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Source: Systematic Botany, 35(4):811-826. 2010.

Published By: The American Society of Plant Taxonomists

URL: <http://www.bioone.org/doi/full/10.1600/036364410X539880>

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## Biogeographic Patterns of Diversification and the Origins of C<sub>4</sub> in *Cleome* (Cleomaceae)

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Communicating Editor: Andrew Hipp

**Abstract**—Photosynthetic pathway innovations have had a large impact on patterns of diversification of angiosperm lineages and the biogeographic distribution of ecological assemblages. C<sub>4</sub> photosynthesis has been one of the most studied processes in plants with respect to function, structure, occurrence, and response to climatic conditions. One of the most promising areas of research of C<sub>4</sub> photosynthesis is in the Cleomaceae. Here we explore the phylogenetic origins of the C<sub>4</sub> pathway in the Cleomaceae using maximum parsimony, maximum likelihood, and Bayesian inference analyses of nrDNA ITS sequences. As has been found previously, commonly recognized genera including *Buhsia*, *Cleomella*, *Dactylaena*, *Gynandropsis*, *Isomeris*, *Oxystylis*, *Podandrogynne*, *Polanisia*, and *Wizlizenia* are derived from within a paraphyletic *Cleome*. The phylogenetic results presented here indicate that there are likely at least five separate origins of carbon concentrating mechanisms in the Cleomaceae, including at least three separate origins of C<sub>4</sub> species. Analyses of historical biogeography suggest Cleomaceae originated in central Asia.

**Keywords**—ancestral state reconstruction, C<sub>4</sub> photosynthesis, Cleomaceae, historical biogeography, ITS, molecular phylogeny.

Photosynthetic pathway innovations, or the mechanisms by which plants fix inorganic carbon, have had a large impact on diversification of angiosperm lineages and the biogeographic distribution of ecological assemblages. One of the most significant of these is the carbon concentrating mechanism (CCM) known as C<sub>4</sub> photosynthesis (Hatch 1971), which has allowed angiosperms to avoid the detrimental effects of the Rubisco oxygenation reaction under relatively high atmospheric concentrations of oxygen in relation to CO<sub>2</sub> (Edwards and Walker 1983). This physiological adaptation has been one of the most studied processes in plants (Sage and Monson 1999; Raghavendra and Sage 2010), and it is a major focus for genetic modification of crop plants for increased productivity (Leegood 2002; Sheehy et al. 2007; Hibberd et al. 2008). Unfortunately, most C<sub>4</sub> crop and model plants have major barriers to detailed study of the genetic basis and the mechanism of C<sub>4</sub> function (Brown et al. 2005).

One of the most promising areas of research of C<sub>4</sub> photosynthesis is in the Cleomaceae, where C<sub>4</sub> photosynthesis has been derived at least once (Kellogg 1999). Cleomaceae is the sister lineage of the Brassicaceae, which includes *Arabidopsis*. There is interest in applying the genomic tools and functional knowledge of *Arabidopsis*, a C<sub>3</sub> species, to a C<sub>4</sub> species in genus *Cleome* (Brown et al. 2005). This would significantly increase our understanding of the genetic basis for structure and function of the C<sub>4</sub> syndrome and provide a context for how the individual elements of C<sub>4</sub> photosynthesis might have been derived phylogenetically.

Despite a number of investigations, outstanding questions remain regarding phylogenetic relationships within Cleomaceae (Hall et al. 2002; Sanchez-Acebo 2005; Hall 2008; Inda et al. 2008). With more than 200 species in the family and a world-wide distribution, the number of origins of the C<sub>4</sub> pathway and their phylogenetic history are unclear. Four phylogenetic analyses have been conducted in Cleomaceae in recent years (Hall et al. 2002; Sanchez-Acebo 2005; Hall 2008; Inda et al. 2008), but none of these studies included more than 40 species, and none of them included any of the potential C<sub>4</sub> or C<sub>3</sub>-C<sub>4</sub> intermediate species other than *Cleome*

(*Gynandropsis gynandra*). Additionally, all four of these studies have shown that many of the genera currently recognized in the Cleomaceae are derived within a paraphyletic *Cleome*. The taxonomic problems with generic boundaries and relatively sparse sampling of potentially C<sub>4</sub> or C<sub>3</sub>-C<sub>4</sub> intermediate lineages needs to be overcome to clarify the patterns of photosynthetic pathway diversification in the family.

Cleomaceae is comprised of three subfamilies of Capparaceae, as traditionally defined (Pax and Hoffmann 1936; Hall 2008), including the genera *Buhsia* Bunge, *Cleome*, *Cleomella* DC., *Dactylaena* Schrad. ex Schult. f., *Dipterygium* Decne., *Gynandropsis* DC., *Haptocarpum* Ule, *Isomeris* Nutt. ex Torr. & Gray, *Oxystylis* Torr. & Frem., *Podandrogynne* Ducke, *Polanisia* Raf., *Puccinonia* Chiov., and *Wizlizenia* Engelm. Previous studies have shown that many of these genera are nested within *Cleome*, and the previously recognized subfamilies do not represent monophyletic groups (Hall et al. 2002; Hall 2008; Inda et al. 2008). These studies lead to efforts to reorganize the New World Cleomaceae classification (Iltis and Cochrane 2007; Iltis and Cochrane, unpublished), including the recognition of the genera *Cleoserrata* Iltis, *Hemiscola* Raf., *Peritoma* DC., and *Tarenaya* Raf. However, all of these studies have focused on New World taxa only, leaving unclear the relationships of the New World and Old World lineages of *Cleome*.

Recently surveys were made in Cleomaceae in an attempt to identify C<sub>4</sub> species or species having C<sub>4</sub> traits (Voznesenskaya et al. 2007; Marshall et al. 2007; Koteyeva et al. unpublished data). Prior to this, only one species had been demonstrated to be C<sub>4</sub>, *C. gynandra*. *Cleome angustifolia* and *C. oxalidea* have also now been identified as C<sub>4</sub> plants based on C<sub>4</sub>-type carbon isotope composition (Voznesenskaya et al. 2007; Marshall et al. 2007), and Kranz type leaf anatomy (Koteyeva et al. unpublished data). The only *Cleome* species identified thus far to function as a C<sub>3</sub>-C<sub>4</sub> intermediate species is *C. paradoxa* (Voznesenskaya et al. 2007). In addition to these C<sub>4</sub> taxa, there are several species that show intermediate or C<sub>4</sub>-like carbon isotope ratios from a survey of 238 samples of 156 species in four genera of Cleomaceae (Voznesenskaya et al. 2007). Limited microscopy studies of leaf anatomy also show that a

few species have some traits suggested to facilitate  $C_4$  evolution (Voznesenskaya et al. 2007; Marshall et al. 2007). More studies are needed on these and related species to identify structural and functional relationships associated with  $C_4$  photosynthesis and to discover additional species that have developed some form of a CCM, either  $C_3$ - $C_4$  intermediates,  $C_4$ -like, or  $C_4$ .

Here we reconstruct the phylogenetic origins of the  $C_4$  pathway in the Cleomaceae using nrDNA ITS sequences analyzed using maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) approaches. Specifically, we address the questions: (1) How many phylogenetic origins of CCMs have occurred in the Cleomaceae? (2) What are the biogeographic origins of  $C_4$  and how does this pattern fit into overall reconstruction of historical biogeography patterns in the family? (3) How do these analyses contribute to our understanding of generic circumscription and species relationships in the Cleomaceae?

## MATERIALS AND METHODS

**Taxon Sampling**—One hundred and fourteen samples of 81 species of Cleomaceae were included to represent as many genera, clades and geographic areas as possible, based on previous studies. Samples were obtained from the Komarov Botanical Institute (LE), Australian National Herbarium (CANB), Washington State University (WS), Missouri Botanical Garden (MO), New York Botanic Garden (NY), and Kew Royal Botanical Gardens (K). Other sources of samples included cultivated species in the greenhouse of the School of Biological Sciences, Washington State University, and NCBI GenBank sequences from previous studies (Inda et al. 2008). Outgroups were chosen from representatives of major lineages of the Brassicaceae (Hall et al. 2002), as this is the sister lineage to the Cleomaceae (Hall et al. 2002; Hall 2008). Here we consider *Cleome* in the broad sense (as in Voznesenskaya et al. 2007; Inda et al. 2008), and where currently unrecognized generic names, or other informal taxon names, are used to refer to clades, they are placed in quotes to note their informal application.

**DNA Extraction, PCR, and Sequencing**—DNA was isolated using a modified  $2 \times$  CTAB buffer method (Doyle and Doyle 1987). Templates of the nrDNA ITS region were prepared using the primers ITS5HP (Suh et al. 1993) and ITS4 (White et al. 1990). Polymerase chain-reaction (PCR) amplifications follow the procedures described by Roalson et al. (2003).

The PCR products were electrophoresed using a 1% TBE agarose gel, stained with ethidium bromide to confirm a single product, and purified prior to sequencing using the Exonuclease enzymatic reaction (New England Biolabs, Ipswich, Massachusetts). Sequencing was performed using an ABI Prism 3730 Genetic Analyzer. Direct cycle-sequencing of purified template DNAs followed manufacturer's specifications, using the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, Foster City, California). The two ITS sequencing primers provide sequences for overlapping fragments that collectively cover the entire spacer and 5.8S rDNA regions along both strands.

Automated DNA sequencing chromatograms were proofed, edited, and contigs were assembled using Sequencher 4.6 (Gene Codes Corporation, Inc., Ann Arbor, Michigan). The ITS sequences were truncated to include only ITS1, 5.8S, and ITS2. Identification of the terminal ends and spacer boundaries of ITS1, 5.8S, ITS2 was based on comparisons with other species of Cleomaceae (Inda et al. 2008). Sequences were aligned using Clustal X (Thompson et al. 1997) with the following parameters: pairwise comparisons- gap opening penalty: 10.00, gap extension penalty: 1.00; and multiple comparisons- gap opening penalty: 10.00, gap extension penalty: 1.00. The resultant alignment was then checked by eye for necessary minor corrections to the alignment. Alternate alignment parameters did not result in significantly different topologies (data not shown). New sequences are deposited in GenBank (Appendix 1), and the aligned data matrix and trees are deposited in TreeBASE (study number S10343).

**Phylogenetic Analyses**—The ITS gene region was analyzed with MP, ML, and BI. Maximum parsimony and ML analyses were performed using PAUP\* 4.0b10 (Swofford 2001). Maximum parsimony analyses used heuristic searches (ACCTRAN, 1,000 random addition cycles, TBR branch swapping; limit of 10,000 rearrangements per addition-sequence replicate; characters equally weighted). Swapping was run to completion for all random addition replicates. Clade support was estimated using 1,000 heuristic

bootstrap replicates (1,000 random addition cycles per replicate, TBR branch swapping; limit of 10,000 rearrangements per addition-sequence replicate; Felsenstein 1985; Hillis and Bull 1993).

