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Molecular phylogenetics and character evolution of the "sacaca" clade: Novel relationships of *Croton* section *Cleodora* (Euphorbiaceae)

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ABSTRACT

Phylogenetic relationships of *Croton* section *Cleodora* (Klotzsch) Baill. were evaluated using the nuclear ribosomal ITS and the chloroplast *trnL-F* and *trnH-psbA* regions. Our results show a strongly supported clade containing most previously recognized section *Cleodora* species, plus some other species morphologically similar to them. Two morphological synapomorphies that support section *Cleodora* as a clade include pistillate flowers in which the sepals overlap to some degree, and styles that are connate at the base to varying degrees. The evolution of vegetative and floral characters that have previously been relied on for taxonomic decisions within this group are evaluated in light of the phylogenetic hypotheses. Within section *Cleodora* there are two well-supported clades, which are proposed here as subsections (subsection *Sphaerogyni* and subsection *Spruceani*). The resulting phylogenetic hypothesis identifies the closest relatives of the medicinally important and essential oil-rich *Croton cajucara* Benth. as candidates for future screening in phytochemical and pharmacological studies.

1. Introduction

Croton L., the second largest genus of Euphorbiaceae, is an important pantropical lineage with several species that are employed in traditional medicine practices in Africa, Asia, and South America (Moreno et al., 2009; Salatino et al., 2007). The genus has over 1200 species (Govaerts et al., 2000), ranking it as the 11th largest angiosperm genus (Frodin, 2004). It is found in tropical regions worldwide, although there are some representatives in subtropical and northern temperate areas as well. The Neotropics is the most species-rich region for the genus, with main centers of diversity in Brazil, the West Indies, and Mexico (Burger and Huft, 1995). *Croton* has great morphological diversity, ranging from herbs to shrubs to trees, rarely lianas, and it occupies a wide range of habitats. The genus is found in almost all types of vegetation, but most species grow in dry or open vegetation, and in disturbed sites.

The first attempts to recognize infrageneric groupings within *Croton* date back to Baillon (1858, 1864), and more recently Webster (1993) recognized 40 sections and five subsections within the genus. Berry et al. (2005) undertook the first molecular phylogenetic study of *Croton* and related groups. They showed that *Croton* was monophyletic once the former section *Astraea* (Klotzsch) Baill. was removed and restored to separate generic status. Since then, several other phylogenetic studies have contributed to building an infrageneric classification of *Croton* that reflects phylogenetic relationships (Riina et al., 2009, 2010; Van Ee and Berry, 2009, 2010, 2011; Van Ee et al., 2008, in press). Nonetheless, the basic relationships within many sections have yet to be explored.

One of the groups in need of critical revision is *Croton* section *Cleodora* (Klotzsch) Baill., which is concentrated in Brazil, but with a few members extending as far north as central Mexico. A species belonging to this section, *Croton cajucara* Benth., popularly known as "sacaca" (from the Tupi language, *sake'ka*, which means "witchcraft" or "spellcasting"; Le Cointe, 1934), has historically been used in the Amazon region to treat diarrhea, diabetes, liver and kidney problems, to lower cholesterol, and for weight loss (Salatino et al., 2007). Guided by the traditional ethnobotanical uses of *C. cajucara*, numerous researchers have explored the phytochemical and pharmacological properties of this species (Carvalho et al., 1996; Farias et al., 1997; Maciel et al., 1998a, 1998b; Souza-Brito et al., 1998; Grynberg et al., 1999; Lemos et al., 1999; Hiruma-Lima et al., 1999a; Maciel et al., 2000; Campos et al., 2002; Hiruma-Lima et al., 2002; Maciel et al., 2002; Grassi-Kassisse et al., 2003; Rosa et al., 2003; Alviano et al.,

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2005; Brito et al., 2006; Santos et al., 2006; Souza et al., 2006; Perazzo et al., 2007). The effectiveness of *C. cajucara* at reducing lipid and glucose levels has been demonstrated in the laboratory by several authors (Farias et al., 1996, 1997; Costa et al., 1999; Grynberg et al., 1999; Hiruma-Lima et al., 1999b; Maciel et al., 2000; Silva et al., 2001a, 2001b). *Croton cajucara* has also been shown to contain high levels of linalool, an alcohol used in the perfume industry (Araújo et al., 1971), which has raised much interest in this species among researchers in recent years (Moreno et al., 2009). Although Müller (1873) implied a close relationship between *C. cajucara*, *C. heterocalyx* Baill., and *C. sphaerogynus* Baill. by placing them together in his key, we are aware of no other publication until Webster (1993) that suggests what the closest relatives of *C. cajucara* might be. All phytochemical and pharmacological investigations within section *Cleodora* had been confined to *C. cajucara* until Moreno et al. (2009), who tested *C. heterocalyx* based on the hypothesis of close relationship implied by the taxonomic treatment of Webster (1993), discovered that it also contains linalool and has antimicrobial properties. Moreno et al. (2009) found 17 chemical compounds in common between the essential oils of *C. heterocalyx* and those found in *C. cajucara* by Lopes et al. (2000). Herein we provide a more comprehensive hypothesis of the phylogenetic relationships of the species related to *C. cajucara* and *C. heterocalyx*, which may be useful for further bioprospecting in this group. We expect that most of the species of section *Cleodora* will be subjected to phytochemical assays in the coming years, and this information can then be overlaid onto the phylogeny to reconstruct patterns of chemical evolution in this group.

Croton section *Cleodora* was defined morphologically by Webster (1993) by the presence of appressed-stellate trichomes, petiolar glands, terminal inflorescences usually with bisexual cymules, staminate flowers with 15–20 stamens, and pistillate flowers with multifid styles and united and/or imbricate sepals. Webster (1993) placed the following species in the section: *Croton cajucara* Benth., *C. calycularis* Huber (=*C. spruceanus* Benth.), *C. hemiargyreus* Müll.Arg., *C. heterocalyx* Baill., *C. hoffmannii* Müll.Arg., *C. maracayuensis* Chodat & Hassl. (=*C. floribundus* Spreng.), *C. seputubensis* Hoehne (=*C. cajucara*), and *C. sphaerogynus* Baill.

In a molecular phylogenetic analysis of relationships within *Croton* section *Cyclostigma* Griseb., Riina et al. (2009) recovered two species that were previously placed in that section, namely *Croton organensis* Baill. and *C. warmingii* Müll.Arg. (=*C. rottlerifolius* Baill.), emerging in a clade with *C. cajucara* and *C. hoffmannii*, both species traditionally placed in section *Cleodora*. This prompted us to increase our sampling beyond the traditional circumscription of section *Cleodora* by adding species such as *C. organensis* and *C. rottlerifolius*, as well as other taxa morphologically suggestive of the section. Given the extensive pharmacological interest in *C. cajucara*, we wish to identify the closest relatives of this medicinally important species to point out new candidate species for pharmacological screening. Within section *Cleodora* we also aim to use molecular methods to reconstruct the phylogenetic relationships of its members, introduce a phylogenetic classification, identify morphological synapomorphies and diagnostic characters, and investigate the evolution of floral characters.

2. Materials and methods

2.1. Taxon sampling and DNA sequencing

Species from section *Cleodora sensu* Webster (1993), and species with a similar morphology but of uncertain sectional placement, were included in our molecular sampling. A selection of taxa belonging to other sections and lineages of *Croton* were used to establish the limits of section *Cleodora*. We used two outgroup taxa, *Astraea lobata* (L.) Klotzsch and *Brasiliocroton mamoninha* Berry & Cordeiro, following the findings of Berry et al. (2005).

DNA was extracted from silica-dried or herbarium tissue of single individuals using the DNeasy Plant Mini kit (Quiagen, Valencia, California) following the manufacturer's instructions. The nuclear ribosomal ITS (ITS1, 5.8s, and ITS2) and the chloroplast *trnL* intron and *trnL-F* intergenic spacer regions (hereafter collectively referred to as "*trnL-F*") were amplified and sequenced employing the same methods as described in Berry et al. (2005). These two loci have been used in all species level phylogenies of *Croton* to date (Berry et al., 2005; Van Ee et al., 2008; Cordeiro et al., 2008; Van Ee and Berry, 2009, 2010, 2011; Riina et al., 2009, 2010). We added the non-coding plastid *trnH-psbA* spacer region, which given its high sequence variability has been shown to be a good candidate for a DNA barcoding region (Kress et al., 2005; Lahaye et al., 2008). The *trnH-psbA* region was amplified and sequenced using primers $trnH^{GUG}$ (Tate and Simpson, 2003) and psbA (Sang et al., 1997).

