Chaco area, with some northward expansion to Central America and the Caribbean, in our sampling represented by the Cuban species *A. pilosella*. The *Monttea/Ourisia* clade apparently originated in the southern Andes, with two independent dispersal events from southern South America (probably Chile) to Tasmania/New Zealand at 6 (3–10) Ma and 3 (1–5) Ma (Fig. 2).

Ancestral state reconstruction of the floral rewards—Figure 1 (pie diagrams at nodes) shows the inferred evolution of floral oil as a reward in the Angelonieae obtained under maximum likelihood trait state reconstruction; parsimony-based reconstructions gave the same result. The most plausible ancestral condition is the absence of floral oil with five later gains, one in the common ancestor of *Angelonia*, one in the common ancestor of *Monttea*, and three in *Basistemon* (represented by 7 of its 8 species), where *B. silvaticus* and *B. spinosus* produce oil, and flowers of *B. pulchellus* have at least a few oil hairs. When this species was coded as oil producing (as shown in Fig. 1), the ancestral state in *Basistemon* was "oil present" with a likelihood of 34%; when it was coded as "oil absent," that likelihood dropped to 32%. Unfortunately, a third oil-producing species, *B. argutus*, know only from the type, could not be sequenced. The alternative scenario, with the ancestral state of the Angelonieae being "oil hairs present" would require one gain in the ancestor, one loss in the common ancestor of *Melosperma*, *Ourisia*, *Monttea*, followed by a regain in *Monttea* and several losses in *Basistemon*, and is thus less parsimonious.

DISCUSSION

Species and genus relationships—Morphological and taxonomic work by Barringer (1985) suggested that *Angelonia*, *Monopera*, and *Basistemon* share a unique common ancestor because of their similar saccate corollas and capitate stigmas. This inference is supported by the molecular phylogenetic results presented here. Likewise, *Monttea* and *Melosperma* were inferred to be closely related based on curved filaments and their similar nectary scales (Rossow, 1985; Sérsic and Cocucci, 1999). However, their sister group relationship to *Ourisia* was unknown, probably due to insufficient taxon sampling as also suggested by Meudt (2006). *Ourisia* has the same nectary scales as *Monttea* and *Melosperma*, but like *Melosperma* lacks oil-producing hairs in its flowers, while the three species of *Monttea* have such hairs and floral oils. We discuss the implications of this for the evolution of the oil-flower syndrome below.

The nesting of *Monopera* in *Angelonia* agrees with the original placement of the two species of this genus. The type species of *Monopera* was described as *Angelonia micrantha* (Bentham, 1846), and the second species, *M. perennis* (Chodat & Hassl.) Barringer, was originally *A. micrantha* Benth. var. *perennis* Chodat & Hassl. The only nomenclatural change needed now is the return of these entities to *Angelonia*. Barringer's (1983) reason for separating *Monopera* was the unisaccate corolla, which he thought was quite different from the bisaccate corolla typical of *Angelonia*, while the molecular tree implies that this is a derived form of corolla. Our species sampling within *Angelonia* is still too sparse to fully assess the monophyly of the three sections of Barringer (1981).

Cross validation of divergences times and southern hemisphere long-distance dispersal—Our dating analysis yielded a crown age of the Angelonieae in the Uppermost Eocene at 35 (26–47) Ma, slightly younger than the age for the Plantaginaceae of 46 (36–57) Ma inferred by Bell et al. (2010), who only included five representatives of this family of ca. 90 genera. The *Centris* clade of oil bees that is most closely associated with the oil-offering Angelonieae is 44 (31–55) Ma old (Martins et al., 2014), which would place the diversification of these bees at roughly the same time as the divergence between the two main clades within the Angelonieae. A recent phylogeographic study of *Monttea chilensis* that used *M. schickendantzii* to root their analyses, dated the split separating these two species to 5.6 (2–6) Ma, with a published plastid substitution rate, or 11 (5–16) Ma with a nuclear substitution rate (Baranzelli et al., 2014), while we inferred an age for this split of 0.8 (0.1–2.6) Ma. This difference is probably due to a combination of the slightly faster plastid and nuclear substitution rates used here compared with their study and their dense population sampling within *Monttea* (they have 13 individuals in their plastid tree and 28 in the nuclear tree). The diversification of the Angelonieae appears to have occurred largely in southern South America (inset in Fig. 2).