Maximum likelihood analyses employed heuristic searches (TBR branch swapping). Clade support was estimated using 700 heuristic bootstrap replicates (10 random addition cycles and 100 total rearrangements per replicate, TBR branch swapping; Felsenstein 1985; Hillis and Bull 1993). Maximum likelihood analysis of the ITS data set employed the symmetrical (SYM) model with proportion of invariant sites (I) and gamma shape (G) parameters (Zharkikh 1994; six substitution types: A/C- 0.86833032, A/G- 1.96300376, A/T- 1.52126422, C/G- 0.51056371, C/T- 3.47308378, G/T- 1.0000; I = 0.25458051; G = 1.440765). This model was chosen based on the results of analysis using DT\_ModSel (Minin et al. 2003). The DT\_ModSel analysis uses a Bayesian information criterion to select a model using branch-length error as a performance measure in a decision theory framework that also includes a penalty for model overfitting. Bayesian inference analyses were performed using MrBayes v. 3.1 (Huelsenbeck and Ronquist 2001) and the model from the ML analysis. Four chains were run for 10,000,000 generations each, and sampled every 10,000 generations. Multiple independent BI analyses were run and the results were compared with AWTY (Wilgenbusch et al. 2004) to test for convergence and mixing.

To assess the potential resolving power of combining the data gathered here with previously published data sets, we combined our data with the cpDNA data previously published (Hall et al. 2002; Hall 2008). Because there is incomplete overlap in taxon sampling between the previous studies and this study, two approaches were taken in data combination. First, a set of 38 species for which there was maximal overlap of the *matK*, *ndhF*, and ITS were analyzed, and, second, the complete ITS data set was analyzed with the sequences available for *matK* and *ndhF*, with a large percentage of missing data present. Both of these combined data sets were analyzed with MP to assess the potential contribution of the combined data for resolving the backbone of the Cleomaceae phylogeny.

**Tests of Alternative Topologies**—Given low support at some nodes deep in the trees, particularly those associated with possible CCM lineages, two different methods were used to assess whether other topologies could be statistically rejected: the Shimodaira-Hasegawa (SH) test, and Bayesian confidence interval. The SH test (Shimodaira and Hasegawa 1999) was implemented in PAUP\* comparing constraint ML topologies with the best ML tree using 5,000 RELL bootstraps. The posterior distribution of trees from the BI analysis was assessed for the proportion of trees in the posterior distribution that had the partitions of interest. As the posterior distribution should reflect a statistical confidence interval, the proportion of trees with the partition to be tested can be compared with the 95% confidence limits to give an indication of whether a particular partition can be statistically rejected. Three alternative topologies were compared to the ML or BI topologies to test whether fewer origins of the  $C_4$  pathway than suggested by the ML tree could be rejected. These were (1) monophyly of the three clades which contain full  $C_4$  species (" $C_4$  test"; monophyly of *C. angustifolia*, *C. gynandra*, *C. luderitziana*, *C. oxalidea*, and *C. paradoxa*); (2) monophyly of the "*Gynandropsis*" and "*C. angustifolia*" clades (excluding *C. oxalidea*; lineages 5 and 8; " $C_4$ -2 test"); and (3) monophyly of the "*Gynandropsis*," "*C. angustifolia*," "*Corynandra*," Clade 4, and Clade 5 clades (lineages 5, 7, and 8; " $C_4$ -reversion test"). The " $C_4$  test" was used to test whether the three lineages with fully  $C_4$  species could be rejected as monophyletic. This test was not done excluding *C. luderitziana* and *C. paradoxa* because of the strong support placing those lineages with *C. angustifolia*. The " $C_4$ -2 test" was conducted because the strong support for the placement of *C. oxalidea* with other Australian species might negatively affect the  $C_4$  test; so, this test would determine whether we could reject monophyly of the "*Gynandropsis*" and "*C. angustifolia*" clades together to the exclusion of *C. oxalidea*. Finally, the " $C_4$ -reversion test" is similar to the  $C_4$ -2 test in not forcing the movement of *C. oxalidea* away from the other Australian species, but rather testing the possibility that the "*Gynandropsis*," "*C. angustifolia*," and Australian lineages (Clade 4, Clade 5, and "*Corynandra*") were together a monophyletic group. No tests were conducted for the monophyly of these lineages with the *C. allamanii*, *C. siliculifera*, and *C. sparsifolia* lineages because of the large number of strongly supported branches that would have to be rearranged to group those species.

**Reconstruction of Biogeographic Patterns**—Historical biogeographic patterns in the Cleomaceae were inferred using three methods: standard Fitch parsimony optimization (FPO); accelerated transformation; as implemented in Mesquite 1.12; Maddison and Maddison 2006), dispersal-vicariance analysis (DIVA; Ronquist 1996, 1997), and the dispersal-extinction-cladogenesis model (DEC; Ree and Smith 2008). As all of these methods require the use of a single resolved phylogeny, the ML tree was

used in each of these reconstruction methods. The FPO method does not allow for polymorphic states at the internal nodes, which is equivalent to treating shifts in geographic distributions are the result of dispersal alone. Polymorphisms are restricted to terminal nodes and ancestral states are calculated by minimizing the number of character state changes on the tree. For these analyses geographic distribution was coded as a single multistate unordered character based on distributions as described in Appendix 1 and analyzed using Mesquite 1.12 (Maddison and Maddison 2006). Distributions were coded based on specimen locality, not total species range. Where collections were obtained from botanical gardens, their locality was based on the locality of the original seed source, as reported by the botanical garden. DIVA analysis, alternatively, assumes that geographic distributions can be the result of dispersal, extinction, and vicariance events. Polymorphic states are not restricted to terminals and ancestral states are calculated by minimizing the number of dispersal events necessary to explain the distribution pattern. Geographic distributions were coded as a single multistate character based on distributions as described in Appendix 1 and the analysis was performed using DIVA 1.1a (Ronquist 1996).

Dispersal-extinction-cladogenesis, a continuous-time model for geographic range evolution, was implemented in Lagrange (v. 2.0.1; Ree and Smith 2008) to infer ancestral areas. Dispersal-extinction-cladogenesis requires a chronogram (or branch lengths that approximate the relative ages of the lineages) to perform its analysis. We used the r8s program (v. 1.7.1; Sanderson 2004) to estimate a chronogram for Cleomaceae based on our ML tree using semiparametric rate smoothing (SPRS) by penalized likelihood and the truncated Newton algorithm (Sanderson 2002). The r8s program requires that all nodes be resolved prior to analysis; therefore, unresolved branches in the ML tree were resolved in PAUP\* to short branch lengths (0.000100). Smoothing parameters were derived using cross-validation (data not shown). The Cleomaceae stem age was constrained to a maximum age of 41 my, based on estimates of the stem age of Brassicaceae (Schranz and Mitchell-Olds 2006). Other estimates of the age of the Brassicaceae vary from 50–18 my (Al-Shehbaz et al. 2006; Wikström et al. 2001). Different maximum constraints on the Cleomaceae stem age did not have major effects on the resultant chronogram (data not shown). We were interested in the chronogram for use in the DEC analysis but not as a strict interpretation of the age of the Cleomaceae, so only the results of one of the r8s analyses are presented.

The ten areas described for FPO, DIVA, and DEC analyses of Cleomaceae were Brazil, North America, Caribbean / Central America, Northern Andes / Guiana Shield, Southern Andes / temperate South America, Australia, Western Europe, Central Asia, Southern Africa (sub-Saharan Africa and south), and Northern Africa / Arabian peninsula. DIVA optimizations were conducted with either an unrestricted maximum number of areas assigned to each node or with the maximum number of areas restricted to two. Dispersal-extinction-cladogenesis analyses were also restricted to a maximum number of two ancestral areas per node, because with ten areas, an unconstrained transition rate matrix includes all possible ranges. In an analysis with ten possible areas, this matrix has a size of 1,024 × 1,024, which is too large for Lagrange to handle at this time. It was reasoned that this approach approximates the biological reality of the situation, as most species of Cleomaceae are generally not found in more than two of the geographic regions described here. Additionally, forcing internal nodes to not include all geographic regions may tell us more about historical biogeography patterns than would suggesting that ancestors were extremely widespread among New and Old World land masses.

Given the lack of strong support for early branching patterns in the family, two alternative topologies were used for DEC reconstructions. These two were chosen to reflect the major alternative topological possibilities from the posterior probability distribution of trees from the Bayesian analyses: (1) the North American clade sister to the rest of the Cleomaceae, and (2) the Australian clade sister to the rest of the Cleomaceae. In each case, one tree from the posterior distribution was chosen with the noted constraint for DEC analysis (hereafter referred to as the “NA-sister tree” and “Australia-sister tree”).

## RESULTS

**Phylogenetic Analyses**—New ITS sequences were obtained for 76 species/accessions belonging to Cleomaceae. In combination with GenBank sequences, 114 ingroup accessions were aligned with six species of Brassicaceae as outgroups. The aligned ITS data matrix of 81 species was 760 base pairs (bp)

long with 513 variable sites (67.5%), of which 441 (58%) were parsimony informative. Two sequences are missing a portion (57–139 bp) of the 5' end of the ITS 1 spacer, and 12 sequences are missing a portion (18–75 bp) of the 3' end of the ITS 2 spacer due to poor sequencing reads of these regions or missing data from GenBank.

Maximum parsimony analysis of the ITS Cleomaceae data set resulted in three most-parsimonious trees (length = 3,287 steps, CI = 0.330, RI = 0.771, RC = 0.254; Figs. 1A, B). The ITS ML analysis resulted in a single tree (-lnL = 16,339.36050; Figs. 2A, B). Multiple independent BI analyses converged on the same posterior probability distribution of trees, as inferred from the posterior probability distributions post burnin. AWTY comparisons suggest that convergence and mixing in the two analyses are reached and that the two analyses are sampling from the same posterior distribution (Suppl. Fig. 1). Results of Bayesian inference are presented as clade probabilities greater than 50% on the ML phylogeny (Figs. 2A, B). Each analysis resolved similar clades, with some differences in how these clades are grouped together (Figs. 1, 2). In both the MP and ML analyses, *Cleome* is paraphyletic with regard to the genera *Cleomella*, *Dactylaena*, *Gynandropsis* (*Cleome gynandra*), *Isomeris* (*Cleome isomeris*), *Oxystylis*, *Podandrogynne*, *Polanisia*, and *Wizlizenia*. *Cleomella* is paraphyletic: *Cleomella brevipes* and *C. plocosperma* are sister to the *Oxystylis*/*Wizlizenia* clade, while *Cleomella hillmanii* is sister to the “*Peritoma*” *Cleome* clade (Figs. 1A, 2A). Combined analyses of cpDNA and ITS sequences both resulted in similar topologies as found with ITS alone or cpDNA alone, with no significant increases in clade resolution or support (Suppl. Figs. 2, 3).

**Tests of Alternative Topologies**—The SH test and Bayesian confidence interval were used to assess the support for three alternative topologies: the “C<sub>4</sub> test”, which enforced monophyly of *C. angustifolia*, *C. gynandra*, *C. luderitziana*, *C. oxalidea*, and *C. paradoxa*; the “C<sub>4</sub>-2 test”, which enforced monophyly of the “*Gynandropsis*” and “*C. angustifolia*” clades (excluding *C. oxalidea*); and the “C<sub>4</sub>-reversion test”, where monophyly of the “*Gynandropsis*,” “*C. angustifolia*,” “*Coryandra*,” Clade 4, and Clade 5 clades was constrained. The SH test was unable to statistically reject any of these alternative topologies, but the Bayesian confidence interval rejected both the C<sub>4</sub> test and the C<sub>4</sub>-reversion test (Table 1).