Polymerase chain reaction (PCR) amplification of the *trnH-psbA* marker was performed with 0.1 U of HotStarTaq DNA Polymerase (Quiagen Inc., Valencia, California, USA), 1× PCR Buffer (50 mM KCL, 10 mM Tris HCl, ph 9), 200 μM of each dNTP, 2.0 mM $MgCl_2$, 0.4 μM of each primer, and 25–90 ng of template DNA in a volume of 25 μL. The PCR profile consisted of an initial denaturing step at 95 °C for 15 min, amplification proceeded at one cycle of 95 °C for 1 min, 65 °C for 1 min, 72 °C for 1 min; one cycle of 95 °C for 1 min, 64 °C for 1 min, 72 °C for 1 min; 1 cycle of 95 °C for 1 min, 63 °C for 1 min, 72 °C for 1 min; one cycle of 95 °C for 1 min, 62 °C for 1 min, 72 °C for 1 min; one cycle of 95 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min; one cycle of 95 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min; then followed by 19 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, with a final 15 min extension at 72 °C. PCR products were cleaned using ExoSAP-IT (USB Corp., Cleveland, Ohio) following the manufacturer's protocol, and sequenced on ABI 3730 sequencers at the University of Michigan DNA Sequencing Core. Sequences were edited and assembled using the Staden Package v. 2003.0b1 (Staden, 1996), and then aligned in Clustal X (Thompson et al., 1997). The programs BioEdit v. 7.0.0 (Hall, 1999) and MEGA4 (Tamura et al., 2007) were used to make manual adjustments to the alignment generated by Clustal X.

The combined data matrix consists of 42 accessions, and although *trnH-psbA* was not sequenced for five species (*C. hircinus* Vent., *C. hirtus* L'Hér., *C. megalodendron* Müll.Arg., *C. pallidulus* Baill. and *C. velutinus* Baill.), they were included in the combined analysis with that portion coded as missing. A list of species sampled, localities, herbarium vouchers, and GenBank numbers for all sequences is provided in Appendix A. All *trnH-psbA* sequences are new, 20 of the *trnL-F* sequences are new, and 18 of the ITS sequences are new.

2.2. Phylogenetic analyses

Congruence between the nuclear and chloroplast loci, as well as between the two different chloroplast loci, was evaluated using the incongruence length difference (ILD) test (Farris et al., 1994) as implemented in PAUP* v. 4.0b10 (Swofford, 2002). The ILD test was conducted using 1000 partition homogeneity replicates of 10 random addition sequence replicates (RASR) each, tree-bisection-reconnection (TBR) branch-swapping, holding one tree at each step, nchuck = 100, and excluding uninformative characters and taxa with missing data.

Maximum parsimony (MP) heuristic searches were performed in PAUP* with 1000 random taxon addition replicates using TBR branch-swapping. All characters were included in the analyses. Characters were equally weighted, and gaps were treated as missing data. MP bootstrap (BS) values for the combined and individual

nuclear and chloroplast loci were estimated using 1000 bootstrap replicates of 100 RASR each, TBR, and nchuck = 100.

Bayesian analyses were conducted in MrBayes v. 3.1.2 (Huelsenbeck and Ronquist, 2001). Bayesian posterior probabilities (PP) were calculated from two Markov chain Monte Carlo (MCMC) analyses, each consisting of four linked chains (heat = 0.02), 1,000,000 generations, and sampling every 50 generations. All characters were included in the analyses, and the data were divided into three partitions (ITS, *trnL-F*, and *trnH-psbA*) with MrBayes estimating the best model parameters for each partition independently. The burn-in period was estimated by visual examination of the likelihood-by-generation plot and, after removing the trees from the burn-in period, PP values were obtained by computing a majority rule consensus of the trees from both MCMC chains.

2.3. Character state mapping

Eight morphological characters were scored from fresh material and herbarium samples for the entire dataset (Table 1). Most of the selected characters have been used in previous systematic studies in *Croton* (Webster, 1993; Caruzo and Cordeiro, 2007; Lima and Pirani, 2008; Van Ee et al., 2008). To assess the patterns of evolution of these characters, we mapped them onto one of the most parsimonious trees obtained from the combined parsimony analysis. We reconstructed ancestral character states using parsimony as implemented in Mesquite v. 2.72 (Maddison and Maddison, 2009). All characters were treated as unordered.

3. Results

3.1. Molecular data sets and congruence

The aligned lengths of the three regions, as well as information regarding the variable and parsimony-informative characters, are given in Table 2. The nuclear ITS (Fig. 1) and *trnL-F* + *trnH-psbA* chloroplast (Fig. 2) phylogenies do not differ from each other, or from the results of the combined analysis (Fig. 3), in any strongly-supported way, with the exception that the chloroplast analyses recovered section *Cyclostigma* apart from section *Adenophylli* (formerly known as section *Cascarilla*), a result discussed in greater detail by Riina et al. (2009). This difference in topology is likely the reason that the ILD test rejects the hypothesis of no meaningful conflict between the nuclear and chloroplast data ($p = 0.004$). In contrast, the ILD test conducted between the two chloroplast regions (*trnL-F* and *trnH-psbA*) failed to reject the hypothesis of no meaningful conflict between them ($p = 0.630$). The phylogenetic relationships within section *Cleodora* inferred from the different molecular markers are very similar to each other. We therefore discuss the phylogenetic relationships among species of section *Cleodora* using the combined data phylogeny (Fig. 3), and refer to the contrasting results obtained from independent analyses of the nuclear and chloroplast data when appropriate.

Table 1
Morphological characters and coding information.

Character	Coding
Trichomes	0 = stellate, 1 = lepidote
Petiolar glands	0 = absent, 1 = present
Bisexual cymules	0 = absent, 1 = present
Connation of pistillate sepals	0 = united at the base, 1 = united half of their length, 2 = united almost to the apex
Pistillate flower aestivation	0 = valvate, 1 = reduplicate-valvate, 2 = imbricate, 3 = quincuncial
Pistillate flower petals	0 = absent, 1 = reduced, 2 = well-developed
Style division	0 = bifid, 1 = four-fid, 2 = multifid
Connation of styles	0 = absent, 1 = present

Table 2
Number of characters and comparative statistics for the maximum parsimony analyses of the molecular datasets.

	ITS	trnL-F	psbA-trnH	Chloroplast-combined
Total characters	743	1155	767	1922
Constant characters	428	901	434	1335
Variable characters	315	254	333	587
Informative characters	229	108	138	246
% Informative	31	9	18	13
Trees retained	220	–	–	209
Tree length	838	–	–	826
CI	0.5561	–	–	0.8257
RI	0.6346	–	–	0.6346

3.2. Analysis of ITS

The parsimony search of the ITS data set retained 220 trees of length (L) = 838, consistency index (CI) = 0.5561, and retention index (RI) = 0.6346. The "sumt" command in MrBayes was used to compute the consensus of the post-burn-in trees and the posterior probability values from the Bayesian analysis of ITS (Fig. 1).

Results from all analytical approaches performed on this data set recovered a new circumscription of section *Cleodora* compared to that of Webster (1993). Three species placed by Webster (1993) in other sections are shown here to belong to section *Cleodora*, namely *C. fragrans* Kunth, previously placed in section *Lasiogyne*; and *C. organensis* and *C. rottlerifolius*, which were previously placed in section *Cyclostigma*. These three species emerge within a strongly supported clade (100% PP, 75% BS) along with all of the sampled section *Cleodora* species (Fig. 1).

In the ITS phylogeny, section *Cleodora* emerges in a trichotomy with two other clades of *Croton* subgenus *Geiseleria* A.Gray (*sensu* Van Ee et al., in press), which together include sections *Lamprocroton*, *Lasiogyne*, *Luntia*, *Eluteria*, *Barhamia*, *Medea*, and *Geiseleria* (Fig. 1). The recently recognized section *Cuneati* (G.L. Webster) Riina & Berry (Riina et al., 2010) is recovered sister to this trichotomy (Fig. 1). Nevertheless, within section *Cleodora*, two highly supported clades (100% PP and 99% BS each) are recovered (Fig. 1), which represent the two subsections of *Cleodora* that we describe here (subsections *Sphaerogyni* and *Spruceani*).

3.3. Analysis of chloroplast trnL-F and trnH-psbA

The parsimony search of the chloroplast data set retained 209 trees of $L = 826$, CI = 0.8257, and RI = 0.7725. The consensus of the post-burn-in trees from the Bayesian analysis of the combined chloroplast MCMC chains is depicted in Fig. 2.

Similar to the ITS phylogeny, the results from all analytical approaches performed on the chloroplast data recovered a newly circumscribed section *Cleodora* compared to that of Webster (1993), with the same species (*C. fragrans*, *C. organensis*, and *C. rottlerifolius*) emerging within a well supported (100% PP, 61% BS) section *Cleodora* clade (Fig. 2). However, the chloroplast phylogeny recovered, with strong Bayesian posterior probability support (94% PP), section *Cuneati* sister to section *Cleodora*. The chloroplast results provide high support (100% PP, 88% BS) for subsection *Spruceani*, but only weak support (68% PP, <50% BS) for subsection *Sphaerogyni* (Fig. 2).