A molecular phylogenetic study by Meudt and Simpson (2006) already showed that *Ourisia*, which has one species in Tasmania, Australia, 12 in New Zealand, and 15 in the South American Andes, originated in southern South America from where it may have dispersed to Tasmania and New Zealand. Their study refrained from molecular clock analysis, and their trees were rooted on more distant Plantaginaceae, instead of the true sister clade *Monttea/Melosperma* (our Figs. 1 and 2), which is native to Chile and Argentina just as the South American species of *Ourisia.* Our study confirms their inference of two long distance dispersal events, perhaps near the Miocene–Pliocene boundary. Similarly recent transoceanic dispersals have been inferred in the legume genus *Sophora* (Hurr et al., 1999, 2–5 Ma), the Atherospermataceae *Laurelia* (Renner et al., 2000, 50–30 Ma), the grass *Oreobolus* (Chacón et al., 2006, 3–4 Ma) and the Alstroemeriaceae genus *Luzuriaga* (Chacón et al., 2012, 3 Ma).

Origins of the oil-offering syndrome in the Angelonieae—Plantaginaceae often have glandular hairs on their corollas, which is the key morphological preadaptation facilitating the transition from nectar to oil as a reward (Fig. 1, photo I). If female oil bees that visited *Ourisia*-like flowers for nectar or pollen would occasionally try to exploit the glandular hair patches on the inner corolla and pollinate the flowers in the process, this would have set up the selective conditions for switching from nectar to oil as the main reward. This scenario is made more plausible by the observation that oil bees sometimes harvest glandular exudates even from nonfloral plant hairs (Melo and Gaglianone, 2005). The flowers of *Monttea*, like those of its closest relatives *Melosperma* and *Ourisia,* still have nectary disks (Meudt, 2006, p. 19), while at the same time possessing oil hair patches and being pollinated by medium-sized oil-collecting *Centris* (Simpson et al., 1990; Tadey, 2012). Bees from the particular subgenera of *Centris* (Centridini) and from the genus *Caenonomada* (Tapinotaspidini) interacting with Angelonieae have pads of soft hairs on their forelegs adapted to absorbing the oil from the trichomes, which are <1 mm high (Simpson et al., 1990; Martins et al., 2013). While exploiting the oil glands, the bees stabilize themselves by holding onto the filaments with their mandibles and their head then comes into contact with the stigma (Martins et al., 2013: their photo 5). Relatively long glandular hairs on the inner flower lip also characterize the genus *Mecardonia* (15 species) in the sister clade of the Angelonieae, the Gratioleae (Cappellari et al., 2009). In that case, however, it is male oil bees that collect oil from these trichomes; there is no nectar.

It is possible that oil hairs in *Basistemon* evolved repeatedly. We included seven of its eight species (Barringer, 1985), including the oil-offering *B. silvaticus* and *B. spinosus*, but our phylogeny lacks the oil-producing *B. argutus*. In addition, *B. pulchellus*, has very few oil hairs, and we therefore coded it once as oil producing, once as not oil producing (Fig. 1 shows it coded as oil producing). Fieldwork is required to understand the within- and among-species variation in oil production in this genus.

Conclusions—Our results reveal that oil as a pollinator reward evolved four or five times within the Angelonieae, a clade that may date to the Eocene, matching the recently inferred crown age of the *Centris* oil bee clade that it is most closely associated with (Martins et al., 2014). We also found that the species and subspecies of *Angelonia micrantha* that were separated and ranked as the genus *Monopera* should be returned to *Angelonia* and that *Ourisia* is sister to *Monttea* and *Melosperma*, with which it shares characteristic nectary scales. The two long-distance dispersal events from Chile to Australasia in *Ourisia* appear to have occurred about 3 to 6 to Ma, adding yet another example of Pliocene transoceanic dispersal among the continents of the southern hemisphere.

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