**Reconstruction of Biogeographic Patterns**—Fitch parsimony optimization (FPO) ancestral area reconstruction resulted in most nodes resolved to a single or two most parsimonious reconstructions (Fig. 3). Exceptions were primarily restricted to the ancestor of the Australian clade (lineage 7) + the rest of *Cleome*, the “*Tarenaya*” clade, and the broadly distributed *C. viscosa* (Fig. 3). DIVA with unrestricted ancestral areas resulted in most interior nodes with a large number of equally parsimonious reconstructions, with many broadly distributed ancestors (3–9 areas; data not shown). When restricted to a maximum of two ancestral areas, most interior nodes were resolved to a single or two equally parsimonious reconstructions (Fig. 3), with the exception of several nodes in the “*Tarenaya*” clade.

Penalized likelihood with the smoothing parameter value set at 3.2 based on cross-validation resulted in a chronogram with the Cleomaceae crown diversifying less than 19 mya (Fig. 4). This chronogram was then used in the DEC analysis of ancestral area reconstruction which is largely congruent with the results from FPO and DIVA (Fig. 3). Particularly, DEC reconstructed the ancestor of the Cleomaceae in central

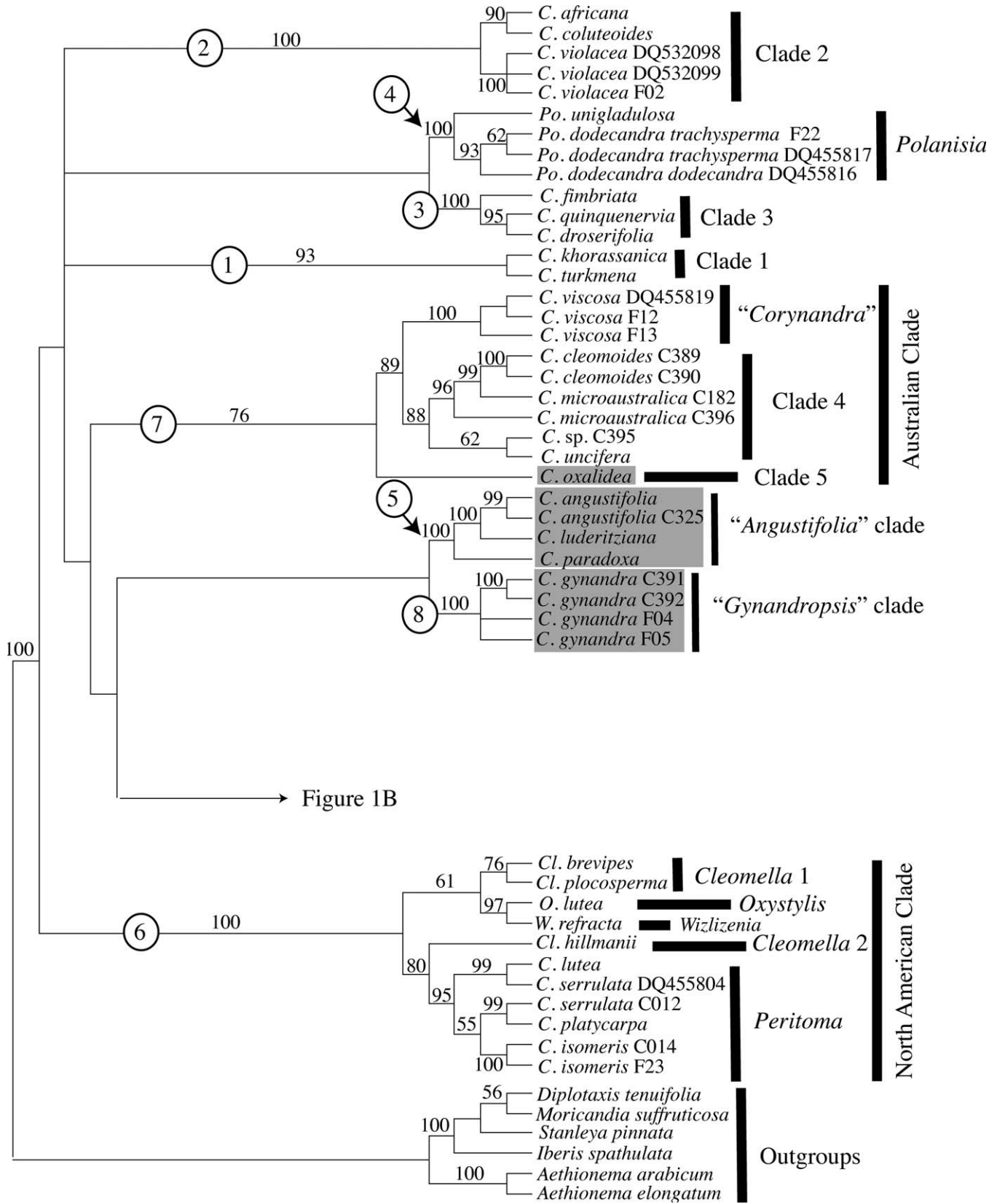


FIG. 1A. Maximum parsimony strict consensus tree of three shortest trees (length = 3,287; CI = 0.330; RI = 0.771; RC = 0.254). Outgroups and early-diverging clades. Numbers above branches reflect maximum parsimony bootstrap values. Species with putative CCMs are in gray boxes. Circled numbers associated with branches refer to the lineages discussed in the text. Generic abbreviations are as follows: C. = *Cleome*, Cl. = *Cleomella*, O. = *Oxystylis*, Po. = *Polanisia*, and W. = *Wizlizenia*.

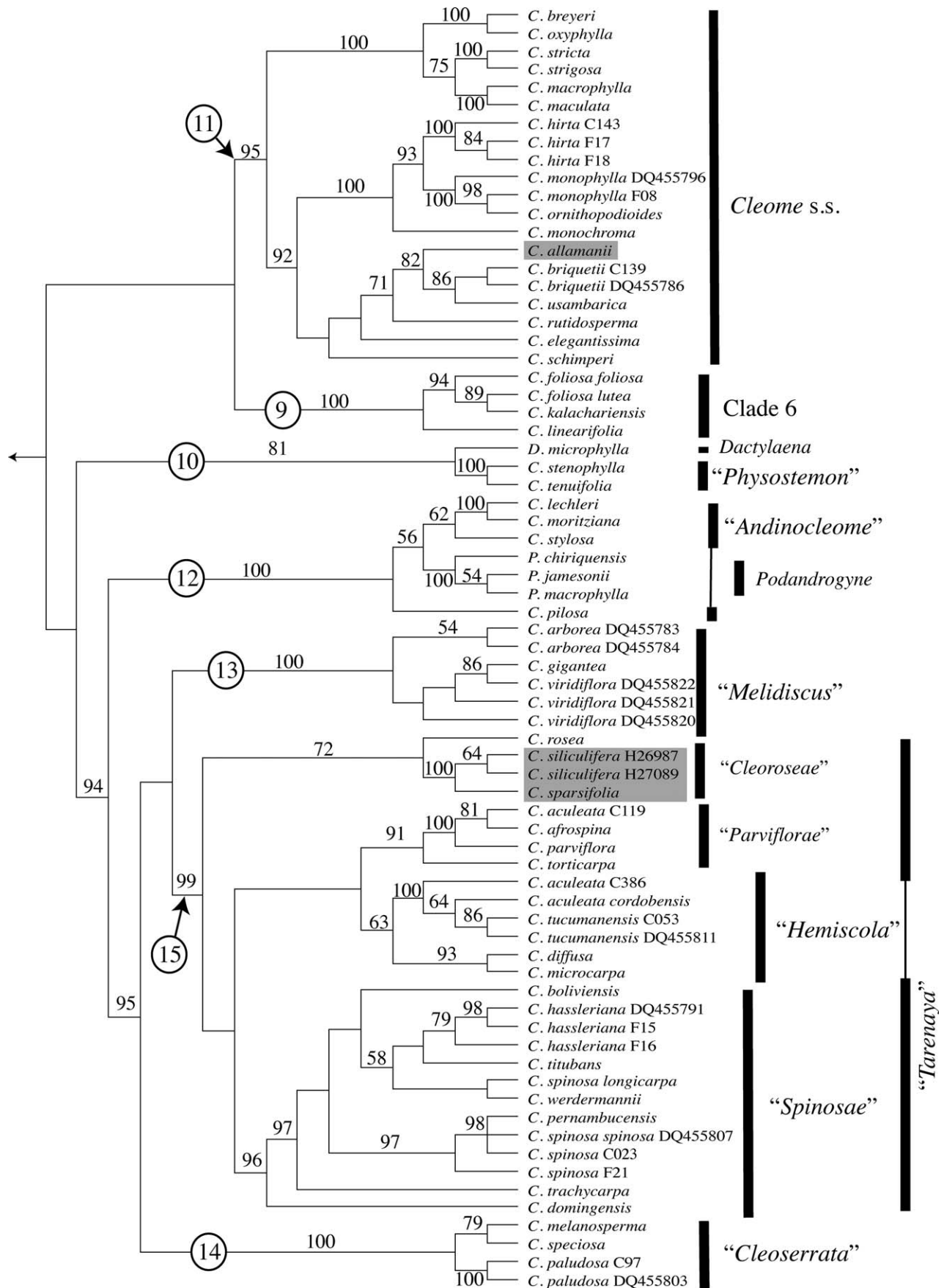
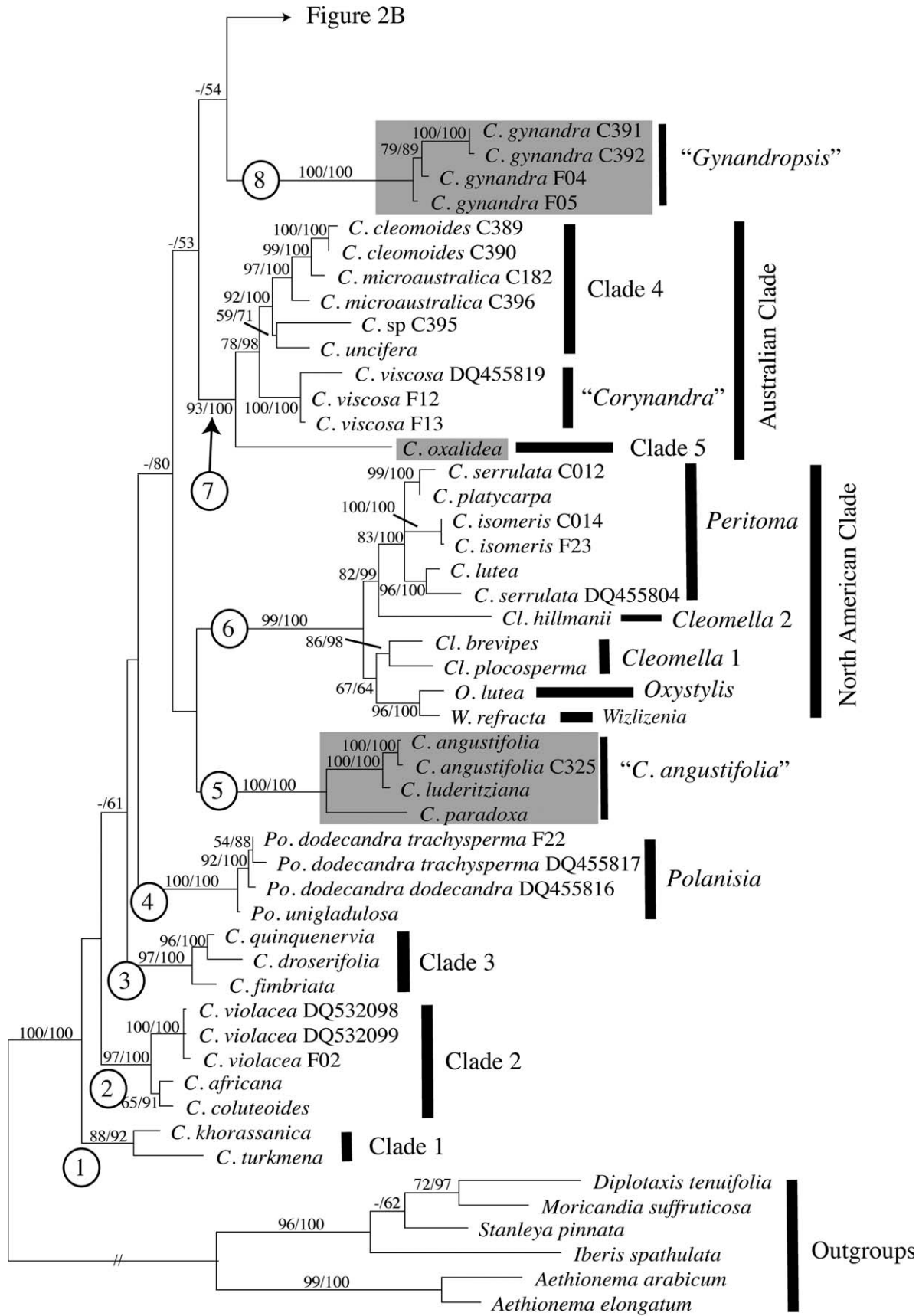