3.4. Combined nuclear and chloroplast analysis

The parsimony search of the combined data set retained four trees of $L = 1686$, CI = 0.6809, and RI = 0.6741. The consensus of

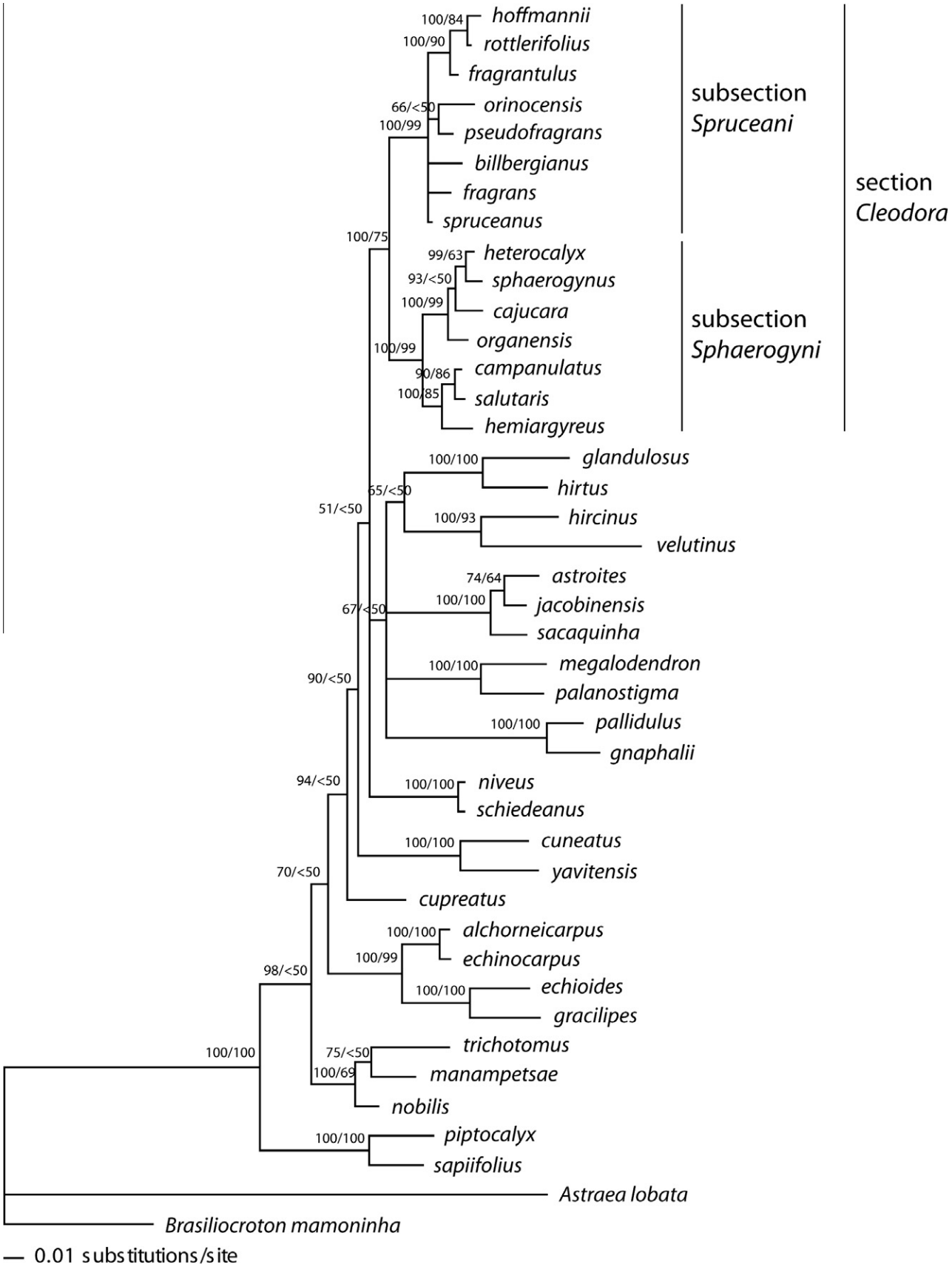

Fig. 1. Phylogram of the Bayesian analysis of ITS data. The numbers represent support values in the following order: Bayesian posterior probability (PP)/ parsimony bootstrap support (BP).

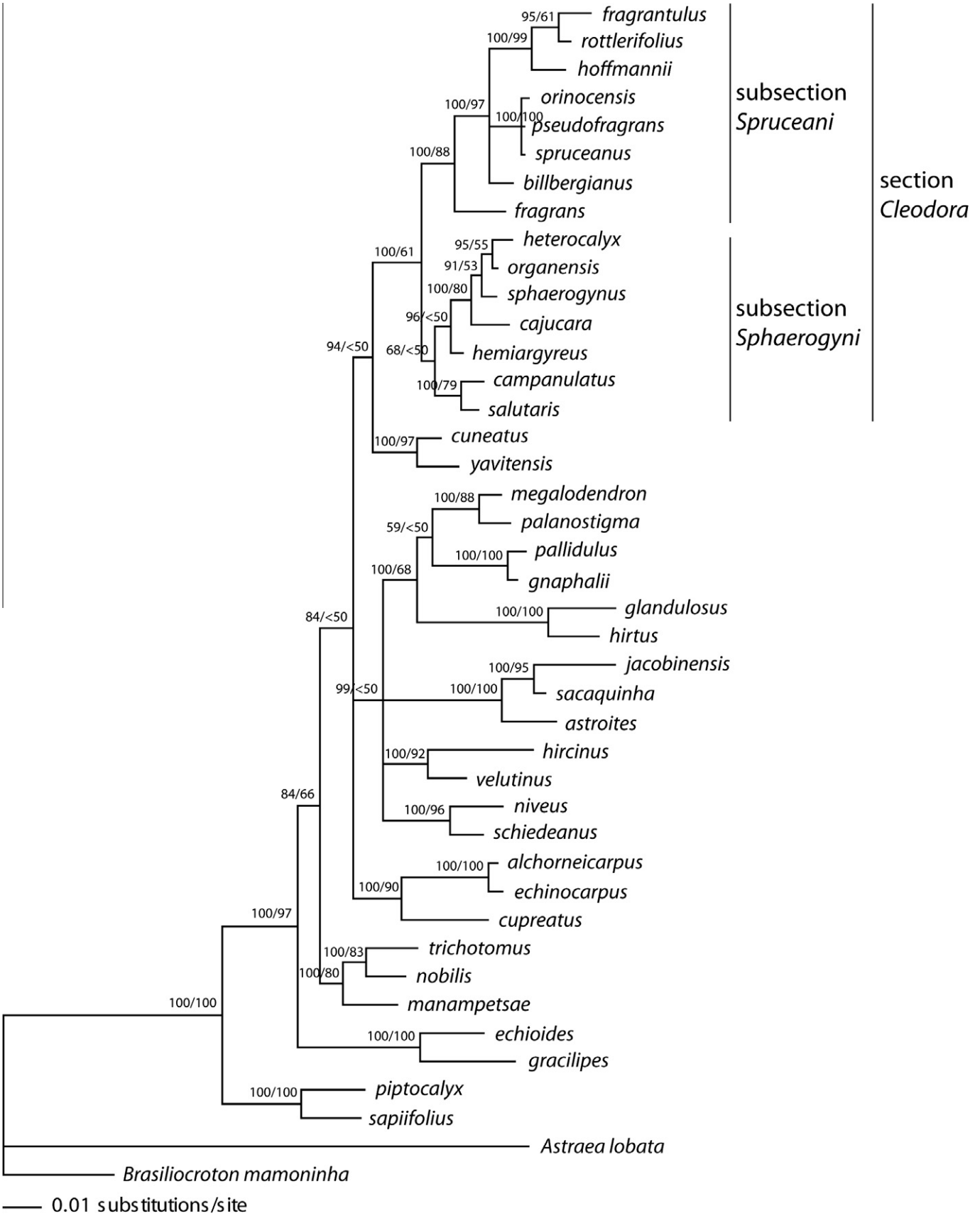

Fig. 2. Phylogram of the Bayesian analysis of combined chloroplast *trnL-F* and *trnH-psbA* data. The numbers represent support values in the following order: Bayesian posterior probability (PP)/parsimony bootstrap support (BP).

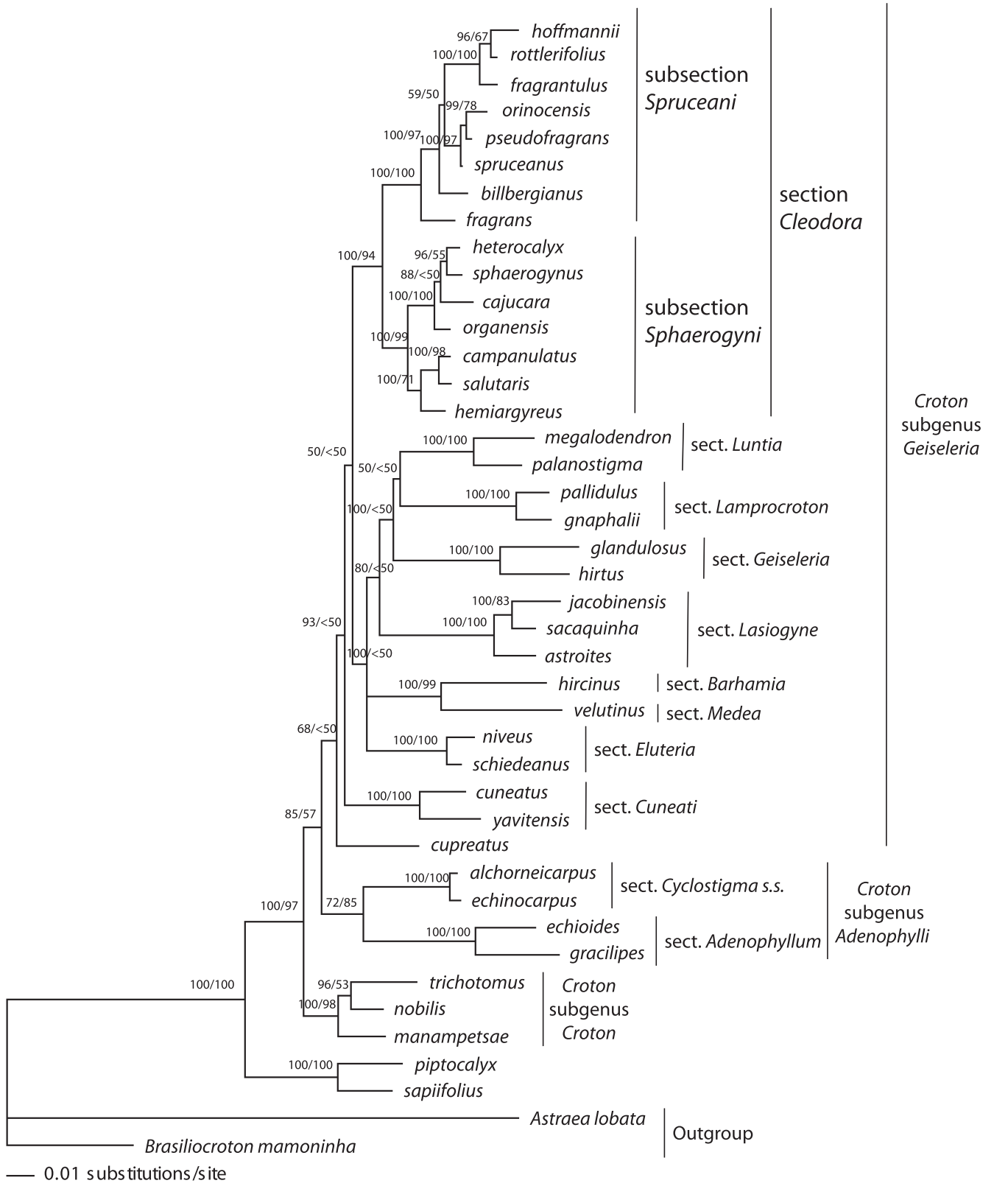

Fig. 3. Phylogram of the Bayesian analysis of combined *trnL-F*, *trnH-psbA* and ITS data. The numbers represent support values in the following order: Bayesian posterior probability (PP)/parsimony bootstrap support (BP). Names on the right of vertical bars represent the sectional or informal clade assignment of the species.

the post-burn-in trees from the Bayesian analysis of the combined data MCMC chains is depicted in Fig. 3. All analyses performed on this data set recovered the same new circumscription of section *Cleodora*, with the same three previously non-section *Cleodora* species (*Croton fragrans*, *C. organensis*, and *C. rottlerifolius*) emerging within a highly supported clade (100% PP, 94% BS) along with the other members of section *Cleodora sensu* Webster (1993) (Fig. 3).

In our combined phylogenetic results, section *Cleodora* is recovered within subgenus *Geiseleria* (Fig. 3). Subgenus *Geiseleria* emerges sister to subgenus *Adenophylli* (Griseb.) Riina, B.W. van Ee and Berry (*sensu* Van Ee et al., in press), and together these are sister to subgenus *Croton* (Fig. 3). The sister group of section *Cleodora*, although with weak support (50% PP, <50% BS), is a portion of the remainder of subgenus *Geiseleria*, which includes groups such as sections *Lamprocroton*, *Geiseleria*, and *Cuneati* (Fig. 3).

Section *Cleodora* is composed of two major sister clades, each of which are strongly supported (100% PP each; 100% and 99% BS), and henceforth will be referred to as subsections. Subsection *Sphaerogyni*, depicted on the bottom in Fig. 3, includes the type of the section (*Croton sphaerogynus*), and subsection *Spruceani* is depicted on the top of Fig. 3.

3.5. Morphological character state mapping

Our results support previous claims that the morphological characters traditionally used in the systematic classification of *Croton* are highly homoplasious (Berry et al., 2005; Van Ee et al., 2008; Riina et al., 2009). Most of the clades, or sections, within *Croton* are defined by a suite of homoplasious characters, rather than by clear synapomorphies. The reconstruction of the evolution of trichome types within section *Cleodora* (Fig. 4A) reveals that the presence of lepidote trichomes is plesiomorphic, and is shared not only by section *Cleodora* but by other New World and Old World groups. Within section *Cleodora*, stellate trichomes appear to have evolved independently on two separate occasions (Fig. 4A).

The presence or absence of extrafloral nectaries on leaves is a frequently used diagnostic character within *Croton*. Although we have a relatively small sampling for tribe Crotoneae in our analysis, the inferred evolution of this character indicates that the presence of petiolar glands (Fig. 4B) is plesiomorphic for *Croton*, and for section *Cleodora*.

Croton flowers are usually arranged in thyrsoid inflorescences in which pistillate flowers are solitary at the lower nodes, and staminate flowers are found in cymules at the upper nodes. However, some species possess inflorescences with bisexual basal cymules, in which pistillate and staminate flowers occur together, as Webster (1993) described for sections *Cleodora* and *Cyclostigma*. The presence of bisexual cymules (Fig. 4C), as already pointed out by Van Ee et al. (2008), is homoplasious across the genus. The presence of inflorescences with bisexual basal cymules, used by Webster (1993) as one of the defining characters of section *Cleodora*, is thus a plesiomorphic character state for the section.

The optimization of the fusion of sepals in pistillate flowers onto the phylogeny (Fig. 4D) implies that the union of sepals only at the base is a plesiomorphic character state for *Croton*, found in almost the entire genus. The fusion of sepals in pistillate flowers up to half of their length has evolved at least three different times, once within section *Cleodora* subsection *Spruceani* (*Croton fragrantulus* Croizat, *C. hoffmannii*, and *C. rottlerifolius*), again in *C. megalodendron* and other members of section *Luntia*, and a third time in *C. astroites* Dryand. and other species of section *Lasiogyne* (Fig. 4D). The fusion of sepals almost to the apex has evolved only once in *Croton*, within section *Cleodora* (Fig. 4D), and it is an autapomorphy for *C. spruceanus*. *Croton spruceanus* is more closely related to species with sepals that are united only at the base, but together these are sister to the members of section *Cleodora* that have sepals fused up to half of their length (Figs. 3, 5A), suggesting that the full fusion of the sepals is probably not entirely independent of the partial fusion within section *Cleodora*.

Styles in *Croton* vary from simple (with three terminal stigmatic tips), to bifid (six terminal tips), four-fid (12 terminal tips), or multifid (more than 12 terminal tips). The inferred evolution of style division indicates that four-fid and multifid styles are highly homoplasious within *Croton*. Multifid styles are found throughout the genus, and therefore, the presence of multifid styles (Fig. 5A), one of the characters used by Webster (1993) to define section *Cleodora*, is plesiomorphic for the clade. A reduction from multifid to four-fid styles appears to have evolved at least twice within the section (Fig. 5A).

Pistillate flowers in *Croton* are generally apetalous, or with greatly reduced petals, and only rarely are conspicuous petals present in them. For practical reasons, in the cases in which small filamentous or glandular structures are present in the position of petals, we refer to these structures as reduced petals. Our results show that the presence of reduced petals is shared by a majority of New World *Croton* species (Fig. 5B), with a loss of this character in members of section *Luntia* (*C. megalodendron* and *C. palanostigma* Klotzsch), and in almost all members of section *Cleodora* except for the small clade consisting of *C. hemiargyreus*, *C. salutaris* Casar., and *C. campanulatus* Caruzo & Cordeiro, which have petals reduced to ovoid glands.

The aestivation of pistillate flowers in *Croton* is generally valvate, or less often reduplicate-valvate (with the adjoining lobes valvate but then folding backwards and outwards at the same time). Reconstructing this character onto the molecular phylogeny indicates that valvate aestivation in pistillate flowers is the ancestral state for *Croton* (Fig. 5C). Overlapping aestivation (imbricate or quincuncial) is present in the pistillate flowers of all members of section *Cleodora*, except for the reduplicate-valvate aestivation in *C. fragrans*, and thus can be regarded as a synapomorphy for the clade.