FIG. 1B. Maximum parsimony strict consensus tree of three shortest trees (length = 3,287; CI = 0.330; RI = 0.771; RC = 0.254). Later-diverging clades. Numbers above branches reflect maximum parsimony bootstrap values. Species with putative CCMs are in gray boxes. Circled numbers associated with branches refer to the lineages discussed in the text. Generic abbreviations are as follows: C. = *Cleome*, D. = *Dactylaena*, and P. = *Podandrogynae*.



– 0.01 substitutions/site

FIG. 2A. Maximum likelihood tree (-lnL = 16,339.36050). Outgroups and early-diverging clades. Numbers above branches reflect maximum likelihood bootstrap values and Bayesian posterior probabilities, respectively. Species with putative CCMs are in gray boxes. Circled numbers associated with branches refer to the lineages discussed in the text. Generic abbreviations follow those in Fig. 1A.

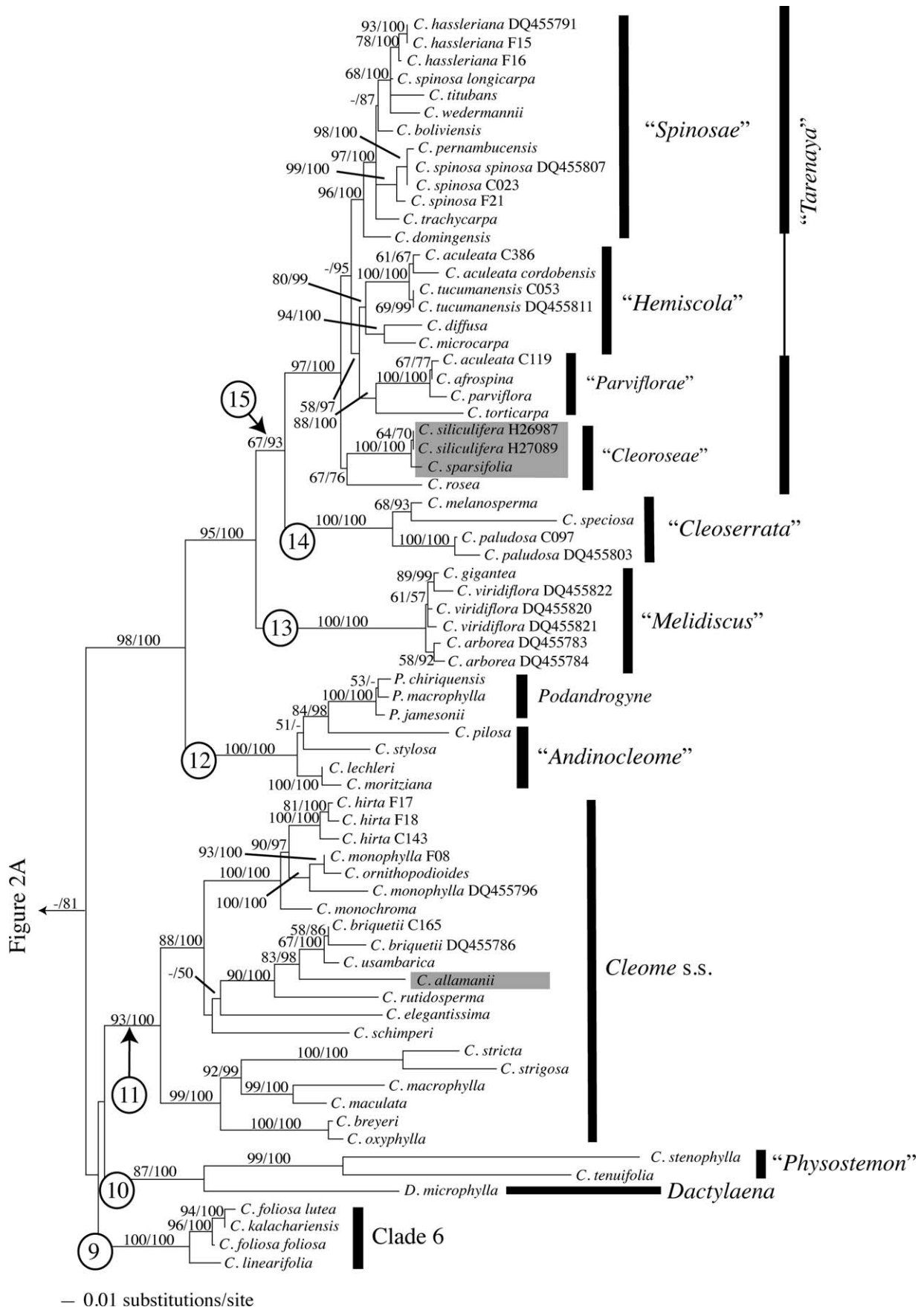


FIG. 2B. Maximum likelihood tree (-lnL = 16,339.36050). Later-diverging clades. Numbers above branches reflect maximum likelihood bootstrap values and Bayesian posterior probabilities, respectively. Species with putative CCMs are in gray boxes. Circled numbers associated with branches refer to the lineages discussed in the text. Generic abbreviations follow those in Fig. 1B.

TABLE 1. Alternative topology tests for monophyly of  $C_4$  lineages. Significant results are noted by asterices.

Test	Best ML tree (-ln L)	Constraint tree (-ln L)	Difference (-ln L)	<i>p</i> value	Proportion of trees in the posterior distribution	Confidence interval
$C_4$	16,339.36050	16,356.76723	17.40674	0.0988	0/800	0.000*
$C_4$ -2	-	16,340.82237	1.46188	0.4424	247/800	0.30875
$C_4$ -reversion	-	16,344.25089	4.89039	0.3234	4/800	0.005*

Asia (node 1, Fig. 4), with subsequent radiations to North America (node 2) and western Europe (node 3), and from North America to South Africa (node 4), and from South Africa to Australia (node 5) and the northern Andes / Guiana Shield (node 6). The reconstruction of these ancestral areas were not dependent on the node ages determined in the r8s analyses, as the relative branch lengths after rate smoothing were the same regardless of the absolute scale placed on the branches. Dispersal-extinction-cladogenesis estimated the dispersal and extinction rates for the Cleomaceae at 0.02153 and 0.0227, respectively. The biogeographic results of the NA-sister tree (Suppl. Fig. 4) and Australia-sister tree (Suppl. Figure 5) are discussed below, but the inferred speciation and extinction rates for these alternative analyses were lower than the ML tree (NA-sister tree: dispersal = 0.004996, extinction = 0.009245; Australia-sister tree: dispersal = 0.004716, extinction = 0.008738).

#### DISCUSSION

Overall, the MP and ML phylogenetic hypotheses correspond well with each other, with previous phylogenetic estimates of relationships in the Cleomaceae (Figs. 1, 2; Hall et al. 2002; Sanchez-Acebo 2005; Hall 2008; Inda et al. 2008), and with interpretations of generic boundaries based on morphology (Iltis and Cochrane 2007; Iltis and Cochrane, unpublished). Phylogenies based on cpDNA (Hall et al. 2002; Hall 2008) have placed the root of Cleomaceae between western North American cleomoids (including *Cleome* in part, *Cleomella*, *Oxystylis*, and *Wizlizenia*) and *Cleome droserifolia* (Hall 2008) and the rest of the family. However, this root placement has had variable support, depending on the type of analysis. Maximum likelihood analysis supports this root placement, but these branches were unresolved in the strict consensus MP phylogeny. Here, MP analyses suggest the root of the Cleomaceae is on the branch between North American cleomoids and the rest of the family (Fig. 1A), however, this branch does not have bootstrap support. Maximum likelihood analyses suggest clades 1–3 and the *Polanisia* clade are the earliest diverging lineages (Fig. 2A; lineages 1–4); but, as with previous studies, with little support. Given the lack of a clear placement of the root in this or previous studies, additional data are necessary to conclusively place the root of the Cleomaceae. It should be noted that in cpDNA phylogenies as well as the ITS phylogeny, MP and ML analyses provide different rootings. This may suggest that one or both of these methods are having difficulties placing the root, possibly due to the significant phylogenetic distance to the outgroups in the Brassicaceae. However, if these inconsistencies are due to a long root issue, the expectation would be that ML analyses would be less influenced by this problem than MP (Felsenstein 1978; Huelsenbeck and Hillis 1993), and would therefore more likely represent the “true” root. For these reasons, the ML phylogenetic hypothesis is used here for interpretation of  $C_4$  origins and ancestral range reconstructions.

Fifteen lineages, as circumscribed here, are strongly supported and correspond in many cases to previously recognized genera or supraspecific classification units within *Cleome* s. l. (referred to as clades, but which may or may not correspond to one of the 15 lineages). In all analyses, lineages 1–8 (clades 1–5, the *Polanisia* clade, the North American clade, the “*Gynandropsis*” clade, and the “*C. angustifolia*” clade) are the earliest diverging branches on the tree. In all analyses, the branches grouping these lineages are not well supported; however, lineages 1–4 and 6 are always the earliest diverging lineages on the trees. The resolution of these branches is critical for clarifying the biogeographic history of the Cleomaceae, as lineages 1–3 have Old World distributions while the *Polanisia* and North American clades (lineages 4 and 6, respectively) have New World distributions. This issue is discussed more in the section on biogeography, below.

Combined analyses of previously published cpDNA (Hall et al. 2002; Hall 2008) and the ITS datasets using a supermatrix approach with both broad taxon sampling and large amounts of missing data, and a truncated taxon sample with nearly complete overlap between the cpDNA and ITS datasets resulted in nearly identical topologies as presented here with little significant increase to the bootstrap support for the early branching events in Cleomaceae (trees not shown). In the second approach, with broad sampling and lots of missing data, MP analysis increased bootstrap support for North America sister to the rest of the family to 77%, but most of the rest of the early nodes are not resolved. As the backbone of the Cleomaceae tree was not resolved in the previous cpDNA analyses (Hall et al. 2002; Hall 2008), this general lack of support is not too surprising. It does not appear that the gene regions sequenced to date exhibit adequate variation at the level necessary to resolve these nodes, either alone or in combination.