4. Discussion

4.1. Phylogenetic relationships

The results of this study indicate that section *Cleodora sensu* Webster (1993) is largely monophyletic. These results corroborate those found by Riina et al. (2009), where *Croton organensis* and *C. rottlerifolius* (treated as *C. warmingii* in their study), both formerly placed in section *Cyclostigma* by Webster (1993), now emerge within a well supported clade with most members of Webster's section *Cleodora*. Besides these two species, *Croton fragrans*, a species placed by Webster (1993) in section *Lasiogyne*, also emerges within section *Cleodora*.

Section *Cleodora* is recovered as part of subgenus *Geiseleria* (Fig. 3), which is equivalent to clades C-5 through C-11 of Berry et al. (2005). Our results show that the chloroplast (*trnL-F* + *trnH-psbA*) and ITS trees are different, but not incompatible, regarding the sister group of section *Cleodora*. The chloroplast phylogeny strongly supports (94% PP) section *Cuneati* as the sister group of section *Cleodora* (Fig. 2), whereas the ITS phylogeny places it in an unresolved position within subgenus *Geiseleria* (Fig. 1). The results from the chloroplast phylogeny are similar to those reported by Riina et al. (2010) in a study of arborescent clades of Neotropical *Croton*. Section *Cuneati* is morphologically similar to section *Cleodora* in its arborescent habit, presence of petiolar glands, bisexual cymules sometimes present, and low stamen number (10–20), but it differs from section *Cleodora* mainly by its discoid glands in the leaf margins (absent in section *Cleodora*), valvate sepals in the pistillate flowers (which are quincuncial, imbricate, or reduplicate-valvate in section *Cleodora*), free styles (which are united in section *Cleodora*), seeds ecarunculate or with a vestigial caruncle (compared to the small and usually reniform caruncle in section *Cleodora*), or sometimes with a distinctive aril (absent in section *Cleodora*).

Our results from the combined ITS and chloroplast data recover a section *Cleodora* clade with high support (100% PP, 94% BS)

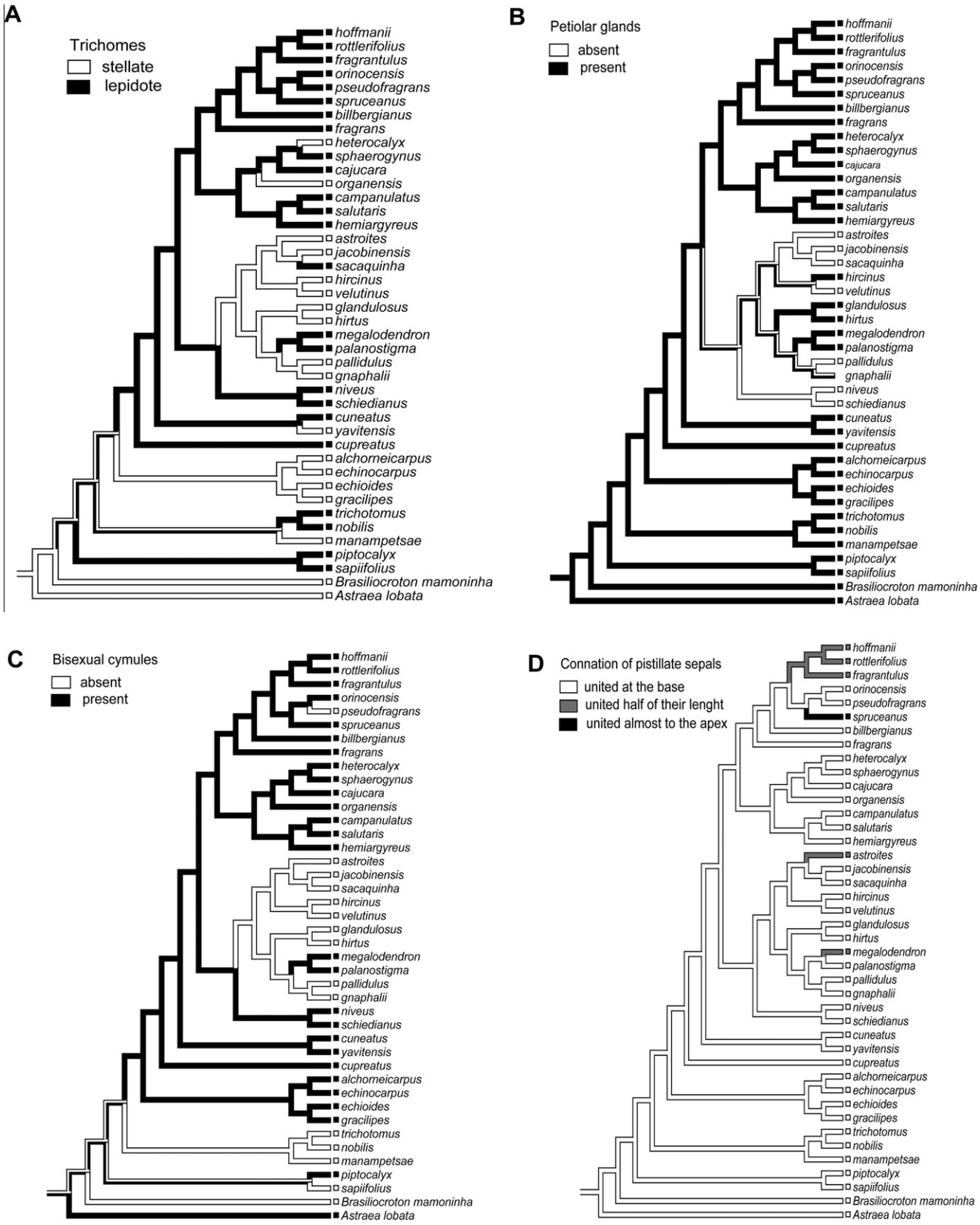

Fig. 4. Evolution patterns of morphological characters mapped onto one of the most parsimonious trees obtained from the combined parsimony analysis. (A) Trichomes. (B) Petiolar glands. (C) Bisexual cymules. (D) Connation of pistillate sepals.

(Fig. 3). This clade has two main morphological synapomorphies: sepals of the pistillate flowers with some kind of overlap (Fig. 5C), and styles united at the base or higher, usually forming a tubular structure or a "crown" (Fig. 5D). All species of section *Cleodora* are shrubs or small trees, with the exception of *Croton organensis*, which is a large tree up to 15 m tall. They grow along moist or dry forest borders, and they usually have reddish or clear latex. Other important features shared by the majority of the

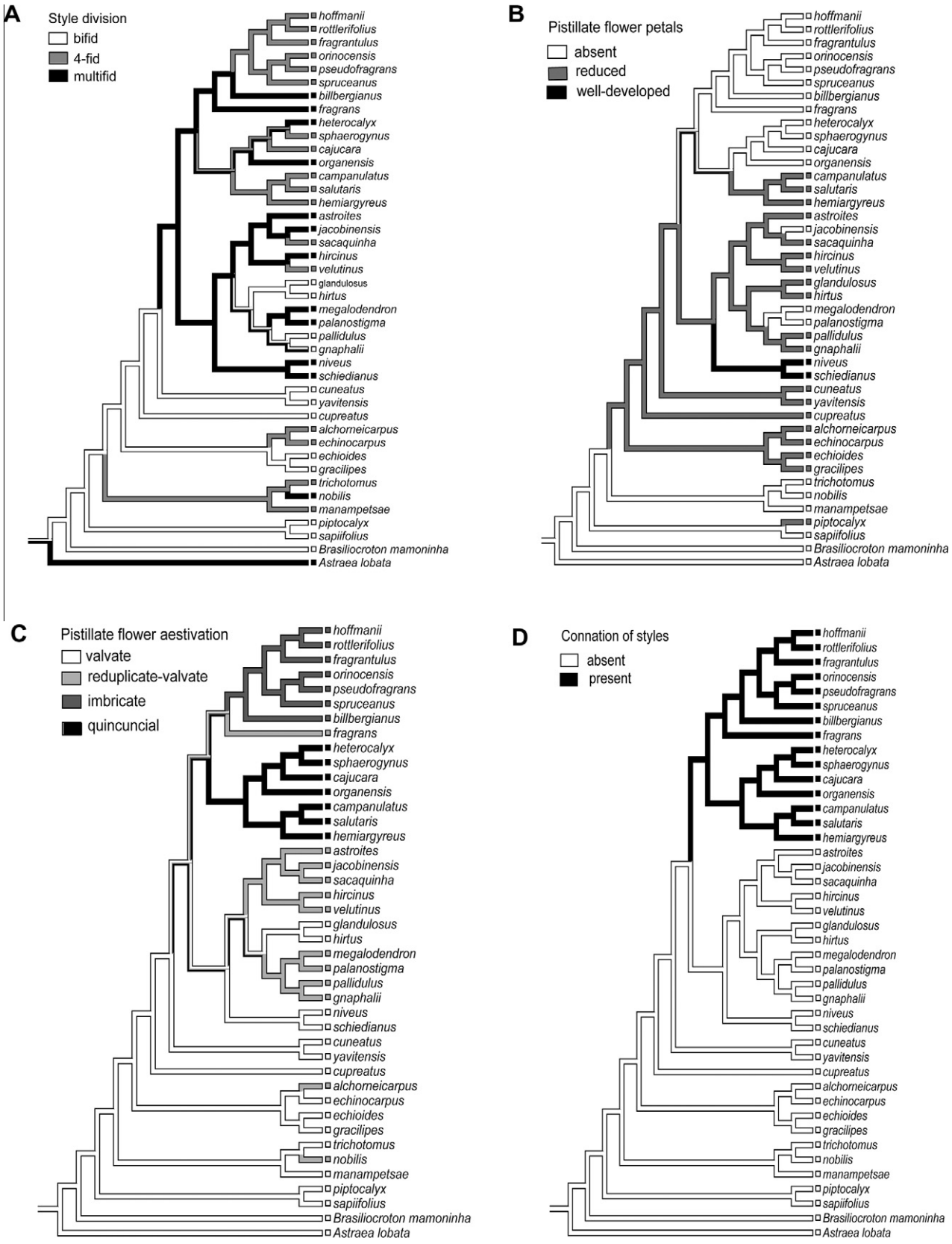

Fig. 5. Evolution patterns of morphological characters mapped onto one of the most parsimonious trees obtained from the combined parsimony analysis. (A) Style division. (B) Pistillate flower petals. (C) Pistillate flower aestivation. (D) Connation of styles.