**Classification of Cleomaceae**—As has been found previously, commonly recognized genera including *Buhsia* (*Cleome coluteoides*), *Cleomella*, *Dactylaena*, *Gynandropsis* (*Cleome gynandra*), *Isomeris* (*Cleome isomeris*), *Oxystylis*, *Podandrogyne*, *Polanisia*, and *Wizlizenia* are derived from within a paraphyletic *Cleome* (Figs. 1, 2; Hall 2008; Inda et al. 2008). Other genera recognized in the family, but not yet sampled in any phylogenetic analysis, include *Haptolepis bahiensis* Ule and *Puccinonia microdenia* Chiov. These monotypic genera are found in Brazil and Somalia, respectively, and need to be added to phylogenetic analyses to test how these generic concepts fit into the overall classification and whether they might apply to any currently unnamed clades. When the nomenclatural changes suggested by Iltis and Cochrane (2007; unpublished) are considered, many, but not all, of the problems with nonmonophyletic genera are resolved. Particularly, the use of *Tarenaya* for much of New World *Cleome* results in this group being paraphyletic (Figs. 1, 2). The problem with *Tarenaya* might be addressed by including *Hemiscola* within *Tarenaya*, or by breaking *Tarenaya* into more genera (possibly according to the sections of *Tarenaya* including *Parviflorae*, *Spinosa*, and

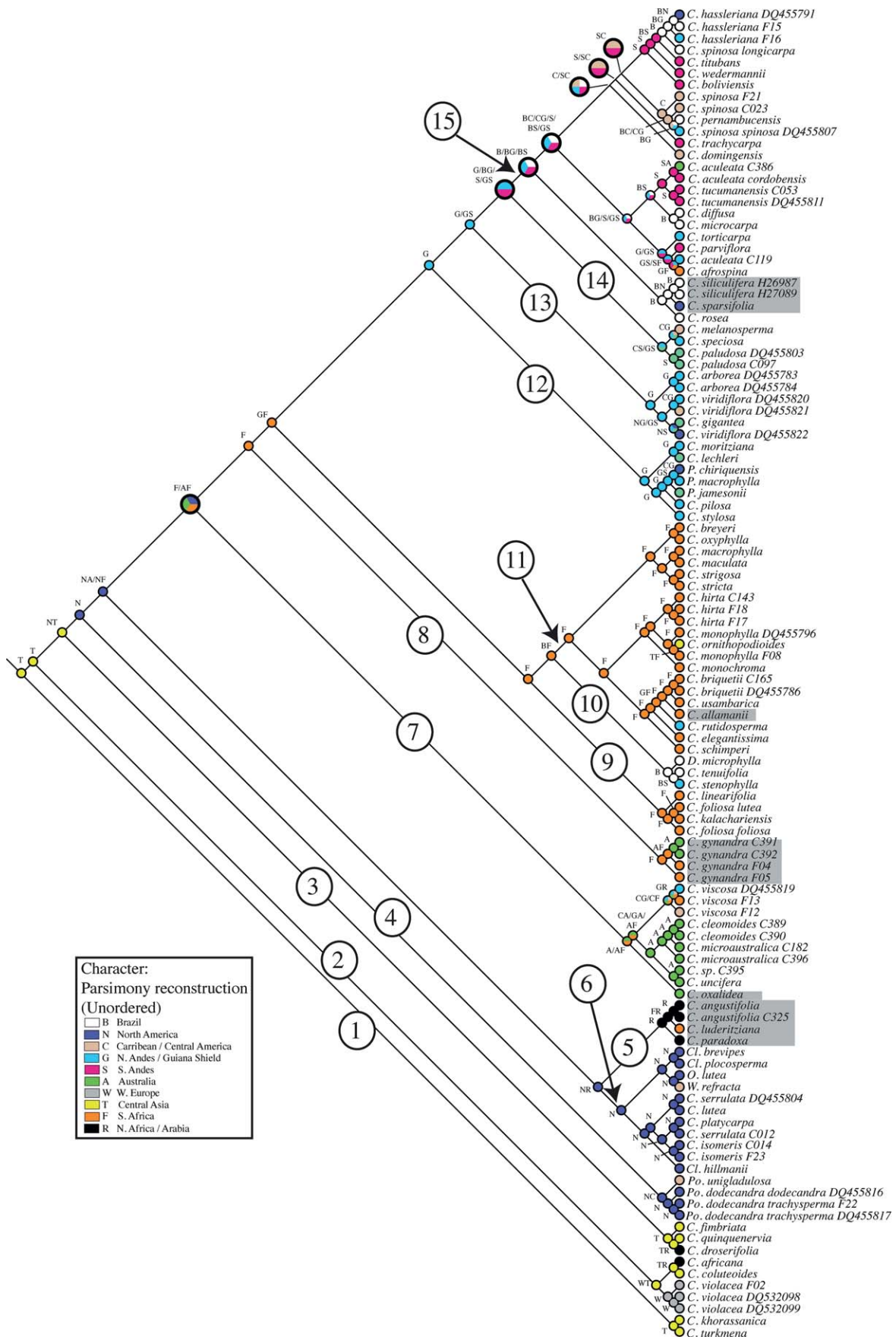


FIG. 3. Reconstruction of ancestral areas using FPO and DIVA restricted to a maximum of two ancestral areas. Colored nodes reflect FPO ancestral area reconstructions whereas letter designations reflect DIVA reconstructions. Letter codes separated by a “/” reflect equally parsimonious reconstructions. Species with putative CCMS are in gray boxes. Circled numbers associated with braches refer to the lineages discussed in the text. Generic abbreviations follow those in Figs. 1A, B.

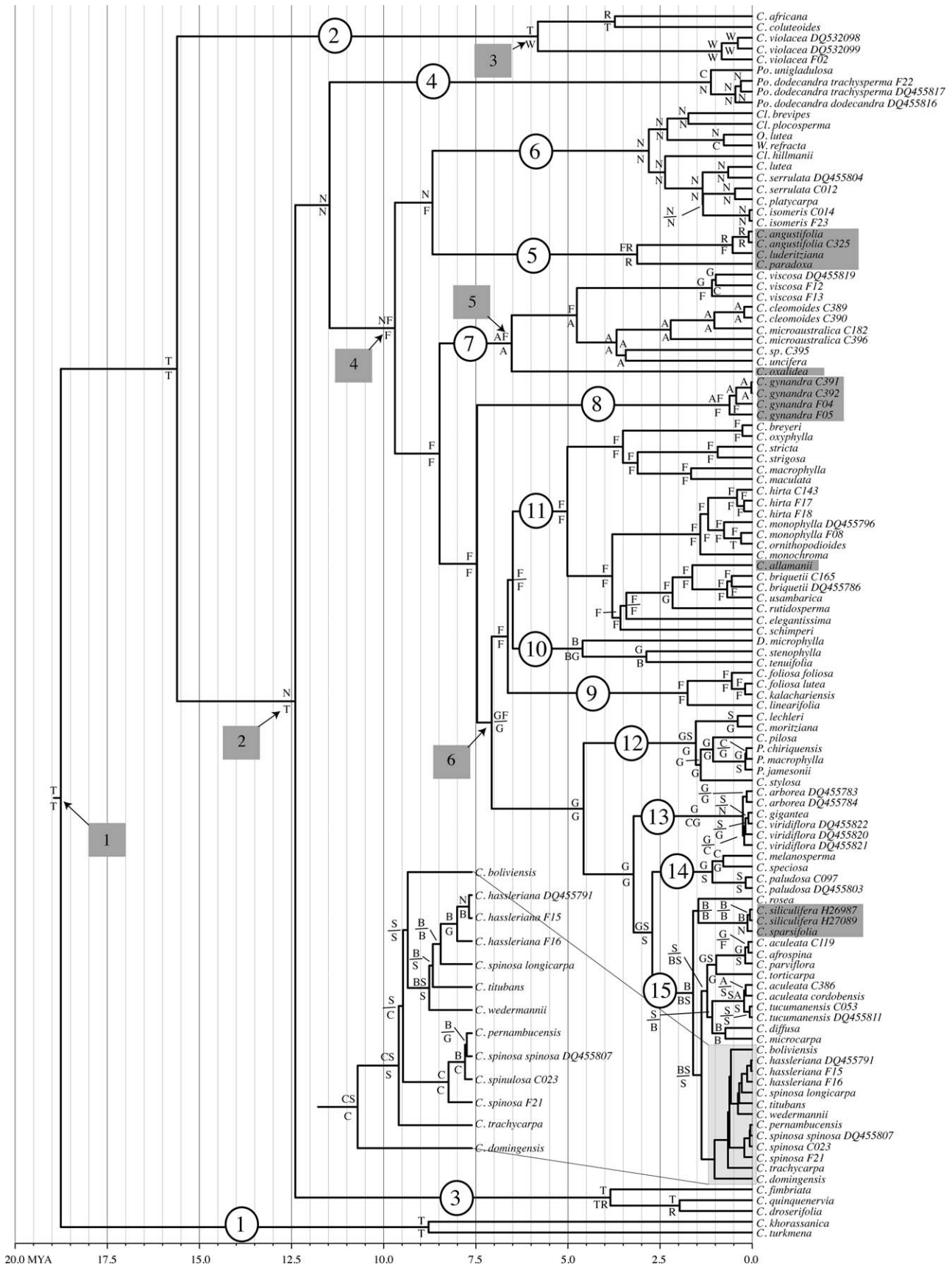


FIG. 4. Cleomaceae chronogram and ancestral area reconstruction using DEC. Ancestral area designations follow the notation used in Fig. 3 for DIVA reconstructions. Areas listed above and below the ancestral branch for a node refer to the areas reconstructed for the daughter branches to the top and bottom, respectively. Numbered nodes (1–6) in gray boxes are referenced in the text. Species with putative CCMs are in gray boxes. Circled numbers (white; 1–15) associated with branches refer to the lineages discussed in the text. Generic abbreviations follow those in Figs. 1A, B.

*Cleorosae* which have been tentatively recognized by H. Ilitis, unpublished). Morphological characters associated with these clades need to be explored in more detail in order to determine the best approach.

More difficult is the generic determination of many of the Old World clades (clades 1–6, Figs. 1, 2). While the *Cleome* type species *C. ornithopodioides* clearly defines what clade should retain the name *Cleome* if it is used in the strict sense, it is not clear at this time whether any generic names apply to the orphan Old World clades associated with the New World genera *Polanisia*, *Cleomella*, *Oxystylis*, and *Wizlizenia* (Figs. 1, 2). Clade 2 (Figs. 1A, 2A) includes *C. coluteoides*, the type for the genus *Buhsia*, and the application of this generic concept needs to be explored.

In addition to the potential revision of *Cleome* circumscription, the organization of other genera in the Cleomaceae also needs to be addressed. Particularly, *Cleomella* is paraphyletic with respect to *Oxystylis*, *Wizlizenia*, and the *Peritoma* / *Isomeris* members of *Cleome* (Figs. 1A, 2A). This result has been found previously (Hall 2008) using a different sampling of *Cleomella* species (*Cleomella longipes* Torr. and *C. obtusifolia* Torr. & Frem.) suggesting that a detailed sampling of the ten *Cleomella* species, six species (eight taxa) of *Peritoma*-type *Cleome*, and the monotypic *Oxystylis* and *Wizlizenia* is needed in order to define generic limits and stabilize the nomenclature of these North American lineages.

**Origin and Distribution of C<sub>4</sub> Photosynthesis**—The phylogenetic results presented here suggest that there are likely at least five separate origins of carbon concentrating mechanisms (CCMs) in the Cleomaceae (Fig. 2A, B). One clear origin of C<sub>4</sub> is *Cleome oxalidea* (Clade 5), which is closely related to other Australian species, and clearly separate from the “*Gynandropsis*” / “*C. angustifolia*” clades (Figs. 1A, 2A). Two other origins of C<sub>4</sub> are suggested, one is the C<sub>4</sub> species *C. gynandra* (the “*Gynandropsis*” clade), and the “*C. angustifolia*” clade. The latter clade in both MP and ML analyses includes the C<sub>4</sub> species *C. angustifolia*, the C<sub>3</sub>-C<sub>4</sub> intermediate *C. paradoxa*, and *C. luderitziana* (Figs. 1, 2). *Cleome luderitziana* has not been studied to characterize its form of photosynthesis, its type of leaf morphology nor its leaf anatomy. It may be C<sub>3</sub> or C<sub>3</sub>-C<sub>4</sub> intermediate according to its δ<sup>13</sup>C value (–23.9‰ [parts per thousand]). *Cleome paradoxa*, like a number of intermediates which have been studied in other families (Edwards and Ku 1987), has a C<sub>3</sub> type δ<sup>13</sup>C value (–23.8‰), similar to that of *C. luderitziana* (Voznesenskaya et al. 2007).

In the MP analysis the *Gynandropsis* and *C. angustifolia* clades are sister to each other, which could be suggestive of one C<sub>4</sub> origin, but the branch connecting them has low support. In the ML analysis they are not sister groups, suggestive of two origins. The leaf form and type of Kranz anatomy is different between *C. gynandra* (planar leaves with Atriplicoid type anatomy) and *C. angustifolia* (semiterete leaves with a single Kranz unit around the periphery of the leaf; Koteyeva et al. unpublished data), which is also supportive of separate origins. In the *C. angustifolia* clade, the intermediate *C. paradoxa*, like *C. angustifolia*, has leaves with thin lobes. Either they may have evolved from a common C<sub>3</sub> ancestor, or there may have been a reversion from C<sub>4</sub> to a C<sub>3</sub>-C<sub>3</sub> intermediate state in *C. paradoxa*. *Cleome luderitziana* could also be a reversion from C<sub>4</sub> to C<sub>3</sub> or C<sub>3</sub>-C<sub>4</sub> intermediate, but further studies will be necessary to determine its pathway type.

Besides evidence for up to three origins of C<sub>4</sub> in the family, there are other apparent origins of intermediate or C<sub>4</sub>-like

photosynthesis based on photosynthetic typing from carbon isotope composition. Previous studies show the values for C<sub>4</sub> plant are typically between –10 to –15‰ and for C<sub>3</sub> plants between –24 to –30‰. Values in C<sub>3</sub> plants can become a few ‰ more positive under arid conditions when photosynthesis becomes more limited by diffusive resistance to CO<sub>2</sub> entrance into the leaf (Cerling 1999). From a comprehensive carbon isotope survey of many species of *Cleome* and other representatives of family Cleomaceae, the δ<sup>13</sup>C values ranged from –12 to –33‰ (Voznesenskaya et al. 2007). Three species in the present phylogenetic study have δ<sup>13</sup>C values which are C<sub>4</sub>-like (e.g. –16 to –17‰) or intermediate (e.g. ~–19‰): *C. allamanii* (–17.1‰ and –19.1‰ from two separate collections), *C. siliculifera* (–16.8‰), and *C. sparsifolia* (–19.7‰ and –22.6‰ from two separate collections). Thus, a fourth CCM origin is suggested in the *Cleome* s. s. clade where the African species *C. allamanii* is located (Figs. 1B, 2B). A fifth apparent origin of a CCM is in the “*Cleoroseae*” clade which includes *C. siliculifera* and *C. sparsifolia* (and the C<sub>3</sub> species *C. rosea*, δ<sup>13</sup>C of –30.5‰).

From limited microscopy studies there is some evidence for a few species of *Cleome* having developed anatomical traits which are considered preconditions for development of Kranz type anatomy and C<sub>4</sub> biochemistry (Voznesenskaya et al. 2007; Marshall et al. 2007). Evidence for this in the course of evolution of C<sub>4</sub> from C<sub>3</sub> species is best demonstrated in the anatomical and phylogenetic study of McKown and Dengler (2007) on the genus *Flaveria*, which has C<sub>3</sub>, C<sub>4</sub>-like, and C<sub>4</sub> species, and a range of intermediates. They outlined a stepwise acquisition of traits in anatomy and patterns of veins prior to development of C<sub>4</sub> biochemistry. This included increased vein density, decreased mesophyll to bundle sheath chlorenchyma, and reductions in layers of ground tissue. Functional C<sub>3</sub>-C<sub>4</sub> intermediates have acquired, as a minimum, well-developed chloroplasts in bundle sheath (BS) cells, specific localization of glycine decarboxylase to mitochondria in BS cells, and reduced CO<sub>2</sub> compensation points which is indicative of reduced photorespiration. The only *Cleome* species currently identified to function as a C<sub>3</sub>-C<sub>4</sub> intermediate species is *C. paradoxa* (Voznesenskaya et al. 2007).

There is evidence that *Cleome africana* and *C. violacea*, which are located in Clade 1 (Fig. 2A), have a few structural traits which can be considered preconditions for development of C<sub>3</sub>-C<sub>4</sub> intermediates and C<sub>4</sub> plants. *Cleome africana* has large BS cells, high leaf venation, and few mesophyll cells between veins, but it has other traits indicative of C<sub>3</sub> photosynthesis, including lack of Kranz anatomy, seven to eight layers of ground tissue, few chloroplasts in BS cells, C<sub>3</sub> type CO<sub>2</sub> compensation point, and a C<sub>3</sub> type δ<sup>13</sup>C value (Voznesenskaya et al. 2007; Marshall et al. 2007). *Cleome violacea* also has increased vein density similar to the C<sub>4</sub> *C. gynandra*, but other features indicative of C<sub>3</sub> photosynthesis: lack of Kranz anatomy, and a C<sub>3</sub> type CO<sub>2</sub> compensation point (Marshall et al. 2007), and C<sub>3</sub> type carbon isotope composition (Voznesenskaya et al. 2007). Likewise, *C. foliosa* which is in Clade 6, had increased leaf venation and a somewhat lower CO<sub>2</sub> compensation point (Marshall et al. 2007), and *C. foliosa* var. *lutea* has a δ<sup>13</sup>C value (–22.8‰) which is on the positive side for C<sub>3</sub> plants (Voznesenskaya et al. 2007). However, *C. foliosa* lacks Kranz anatomy and has few chloroplasts in BS cells.

With the exception of *C. sparsifolia* and *C. siliculifera*, all species with CCMs are of Old World origin, although the C<sub>4</sub> species *C. gynandra* is now a world-wide weed. If the increased

vein density found by Marshall et al. (2007) in *Cleome africana*, *C. foliosa*, and *C. violacea* is interpreted as a progression towards developing  $C_4$ , this could represent potential additional origins of a CCM, as none of these three species are closely related to other origins discussed above. However, *C. africana* and *C. violacea* are closely related, and they might represent a single transition to increased vein density (Figs. 1A, 2A); *C. coluteoides*, the species sister to *C. africana*, needs to be assessed to determine if it also has increased vein density or other  $C_4$  traits.

Multiple origins of CCMs within lineages has been documented elsewhere including the Poaceae (Giussani et al. 2001; Christin et al. 2008; Vicentini et al. 2008), *Eleocharis* (Cyperaceae; Roalson and Friar 2000; Roalson and Hinchliff 2007; Roalson et al. 2010), Suaedoideae (Chenopodiaceae; Schütze et al. 2003; Kapralov et al. 2006), Salsoloideae (Chenopodiaceae; Akhani et al. 2007), and Amaranthaceae s. s. (Sage et al. 2007), among others (see Sage 2004). The origins of CCMs in *Cleome* need to be further evaluated by analyses of anatomy and biochemistry of additional species in  $C_4$  clades and in clades suggested to have  $C_4$  traits, to determine whether there are more  $C_3$ - $C_4$  intermediates, and to clarify relationships deep in the phylogeny where branch support is weak.

**Biogeography**—Three reconstruction methods (FPO, DIVA, and DEC) were analyzed here to assess the historical biogeographic patterns in the Cleomaceae. Each of these methods depends on a different set of assumptions, and it has been shown that the appropriateness of different methods under different scenarios varies greatly (Clark et al. 2008). Particularly, the different methods here applied differ in their allowance of multiple areas (polymorphisms) at ancestral nodes, their allowance (or favoring) of different modes of ancestral area changes, dispersal, vicariance, or both, and whether time scale is explicitly incorporated in the assessment of ancestral area reconstructions.

The historical biogeography of the Cleomaceae reflects a combination of older (early diverging nodes in the tree) and younger (tip clades in the tree) dispersal or vicariance events (Figs. 3, 4). While many of the younger biogeographic events seem most likely to be dispersal events, such as the sister relationship of *C. afrospina*, from Gabon (Africa), and *C. aculeata*, from French Guiana (NE South America), the deeper reconstructions are less clear. Reconstruction of whether vicariance or dispersal is more likely is dependent on two lines of evidence: the age of the clades in the Cleomaceae and the position of the continents at those ages. The time period when Cleomaceae diversified to both New and Old World land masses affects whether we expect vicariance or dispersal scenarios to be more likely. There are limited fossils attributed to the Capparaceae / Cleomaceae / Brassicaceae clade (Muller 1981; Raven and Axelrod 1974), and the age estimates of the stem Brassicaceae range from 22–18 my (Wikström et al. 2001) to 41 my (Schranz and Mitchell-Olds 2006), and 50 my (Al-Shehbaz et al. 2006). As the Cleomaceae is the sister lineage to the Brassicaceae, these stem ages can be attributed to it as well, however, the range in estimates (50–18 my) is extensive. One fossil likely associated with the Cleomaceae stem lineage is *Palaeocleome lakensis* M.Chandler from the Pipe-Clay Series of the Lower Bagshot Beds of Dorset (Chandler 1962). This fossil seed shares some characters with *Cleome*, but Chandler clearly didn't consider it similar enough to be included within *Cleome*. The age of these fossils is unclear as the paucity of animal fossils in these beds make it difficult

to compare them to fossil beds from continental Europe, and there is some disagreement whether the clay pipes are younger or of the same age as the surrounding sandy matrix (Chandler 1962). When continental positions are taken into consideration, though, the plate positions across this time series all suggest that movements among central Asia, North America, southern Africa, Australia, and the northern Andes / Guiana Shield would have required dispersal events (where most of the earliest Cleomaceae nodes are reconstructed; nodes 2, 4–6, Fig. 4). These reconstructions represent an origin of Cleomaceae in Laurasia, but since Laurasia separated into North America, Greenland, and Eurasia at approximately 60 my, a significantly older age of the Cleomaceae would be required to infer vicariance-driven diversification of these early-diverging Cleomaceae lineages.

While these early nodes are reconstructed, regardless of method, as central Asian and North American, subsequent nodes are reconstructed entirely or in part as Australian, southern African, and northern Andes/Guiana Shield (Figs. 3, 4). These areas reflect all major continental areas of the northern and southern hemispheres, and given the time periods involved (likely less than 20 mya for northern hemisphere nodes and less than 10 mya for southern hemisphere nodes), long distance dispersal among continents seems most plausible.