species belonging to this clade (Figs. 6A–H) are the presence of lepidote trichomes of all subtypes (*sensu* Webster et al., 1996), leaves with a pair of glands at the apex of the petiole (acropetiolar glands) or near the base of the lamina (basilaminar glands), inflorescences with basal bisexual cymules (a plesiomorphic characteristic for the genus), sepals of the staminate flowers usually

united half of their length, 15–25 stamens, sepals of the pistillate flowers united at the base or higher, and four-fid (12 terminal tips) or multifid (>12 terminal tips) styles.

Within section *Cleodora* it is possible to identify two major, well-supported clades which we treat as subsections (Fig. 3). Subsection *Sphaerogyni* is predominantly Brazilian, with most species occurring in the Atlantic Rainforest of eastern Brazil, with the exception of *Croton cajucara*, an Amazonian species from lowland moist forests. Subsection *Spruceani* has species that occur in central and northwestern South America and southern Central America (with *Croton billbergianus* Müll.Arg. extending up to central Mexico), with the exception of *C. rottlerifolius*, which occurs in deciduous forests of Brazil, and *C. fragrantulus*, which occurs in similar vegetation in eastern Bolivia and central Peru.

Subsection *Sphaerogyni*, which includes the type species of section *Cleodora* (*Croton sphaerogynus*), is supported by a morphological synapomorphy, namely, the sepals of the pistillate flowers with quincuncial aestivation. Other features shared among species within subsection *Sphaerogyni* are globose fruits, sepals of pistillate flowers that are usually fleshy at the base, and pistillate flowers with a disk that is usually segmented.

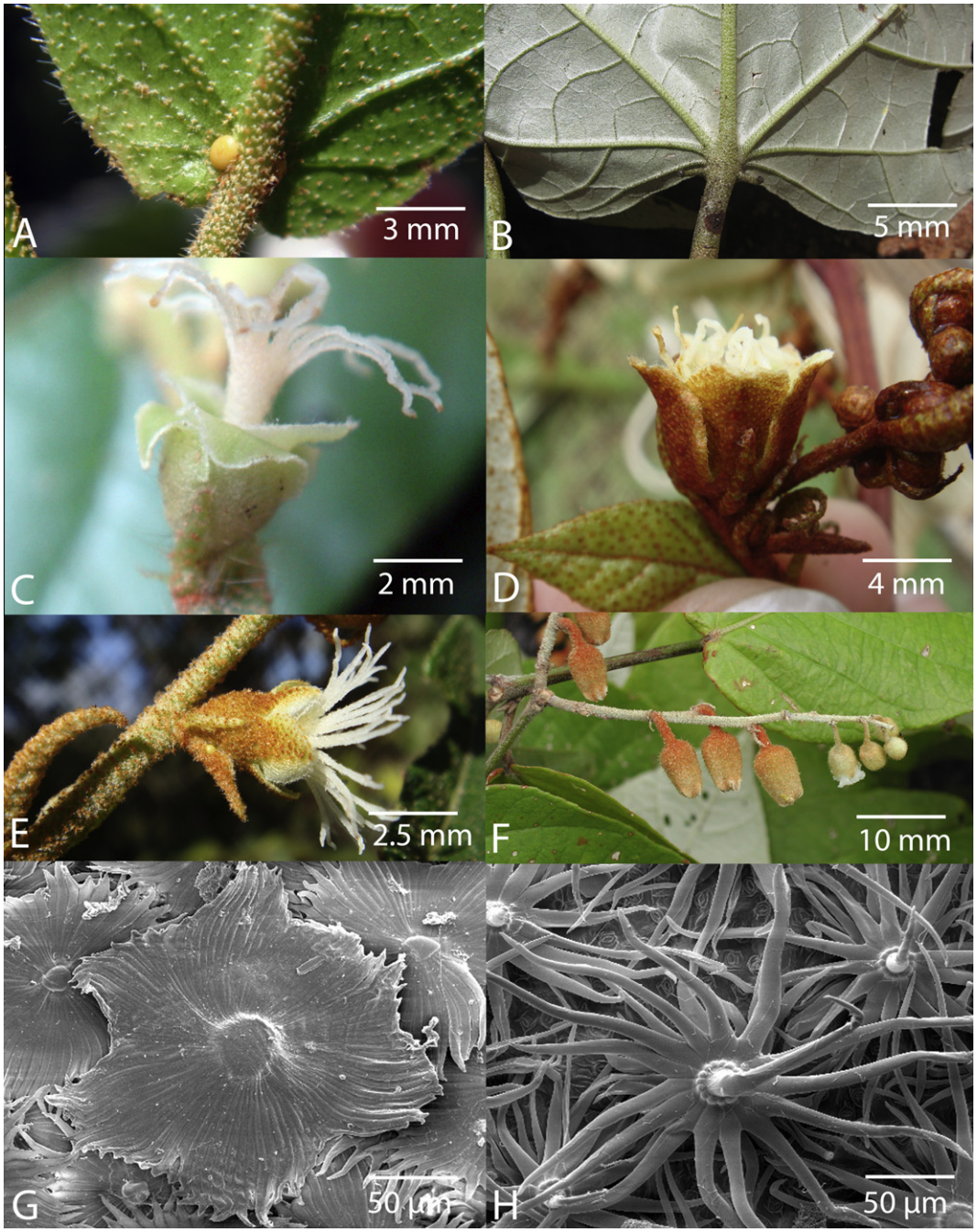

Fig. 6. Morphological features of some members of *Croton* section *Cleodora*. (A) Basilaminar glands of *C. sphaerogynus* (*Caruzo* et al., *65*). (B) Acropetiolar glands of *C. hemiargyreus* (*Caruzo and Ferro, 116*). (C) Pistillate flower of *C. sphaerogynus*, showing stylar column, and 12 terminal tips (*Caruzo and Lima, 121*). (D) Pistillate flower of *C. campanulatus* showing quincuncial aestivation (*Caruzo and Lima, 123*). (E) Pistillate flower of *C. stellatoferrugineus* showing multifid styles (with more than 12 terminal tips) (*Cordeiro* et al., *3033*). (F) Pistillate flowers of *C. spruceanus* showing sepals united almost to the apex (*Caruzo and Riina, 101*). (G) Lepidote subintire trichome of *C. campanulatus* (*Caruzo* et al., *93*). (H) Adpressed-stellate trichomes of *C. spruceanus* with a porrect central radius (*Caruzo and Riina, 101*). (all images by the first author).

Within subsection *Sphaerogyni*, two highly supported groups (each with 100% BS and PP) can be recognized (Fig. 3): the first (bottom) is an entirely lepidote clade with the typical subentire scales (*Croton salutaris*, *C. campanulatus*, and *C. hemiargyreus*), and the other one (top) consists of species that have appressed-stellate or multiradiate trichomes and lack lepidote subentire trichomes (*C. heterocalyx*, *C. sphaerogynus*, *C. cajucara*, and *C. organensis*).

Our results show that the nuclear ITS and chloroplast trees are discordant about the exact placement of *Croton hemiargyreus* within subsection *Sphaerogyni* (Figs. 1 and 2). ITS data (Fig. 1) support *C. hemiargyreus* as sister to *C. salutaris* + *C. campanulatus*, whereas the chloroplast data (Fig. 2) place it sister to the other members of the subsection. However, morphology is consistent with the placement of this species as sister to *C. salutaris* + *C. campanulatus*, as recovered in the ITS (Fig. 1) and combined (Fig. 3) phylogenies. The group consisting of *C. hemiargyreus*, *C. salutaris*, and *C. campanulatus* is strictly arborescent, and is the only one within section *Cleodora* that is characterized by subentire lepidote trichomes, long stipitate acropetiolar glands, and apparently monochlamydeous pistillate flowers with petals reduced to ovoid glands.

The second group in subsection *Sphaerogyni* as recovered in the combined phylogeny (*Croton organensis*, *C. cajucara*, *C. heterocalyx*, and *C. sphaerogynus*; Fig. 3) shares appressed-stellate, multiradiate, and stellate trichomes, as well as the presence of sessile basilaminar glands. Moreover, with the exception of *C. organensis*, these species are usually found in places with sandy soils. *Croton organensis* is supported as the sister species to the rest of this clade, followed by *C. cajucara*, which is recovered sister to *C. heterocalyx* and *C. sphaerogynus*. Within this group, both *C. cajucara* and *C. heterocalyx* have been reported to contain linalool, an alcohol with antimicrobial properties widely used in the perfume industry, making this clade interesting for bioprospecting studies. Since our results indicate that *C. sphaerogynus* is one of the closest relatives of *C. cajucara*, we suggest that it is an excellent candidate for future bioprospecting studies.

Subsection *Spruceani* is composed of species from moist to seasonally dry forests of northern and western South America, Central America, and Mexico, as well as two from seasonally dry forests of eastern Brazil, Peru, and Bolivia. This subsection can be generally characterized by the presence of appressed-stellate trichomes sometimes associated with other types of trichomes, imbricate aestivation in the pistillate flowers, sepals of the pistillate flowers that are usually united at least half of their length, and subglobose to ellipsoid fruits. *Croton fragrans*, followed by *C. billbergianus*, are supported as sister to the rest of the species (Fig. 3). These remaining species share four-fid styles, and are clustered into two highly supported subclades (each with 100% BS and PP): the first (top) contains species of seasonally dry forests of South America and Central America (*C. fragrantulus*, *C. rottlerifolius*, and *C. hoffmannii*) whose distributions fall within the area of the dry "Pleistocenic arc" reported by Pennington et al. (2006) and Prado (2000). The species of this group share the presence of stellate and appressed-stellate trichomes, acropetiolar leaf glands, and campanulate pistillate flowers. The second clade (bottom) is composed of species from lowland Amazon rainforests (*C. spruceanus*, *C. orinocensis*, and *C. pseudofragrans*).

Relationships among species of the first group (*Croton fragrantulus*, *C. rottlerifolius*, and *C. hoffmannii*) are not congruent between the ITS and chloroplast phylogenies. ITS (Fig. 1) supports *C. rottlerifolius* as sister to *C. hoffmannii*, while the chloroplast data (Fig. 2) place *C. fragrantulus* sister to *C. rottlerifolius*. Morphology is consistent with the placement of *C. rottlerifolius* as the sister species of *C. hoffmannii* as recovered in the ITS and combined phylogenies. These two species (*C. rottlerifolius* and *C. hoffmannii*) are morphologically more similar to each other than either one is to *C. fragrantulus*. Some of the morphological characteristics shared between *C. rottlerifolius* and *C. hoffmannii* are the presence of cylindrical branches (vs. flat branches in *C. fragrantulus*), the presence of blackened trichomes on the pistillate flowers, and sepals of the pistillate flowers with a distinct texture at the margin.

4.2. Character evolution

Trichome types have been widely used in the systematic classification of *Croton* (Webster et al., 1996). Trichomes within the genus vary between two basic types, stellate and lepidote, although there are intermediate forms. Trichome type has transitioned between stellate and lepidote multiple times during the evolution of *Croton* (Fig. 4A).

The total fusion of the sepals in *Croton spruceanus* (Fig. 6F) might have some implications for its pollination biology, but field studies need to be done to determine its pollination system. Most *Croton* species are visited by different kinds of insects, and their flowers are either insect or wind pollinated (Domínguez and Bullock, 1989; Bullock, 1994; Decker and Pilson, 2000; Freitas et al., 2001), so it will be interesting to study the reproductive biology of *C. spruceanus* to determine whether its unique calyx morphology is associated with a more specific pollinator, such as hummingbirds.

Styles in *Croton* are usually free, but connation of the styles at the base or higher is present in all members of section *Cleodora*. Despite being a synapomorphy for the entire section *Cleodora* clade, the connation of styles can be found in other *Croton* species not sampled in our study, such as in some Malagasy species (e.g. *C. argyrodaphne* Baill.), and some Neotropical species belonging to section *Argyroglossum* (e.g. *C. blanchetianus* Baill.).

Two different types of overlapping aestivation in pistillate flowers are present within section *Cleodora*, quincuncial aestivation in subsection *Sphaerogyni*, and imbricate aestivation in subsection *Spruceani*, with one species in subsection *Spruceani*, *Croton fragrans*, displaying reduplicate-valvate aestivation. The clade sister to section *Cleodora* has valvate and reduplicate-valvate aestivation. Therefore, our results support an evolutionary trend within *Croton* from pistillate flowers with valvate or reduplicate-valvate aestivation to pistillate flowers with imbricate or quincuncial aestivation in species of section *Cleodora* (Fig. 5C).

4.3. A new circumscription for the section

Croton section ***Cleodora*** (Klotzsch) Baill., Étude Euphorb.: 369. 1858. ≡ *Cleodora* Klotzsch, Arch. Naturgesch 7: 196. 1841. Type: *Cleodora sellowiana* Klotzsch (=*Croton sphaerogynus* Baill.).

=*Croton* section *Stolidanthus* Baill., Adansonia 4: 323. 1864. Type: *Croton heterocalyx* Baill. (lectotype, designated by Webster, 1993: 800).

Trees or shrubs, monoecious, generally laticiferous, latex clear to reddish; covered with appressed-stellate, stellate-lepidote, or lepidote subentire trichomes, rarely stellate or multiradiate trichomes. Leaves alternate, with a pair of acropetiolar or basilaminar, usually sessile, rarely stipitate, glands. Inflorescences terminal, rarely axillary, basal cymules bisexual, rarely unisexual or falsely unisexual; staminate flowers dichlamydeous, campanulate, rarely subcampanulate, valvate or slightly imbricate aestivation, sepals usually united up to half of their length, stamens 15–25; pistillate flowers monochlamydeous, rarely with petals reduced to glands, slightly zygomorphic or actinomorphic, sessile or pedicellate, campanulate, flask-shaped or urceolate, imbricate or quincuncial aestivation, styles four-fid or multifid, united at the base or further up, usually forming a "crown" or a column. Fruits globose, ellipsoid or subglobose (usually trigonal), with calyx persistent and sometimes strongly accrescent; seeds with a small caruncle, without aril.

Species included: *Croton billbergianus* Müll.Arg., *C. cajucara* Benth., *C. campanulatus* Caruzo & Cordeiro, *C. sexmetralis* Croizat,

C. fragrans Kunth, *C. fragrantulus* Croizat, *C. hemiargyreus* Müll.Arg., *C. heterocalyx* Baill., *C. hoffmannii* Müll.Arg., *C. organensis* Baill., *C. orinocensis* Müll.Arg., *C. pseudofragrans* Croizat, *C. rottlerifolius* Baill., *C. rufolepidotus* Caruzo & Riina, *C. salutaris* Casar., *C. sphaerogynus* Baill., *C. spruceanus* Benth., *C. stellatoferrugineus* Caruzo and Cordeiro.

There are two species excluded from section *Cleodora* in its new circumscription, namely *Croton maracayuensis* Chodat & Hassl. (=*C. floribundus* Spreng.), which was placed in section *Cleodora* by Webster (1993), and *Croton velutinus* Baill., which was placed by Baillon (1864) in his section *Stolidanthus*. *Croton maracayuensis* was first treated as a synonym of *C. floribundus* by Bernardi (1984). Webster (1993) placed *Croton floribundus* in section *Argyroglossum* mainly due to its leaves without glands, inflorescences without bisexual cymules, 10–15 stamens, and reduplicate-valvate sepals in the pistillate flowers, which are all characteristics that exclude this species from section *Cleodora*. Although Webster (1993) had synonymized section *Stolidanthus* under section *Cleodora*, he did not include *Croton velutinus* in the latter section, or in any other section recognized in his synopsis. The subshrubby habit, stellate indumentum, leaves without petiolar or basilaminar glands, inflorescences without bisexual cymules, between 10–12 stamens, and reduplicate-valvate sepals of the pistillate flowers of this species exclude it from section *Cleodora*. The best placement for *C. velutinus*, considering all the features above is in section *Barhamia* (Klotzsch) Baill.

Species of *Croton* section *Cleodora* fall into two distinct groups, recognized here as subsections:

Croton subsection ***Sphaerogyni*** Caruzo, **subsect. nov.** – Aestivatio sepalis floribus femineis quincuncialis, sepalis saepe carnosis, fructibus globosis vel ellipsoideus. – Type: *Croton sphaerogynus* Baill.

Sepals of pistillate flowers with quincuncial aestivation, free or united only at the base; sepals usually fleshy at the base; disk usually segmented. Fruits globose or ellipsoid.