Extant ranges of Cleomaceae species also suggest that long distance dispersal is possible, given the large distributions of species including *C. gynandra* (Africa, Asia, Australia, Europe, North America, South America, and elsewhere), *C. viscosa* (Africa, North America, South America, and Australia), and *C. viridiflora* (North and South America). Whether these dispersal events are directly leading to speciation, or vicariance processes are driving diversification of broadly dispersed species at continental and regional levels in more recent diversifications, will require additional studies. Without a better calibration of the phylogeny to absolute time, however, it is difficult to clarify the likelihood of these different biogeographic processes on regional scales.

While FPO, DIVA restricted to two ancestral areas, and DEC restricted to two ancestral areas are largely congruent (Figs. 3, 4), their assumptions and the interpretation of reconstructed nodes and modes of ancestral range inheritance differ significantly (Clark et al. 2008). Fitch parsimony optimization results in several most parsimonious reconstructions at several nodes, particularly in the "*Tarenaya*" clade. Additionally, FPO is limited to a single area reconstruction at any given internal node. As widespread taxa are known for the Cleomaceae, this limitation is not biologically realistic. Fitch parsimony optimization also assumes a dispersal-mediated biogeographic history, which for the Cleomaceae may well represent the mode of ancestral area inheritance, but the limitation of this mode of inheritance makes it difficult to assess the process of biogeographic range inheritance where vicariance might be plausible, such as in "*Tarenaya*" where closely related species are distributed in the northern and southern Andes, Brazil, and Central America (Fig. 3). Interestingly, FPO is more conclusive in its reconstruction of interior nodes here than in other similarly complex systems where a dispersal-mediated allopatric speciation (as is inferred using FPO) is the expected mode of ancestral area inheritance (Clark et al. 2008).

Dispersal-extinction-cladogenesis, while providing similar ancestral area reconstructions for many nodes as FPO and

DIVA, appears to provide the most robust estimate of ancestral area distributions of the three methods (Fig. 4). Advantages of the DEC method include taking into account time scale, as inferred by the chronogram, in assessing the likelihood of ancestral area reconstructions, as well as allowing ancestors to have multiple area distributions. Further, DEC allows for the integration of probability distributions for different area reconstructions during different time periods, which should allow the integration of more a priori knowledge of geological and lineage histories in future analyses (Ree and Smith 2008). The greatest limitation to this is the lack of fossils within the Cleomaceae. The *Palaeocleome* fossil might be effective for placing an upper bound on the crown age of the Cleomaceae, but the lack of a clear age for that fossil, and no clear fossils for lineages within the family, significantly limit our ability to calibrate the tree. As an alternative, geological events, and the inclusion of deeper nodes (which might include more fossil calibration points) might better allow for a more robust assessment of node ages in the Cleomaceae (Roalson et al. 2008; Clark et al. 2008).

As with some of the other methods implemented, DEC reconstructs the origin of Cleomaceae in central Asia, with dispersal and lineage divergence from central Asia into North America, western Europe, and northern Africa / Arabian peninsula (Fig. 4). While some ancestral area inheritance scenarios seem most likely explained as dispersal-mediated allopatric diversification (node 2, Fig. 4; Clark et al. 2008), others seem likely to be vicariance-driven lineage diversification with daughter lineages inheriting subsets of the range of widespread ancestors (nodes 4 and 5, Fig. 4). The flexibility of DEC to allow multiple modes of ancestral area inheritance (dispersal and vicariance) is a particular strength of this method (Ree and Smith 2008).

Two alternative topologies were chosen from the Bayesian posterior distribution to assess the sensitivity of the DEC reconstructions to early branching events. These two trees represent the two most divergent patterns of early tree shape in the distribution from the ML topology (at least with regards to biogeography): where the North American clade is sister to the rest of the Cleomaceae and where the Australian clade is sister to the rest of the family (Suppl. Figures 4, 5). It should be noted that while the NA-sister shape is reasonably frequent in the posterior (77 of 800 trees; 9.625%), the Australia-sister shape is not (seven of 800; 0.875%), making it a fairly improbable topology. While the ML topology DEC analysis resulted in the origin of Cleomaceae in central Asia with movement from there to North American and South Africa, the NA-sister reconstruction suggests Cleomaceae originated in North America with movements from there to South Africa, central Asia, and northern South America. The Australia-sister tree was similar in that early nodes are reconstructed as present in South Africa. One pattern commonly found in different places of all three trees is the movement either from North America to South Africa or vice versa. This is a surprising pattern, as a direct connection between the floras of South Africa and North America is not known (Galley and Linder 2006), although connections have been suggested between the New World and Macaronesian islands (Panero et al. 1999; Trusty et al. 2004) and South America and Africa (Galley and Linder 2006, among others).

Addition of samples from undersampled (or unsampled) lineages might also help clarify these ancestral area reconstructions. Further refinement of the ancestral area transition

matrix, support for internal nodes of the Cleomaceae phylogeny, and integration of additional calibration points to the chronogram should allow for more detailed assessment of ancestral area reconstructions in the future. The phylogenetic hypotheses presented here continue to support a need for major restructuring of generic concepts in the Cleomaceae; they suggest a combination of both dispersal- and vicariance-mediated lineage diversification, and provide support for five or more origins of C<sub>4</sub> and C<sub>3</sub>-C<sub>4</sub> intermediate CCMs in the family. However, several branches of this phylogenetic hypothesis have low support and this hypothesis is based on a single gene region, necessitating more phylogenetic data to verify clade relationships and increase overall confidence in branching structure.

**ACKNOWLEDGMENTS.** This article is the result of a sabbatical leave of TAF to the lab of EHR supported by Civilian Research and Development Foundation grant RUB1-2829-ST-06. TAF acknowledges Carmen M. Vallejo-Roman, G. V. Degtiareva, M. D. Logacheva, and T. H. Samigullin of plant genosystematics laboratory (MSU). Thanks go to Rick Ree and John R. Clark for help in running Lagrange, Hugh Iltis for sharing unpublished manuscripts, and Cody E. Hinchliff and two anonymous reviewers for comments on a previous version of this manuscript.

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APPENDIX 1. Taxon list for all samples used in the current study. Species are organized alphabetically. Herbarium acronyms follow Holmgren and Holmgren (1998), and are provided when known (missing from GenBank accessions from some previous studies). Samples from GenBank are provided with the locality and collection material available. Missing carbon isotope ratios are noted by “—”. Format: species; country; voucher; carbon isotope ratio; ITS GenBank number.

*Aethionema arabicum* (L.) Rothm.; [no voucher listed];—; AY254539. *Aethionema elongatum* Boiss.; [no voucher listed];—; DQ518386. *Cleome aculeata* L. [C386]; Queensland, Australia; L. Hucks s. n. (CANB); -29.9; HM044289. *Cleome aculeata* [C119]; French Guiana; F. Billiet & B. Jadin 7445 (MO); -31.1; HM044288. *Cleome aculeata* var. *cordobensis* (Eichler ex Grisebach) Kuntze; Bolivia: Tarija; R. Ehrlich 8 (LPB);—; DQ455787. *Cleome africana* Botch.; Egypt (WSUG); E. Voznesenskaya 1 (WS); -30.1; HM044222. *Cleome afrospina* H. H. Iltis; Gabon; F. J. Breteler 696 (MO); -28.3; HM044290. *Cleome allamanii* Chiov.; Kenya; Agnew et al. 10879 (MO); -17.1; HM044270. *Cleome angustifolia* Forssk.; South Africa, National Park Kruger; O. Maurin s. n. (WS);—; HM044250. *Cleome angustifolia* [C325]; Ethiopia; I. Friis et al. 8609 (K); -13.2; HM044251. *Cleome arborea* Kunth [DQ455783]; Venezuela: Las Chorreras de las Gonzalez, Merida; T. Ruiz y L. Hernandez 4981 (MY);—; DQ455783. *Cleome arborea* [DQ455784]; Venezuela: Merida; C. E. Benitez de Rojas 6371 (MY);—; DQ455784. *Cleome boliviensis* H.H. Iltis; Bolivia: La Paz; L. Sanchez 1132 (MO);—; DQ455785. *Cleome breyeri* B. Davy; Norcot, South Africa; A. O. D. Mogg et al. 19159 (MO); -25.3; HM044258. *Cleome briquetii* Polhill [C165]; Tanzania; J. A. Mlangwa 285 (MO); -30.6; HM044271. *Cleome briquetii* [DQ455786]; Tanzania: Kilimanjaro; J. Malangwa 285 (MO);—; DQ455786. *Cleome cleomoides* (F. Muell.) H. H. Iltis [C389]; Western Australia, Australia; R. W. Purdie s. n. (CANB); -25.4; HM044243. *Cleome cleomoides* [C390]; Northern Territory, Australia; D. B. Foreman s. n. (CANB); -27.6; HM044244. *Cleome coluteoides* Boiss.; Turkmenistan; V. Botchanzev 136 (LE); -27.0; HM044224. *Cleome diffusa* Banks ex DC.; Brazil: Bahia; R. Harley et al. 17276 (MO);—; DQ455789. *Cleome domingensis* H. H. Iltis; Dominican Republic; A. Grable s. n. (WS); -26.8; HM044292. *Cleome droserifolia* (Forssk.) Delile; Egypt (WSUG); E. Voznesenskaya 41 (WS); -30.0; HM044229. *Cleome elegantissima* Briq.; Angola; L. E. Kers 3651 (MO); -26.7; HM044272. *Cleome fimbriata* Vairy; Uzbekistan; V. Botchanzev 159a (LE); -25.7; HM044227. *Cleome foliosa* Hook. f. var. *foliosa*; Namibia; Leistner et al. 88 (K); -24.1; HM044275. *Cleome foliosa* var. *lutea* (Sond.) Codd & Kers; South Africa; G. Germishuizen 5342 (MO); -22.8; HM044276. *Cleome gigantea* L.; Prague Bot. Garden (WSUG); M. Smith s. n. (WS); -23.4; HM044283. *Cleome gynandra* L. [F4]; Zambia (WSUG); E. Voznesenskaya 42 (WS); -14.5; HM044254. *Cleome gynandra* [F5]; Kenya (WSUG); E. Voznesenskaya 2 (WS); -14.6; HM044255. *Cleome gynandra* [C391]; Queensland, Australia; B. M. Waterhouse s. n. (CANB); -13.9; HM044252. *Cleome gynandra* [C392]; Queensland, Australia; I. D. Cowie s. n. (CANB); -13.5; HM044253. *Cleome hassleriana* Chodat [F15]; Harris Seeds #2285, Rochester, NY (WSUG); E. Voznesenskaya 6 (WS); -25.0; HM044293. *Cleome hassleriana* [F16]; Kiev Bot. Garden (WSUG); E. Voznesenskaya 5 (WS); -26.4; HM044294. *Cleome hassleriana* [DQ455791]; U. S. A.; Missouri, St. Louis; L. Sanchez s. n. (MO);—; DQ455791. *Cleome hirta* (Klotzsch) Oliv. [F17]; Zambia (WSUG); E. Voznesenskaya 9 (WS); -27.9; HM044265. *Cleome hirta* [F18]; Zambia (WSUG); E. Voznesenskaya 10 (WS); -28.1; HM044266. *Cleome hirta* [C143]; Tanzania; N. A. Mwangulango 791 (MO); -28.8; HM044264. *Cleome isomeris* Greene [F23]; E. Voznesenskaya 62 (WS);—; HM044239. *Cleome isomeris* [C014]; California, U. S. A.; R. R. Halse 6133 (WS); -25.2; HM044238. *Cleome kalachariensis* (Schinz) Gilg. & Ben.; Namibia; P. M. Burgoyne & N. Snow 4984 (MO); -27.4; HM044277. *Cleome khorassanica* Bunge & Bien. ex Boiss.; Afghanistan; D. Bukinich s. n. (LE); -27.2; HM044230. *Cleome lechleri* Eichler; Bolivia: La Paz; L. Sanchez 1129 (MO);—; DQ455792. *Cleome linearifolia* (Stephens) Dinter; Namibia; W. Giess et al. 5785 (MO); -26.4; HM044278. *Cleome luderitziana* Schinz; Namibia; M. Bourele et al. 2827 (MO); -23.9; HM044256. *Cleome lutea* Hook.; [no country listed]; O’Kane 3773 (ISTC);—; AF137588. *Cleome macrophylla* (Klotzsch) Briq. var. *macrophylla*; Zambia; H. H. Schmidt et al. 2346 (MO); -29.2; HM044262. *Cleome maculata* (Sond.) Szyszyl.; South Africa: Transvaal; Balkwill et al. 5421 (MO); -25.7; HM044263. *Cleome melanoperma* S. Wats.; Sonora, Mexico; A. L. Reina G. 98-853 (NY);—; HM044284. *Cleome microaustralis* H. H. Iltis [C182]; Northern Territory, Australia; D. J. Dixon & R. K. Harwood 1007 (MO); -29.3; HM044245. *Cleome microaustralis* [C396]; Northern Territory, Australia; A.V. Slee s. n. (CANB); -27.5; HM044246.

*Cleome microcarpa* Ule; Brazil; R. M. Harley 27228 (MO); -28.3; DQ455793. *Cleome monochroma* J. F. Macbr.; Tanzania; P. Kuchar 23051 (MO); -28.9; HM044267. *Cleome monophylla* L. [F8]; Prague Bot. Garden (WSUG); E. Voznesenskaya 11 (WS); -27.8; HM044268. *Cleome monophylla* [DQ455796]; Senegal: Bondia; A. Driort 42 (MO);—; DQ455796. *Cleome moritziana* Klotzsch ex Eichler; Venezuela: Merida; T. Ruiz y L. Hernandez 4984 (MY);—; DQ455794. *Cleome ornithopodioides* L.; Copenhagen Bot. Garden (WSUG); E. Voznesenskaya 12 (WS); -29.7; HM044269. *Cleome oxalidea* F. Muell.; Western Australia, Australia; P. A. Fryxell 3958 (NY);—; HM044247. *Cleome oxyphylla* Burch. var. *oxyphylla*; Namibia; P. M. Burgoyne & N. Snow 3607 (MO); -27.6; HM044261. *Cleome paludosa* Willd. ex Eichler [C97]; Argentina; R. H. Fortunato 2874 (MO); -28.6; HM044285. *Cleome paludosa* [DQ455803]; Bolivia: Santa Cruz; L. Sanchez 1136 (MO);—; DQ455803. *Cleome paradoxa* R. Br. ex DC.; Yemen (WSUG); E. Voznesenskaya 43 (WS); -26.0; HM044257. *Cleome parviflora* Kunth; Bolivia: Santa Cruz; R. Guillen 5317;—; DQ455797. *Cleome pernambucensis* H. H. Iltis, Costa & Silva, ined.; Brazil: Pernambuco; Costa e Silva 1529 (MO);—; DQ455798. *Cleome pilosa* Benth.; Venezuela: Aragua; T. Ruiz 4720 (MY);—; DQ455799. *Cleome platycarpa* Torr.; Nevada, U. S. A.; A. Tiehm 8030 (WS); -24.3; HM044240. *Cleome quinquenervia* DC.; Turkmenistan; E. Leontieva 127 (LE); -25.2; HM044228. *Cleome rosea* Vahl ex DC.; Brazil: Rio de Janeiro; Costa e Silva 1530 (MO);—; DQ455801. *Cleome rutidosperma* DC.; Venezuela: Maracay; T. Ruiz 4360 (MY);—; DQ455802. *Cleome schimperii* Pax; Tanzania; L. Festo & W. Bayona 1729 (MO); -26.0; HM044273. *Cleome serrulata* Pursh [C012]; Washington, U. S. A.; G. H. Watt s. n. (WS); -22.4; HM044237. *Cleome serrulata* [DQ455804]; U. S. A.; Colorado; D. Clark 1640 (COLO);—; DQ455804. *Cleome siliculifera* Eichler [H26987]; Bahia, Brazil; R. M. Harley 26987 (NY);—; HM044286. *Cleome siliculifera* [H27089]; Bahia, Brazil; R. M. Harley 27089 (NY);—; HM044287. *Cleome sparsifolia* S.Wats.; U. S. A.; Nevada;—; DQ455805. *Cleome speciosa* Raf.; Venezuela: Tachira; T. Ruiz y L. Hernandez 4978 (MY);—; DQ455806. *Cleome spinosa* Jacq. [F21]; Nicaragua (WSUG); E. Voznesenskaya 13 (WS); -26.7; HM044295. *Cleome spinosa* [C023]; Puerto Rico; A. Grable 11178 (WS); -30.8; HM044296. *Cleome spinosa* ssp. *spinosa* [DQ455807]; Venezuela: Aragua; T. Ruiz 4702 (MY);—; DQ455807. *Cleome spinosa* ssp. *longicarpa* H.H. Iltis [DQ455808]; Brazil: Pernambuco; Costa e Silva 1522 (MO);—; DQ455808. *Cleome stenophylla* Klotzsch ex Urb.; Venezuela: Guarico; T. Ruiz y R. Villafane 4987 (MY);—; DQ455814. *Cleome stricta* (Klotzsch.) R. A. Graham; Mozambique; M. Elizabeth et al. 555 (MO); -26.7; HM044259. *Cleome strigosa* (Boj.) Oliv.; Tanzania; R. K. Brummitt et al. 17952 (MO); -28.0; HM044260. *Cleome stylosa* Eichler; Venezuela: Tachira; T. Ruiz y L. Hernandez 4977 (MY);—; DQ455812. *Cleome tenuifolia* (Mart. & Zucc.) H. H. Iltis; Bahia, Brazil; R. M. Harley 16325 (NY);—; HM044280. *Cleome titubans* Spegaz.; Argentina: Buenos Aires; A. Krapovickas 2897 (MO);—; DQ455813. *Cleome torticarpa* H. H. Iltis & T. Ruiz Zapata; Venezuela: Falcon; T. Ruiz y R. Villafane 5011 (MY);—; DQ455810. *Cleome trachycarpa* Klotzsch ex Eichler; Argentina; A. Krapovickas & C. L. Cristobal 46421 (MO); -28.9; HM044297. *Cleome tucumanensis* H. H. Iltis [C053]; Argentina; R. Fortunato 6639 (MO); -29.5; HM044291. *Cleome tucumanensis* [DQ455811]; Bolivia: Santa Cruz; L. Sanchez 1114 (USZ);—; DQ455811. *Cleome turkmena* Bobrov; Turkmenistan; D. Kurbanov 1055 (MO); -26.5; HM044231. *Cleome uncinifera* Kers.; Western Australia, Australia; B. J. Lepshi & L. A. Craven 5624 (CANB); -27.3; HM044249. *Cleome usambarica* Pax; Tanzania; M. A. Mwangoka 2967 (MO); -30.5; HM044274. *Cleome violacea* L. [F02]; Prague Bot. Garden (WSUG); E. Voznesenskaya 14 (WS); -29.5; HM044223. *Cleome violacea* [DQ532098]; Spain: Sevilla, El Ronquillo; A. Ortega Olivencia 7-4-2006;—; DQ532098. *Cleome violacea* [DQ532099]; Spain: Sevilla, Guillena; A. Ortega Olivencia 7-4-06;—; DQ532099. *Cleome viridiflora* Schreb. [DQ455820]; Venezuela: Barinitas, Barinas; T. Ruiz y L. Hernandez 4987 (MY);—; DQ455820. *Cleome viridiflora* [DQ455821]; Mexico: Tabasco; Hubbuch s. n. (MO);—; DQ455821. *Cleome viridiflora* [DQ455822]; U. S. A.; Botresarch (USA) Ind. Semo\*82302 accno 61-135-9807;—; DQ455822. *Cleome viscosa* L. [F12]; Mexico (WSUG); E. Voznesenskaya s. n. (WS); -27.1; HM044241. *Cleome viscosa* [F13]; Burkina Faso (WSUG); E. Voznesenskaya 15 (WS); -26.4; HM044242. *Cleome viscosa* [DQ455819]; Venezuela: Portuguesa; T. Ruiz y L. Hernandez 4963 (MY);—; DQ455819. *Cleome werdermannii* Ernst; Bolivia: Santa Cruz; L. Sanchez 1111a (MO);—; DQ455809. *Cleome* sp. nov. [C395]; Australia (CANB 574200); -26.8; HM044248. *Cleomella brevipes* S. Wats.; Nevada, U. S. A.; A. Tiehm 10867 (WS);—; HM044232. *Cleomella hillmanii* A. Nels.; Nevada, U. S. A.; A. Tiehm 10292 (WS); -24.5; HM044233. *Cleomella plocosperma* S. Wats.; Nevada, U. S. A.; A. Tiehm 10871 (WS);—; HM044234. *Dactylaena microphylla* Eichler; Bahia, Brazil; R. Callejas & A. M. de Carvalho 1729 (NY);—; HM044279. *Diploaxis tenuifolia* (L.) DC.; UPM-GERM-6666;—; EF601913. *Iberis spathulata* Lag. ex Willk. & Lange; [no voucher listed];—; AJ440312. *Moricandia suffruticosa* (Desf.) Coss. & Durieu; UPM-GERM-9274;—; EF601908. *Oxystylis lutea* Torr. & Frem.; California, U. S. A.; P. H. Raven 12874 (WS); -23.1; HM044235. *Podandrogyne chiriquensis* (Standl.) Woodson; Costa Rica; J. & K. Utley 4533

(MO); -28.0; HM044281. *Podandrogynne jamesonii* (Briq.) T. S. Cochrane; Ecuador; G. P. Lewis et al. 3438 (MO); -33.5; HM044282. *Podandrogynne macrophylla* (Turcz.) Woodson; Venezuela: Merida; T. Ruiz y L. Hernandez 4982 (MY);—; DQ455815. *Polanisia dodecandra* Britton, Stearns & Poggenb. subsp. *dodecandra* [DQ455816]; U. S. A.; Missouri, St. Louis; P. Stevens s. n. (MO);—; DQ455816. *Polanisia dodecandra* subsp. *trachysperma* (Torr. & A. Gray) H. H. Iltis [F22]; Kiev Bot. Garden (WSUG); E. Voznesenskaya 17

(WS); -26.9; HM044226. *Polanisia dodecandra* subsp. *trachysperma* (Torr. & A. Gray) H. H. Iltis [DQ455817]; [no country listed]; Botresearch U. S. A., Ind. Sem.\*82764 accno -4204-2077;—; DQ455817. *Polanisia uniglandulosa* DC.; Mexico; Stanford et al. 2098 (WS); -24.5; HM044225. *Stanleya pinnata* (Pursh) Britton; R. Price s. n. (GA);—; AF531620. *Wislizenia refracta* Engelm. subsp. *palmeri* (A. Gray) R. Keller; Mexico; R. S. Felger & J. Russell 85492 (MO); -25.6; HM044236.