Species included here occur in eastern Brazil, with the exception of *Croton cajucara*, an Amazonian species occurring in Bolivia, Brazil, Peru, and Venezuela. Members of subsection *Sphaerogyni* typically occupy moist forests, except for *C. heterocalyx*, which occurs in seasonally dry forests and in "restinga" forests in eastern Brazil.

Species included: *Croton cajucara* Benth., *C. campanulatus* Caruzo & Cordeiro, *C. hemiargyreus* Müll.Arg., *C. heterocalyx* Baill., *C. organensis* Baill., *C. rufolepidotus* Caruzo & Riina, *C. salutaris* Casar., *C. sphaerogynus* Baill., *C. stellatoferrugineus* Caruzo and Cordeiro.

Croton rufolepidotus Caruzo and Riina, a recently described species from Antioquia, Colombia is a member of this subsection based on its similarity with *C. salutaris* (Caruzo et al., 2010b). Similarly, another recently described species, *C. stellatoferrugineus* Caruzo & Cordeiro (Caruzo et al., 2010a), fits the morphological definition of this subsection and therefore is included as well.

Croton subsection ***Spruceani*** Caruzo, **subsect. nov.** – Aestivatio sepalis floribus femineis imbricatis, sepalis non carnosis, fructibus subglobosis. – Type: *Croton spruceanus* Benth.

Sepals of pistillate flowers with imbricate aestivation, usually united at least half of their length; sepals not fleshy; disk entire. Fruits subglobose, usually trigonal.

Species of subsection *Spruceani* occur somewhat disjunctly in moist habitats of central and northwestern South America, Central America up to central Mexico, and then in semideciduous forests of eastern Bolivia, central Peru and Brazil (*Croton fragrantulus* and *C. rottlerifolius*).

Species included: *Croton billbergianus* Müll.Arg., *C. sexmetralis* Croizat, *C. fragrans* Kunth, *C. fragrantulus* Croizat, *C. hoffmannii* Müll.Arg., *C. orinocensis* Müll.Arg., *C. pseudofragrans* Croizat, *C. rottlerifolius* Baill., *C. spruceanus* Benth.

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Appendix A

Taxa, localities, vouchers, and GenBank accession numbers for all sequences analyzed. *Taxon*, Origin, *Voucher*, GenBank accession numbers: (ITS; *trnH-psbA*; *trnL-F*). Missing data: –.

Astraea lobata (L.) Klotzsch, BRAZIL, Bahia, *Van Ee 486* (WIS), (EU586945; –; –); *A. lobata*, BRAZIL, Bahia, *Van Ee 487* (WIS), (–; –; HM044768); *A. lobata*, PUERTO RICO, *Van Ee 549* (WIS), (–; HM044809; –); *Brasiliocroton mamoninha* P.E.Berry & Cordeiro, BRAZIL, Espirito Santo, *Pirani 4947* (SPF), (EU586944; HM044810; EU586998); *Croton alchorneicarpus* Croizat, BRAZIl, São Paulo, *Caruzo 71* (SP), (HM044788; –; HM044769); *C. alchorneicarpus*, BRAZIL, Rio de Janeiro, *Riina 1529* (WIS), (–; HM044811; –); *C. astroites* Dryand., PUERTO RICO, *Van Ee 537* (WIS), (EU586902; HM044812; EU586955); *C. billbergianus* Müll.Arg., COSTA RICA, Alajuela, *Van Ee 342* (WIS), (EU477998; HM044813; EU478148); *C. cajucara*, BRAZIL, *Caruzo 96* (SP), (HM044789; HM044814; HM044770); *C. campanulatus* Caruzo & Cordeiro, BRAZIL, Rio de Janeiro, *Caruzo 93* (SP), (HM044790; HM044815; HM044771); *C. cuneatus* Klotzsch, PERU, *Riina 1491* (WIS), (EU497735; HM044816; AY794698); *C. cupreatus* Croizat, ECUADOR, Pichincha, *Riina 1408* (WIS), (EU586919; HM044817; EU586974); *C. echioides* Baill., BRAZIL, Piauí, Carneiro-Torres *795* (HUEFS), (EU586907; HM044818; EU586967); *C. echinocarpus* Müll.Arg., BRAZIL, Minas Gerais, *Riina 1371* (WIS), (EU586922; –; EU586979); *C. echinocarpus*, BRAZIL, Rio de Janeiro, *Riina 1519* (WIS), (–; HM044819; –); *C. fragrans*, VENEZUELA, Cojedes, *Riina 1824* (MICH), (HM044791; HM044820; HM044772); *C. fragrantulus* Croizat, BOLIVIA, Santa Cruz, *Foster 424* (MO), (HM044791; HM044821; HM044773); *C. glandulosus* L., U.S.A., Wisconsin, *Van Ee 512* (WIS), (EU478066; HM044822; EU497713); *C. gnaphalii* Baill., ARGENTINA, Entre Rios, *Belgrano 423* (SI), (EU586940; HM044846; EU586994); *C. gracilipes* Baill., BOLIVIA, Santa Cruz, *Nee 47412* (NY), (EU586909; HM044823; EU586962); *C. hemiargyreus* Müll.Arg., BRAZIL, Minas Gerais, *Caruzo 114* (SP), (HM044793; HM044824; HM044774); *C. heterocalyx* Baill., BRAZIL, Bahia, *Caruzo 108* (SP), (HM044794; HM044825; HM044775); *C. hircinus* Vent., VENEZUELA, Caracas, *Riina 1291* (WIS), (EU477889; –; EU478127); *C. hirtus* L'Her., BRAZIL, *Lima 345* (SPF), (EU478070; –; EU478160); *C. hoffmannii* Müll.Arg., COSTA RICA, Cartago, *Van Ee 598* (WIS), (EF421773; HM044826; EF408111); *C. jacobinensis* Baill., BRAZIL, Bahia, *Carneiro-Torres 789* (HUEFS), (HM044795; HM044827; HM044776); *C. malambo* Karst., COLOMBIA, Bolivar, *Zarucchi 3856* (MO), (–; HM044828; –); *C. manampetsae* Leandri, MADAGASCAR, Toliar, *Van Ee 950* (MICH), (HM044796; HM044829; HM044777); *C. niveus* Jacq., COSTA RICA, Guanacaste, *Van Ee 284* (WIS), (EU478046; –; EU478155); *C. niveus*, MEXICO, Oaxaca, *León 52* (DAV), (–; HM044830; –); *C. nobilis* Baill., MADAGASCAR, Toliar, *Van Ee 938* (MICH), (HM044797; HM044831; HM044778); *C. organensis* Baill., BRAZIL, Rio de Janeiro, *Caruzo 90* (WIS), (EU586914; HM044832; EU586969); *C. orinocensis* Müll.Arg., VENEZUELA, Amazonas, *Riina*

1818 (MICH), (HM044799; HM044834; HM044779); *C. palanostigma* Klotzsch, PERU, Loreto, *Riina 1492* (WIS), (EU586943; HM044835; EU586997); *C. pallidulus* Baill., BRAZIl, São Paulo, *Caruzo 31* (SP), (EU586939; – EU586993); *C. piptocalyx* Müll.Arg., BRAZIL, São Paulo, *Caruzo 54* (SP), (EF421791; HM044836; –); *C. piptocalyx*, BRAZIL, São Paulo, *Riina 1533* (–; –; EF408148); *C. pseudofragrans* Croizat, PERU, Loreto, *Woodward s.n.* (MICH), (HM044800; HM044837; HM044780); *C. rottlerifolius* Baill., BRAZIL, São Paulo, *Caruzo 56* (SP), (–; HM044838; –); *C. rottlerifolius*, BRAZIL, São Paulo, *Riina 1534* (WIS), (HM044801; –; HM044781); *C. sacaquinha* Croizat, BRAZIL, Pará, *Caruzo 97* (SP), (HM044802; HM044839; HM044782); *C. salutaris* Casar., BRAZIl, Rio de Janeiro, *Caruzo 89* (SP), (HM044804; HM044840; HM044783); *C. sapiifolius* Müll.Arg., BRAZIL, Bahia, *Lima 667* (CEPEC), (EF421754; HM044841; EF408150); *C. schiedeanus* Schltdl., MEXICO, Yucatan, *Van Ee 458* (WIS), (EU478051; HM044842; EU478156); *C. sphaerogynus* Baill., BRAZIL, Rio de Janeiro, *Van Ee 505* (WIS), (HM044805; HM044843; HM044784); *C. spruceanus* Benth., BRAZIL, Pará, *Caruzo 101* (SP), (–; HM044844; HM044785); *C. spruceanus*, COLOMBIA, Valle del Cauca, *Baker 6493* (MO), (HM044806; –;–); *C. velutinus* Baill., BRAZIL, Bahia, *Carneiro-Torres 902* (HUEFS), (HM044807; –; HM044786); *C. trichotomus* Geiseler, MADAGASCAR, Toamasina, *Van Ee 991* (MICH), (HM044808; HM044845; HM044787); *C. yavitensis* Croizat, BOLIVIA, Beni, *Beck 5710* (LPB), (EU586918; HM044847; EU586973).

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