

Phylogeny of the Neotropical element of the *Randia* clade (Gardenieae, Rubiaceae, Gentianales)

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Background and aims – Generic limits of the tropical tribe Gardenieae (Ixoroideae, Rubiaceae) have partly remained unsettled. We produced a new phylogeny of the *Randia* clade, with emphasis on its Neotropical clade comprising five genera (*Casasia*, *Randia*, *Rosenbergiodendron*, *Sphinctanthus*, and *Tocoyena*). The result was subsequently used to evaluate and discuss: a) the respective monophyly of the above-mentioned genera and their interrelationships; b) relationships within *Tocoyena* and the evolutionary relevance of its subgeneric classification; and c) the monophyly of the morphologically variable *T. formosa*.

Material and methods – We examined the phylogeny of the *Randia* clade based on maximum likelihood and Bayesian analyses of sequence data from two nuclear (ETS and *Xdh*) and two plastid (*petB-petD* and *trnT-F*) DNA regions from 59 individuals (including seven representatives from the remaining Ixoroideae).

Key results – The Neotropical clade of the *Randia* clade comprises three major lineages, the *Randia armata* subclade, the *Randia-Casasia* subclade and the *Rosenbergiodendron* subclade. Neither *Casasia* nor *Randia* is monophyletic. *Tocoyena* is sister to *Rosenbergiodendron* + *Sphinctanthus* and is subdivided into three lineages: the *Tocoyena pittieri* group, the *Tocoyena guianensis* group, and the core *Tocoyena*. *Tocoyena williamsii* is paraphyletic with respect to *T. pittieri*. *Tocoyena formosa* is polyphyletic and should be re-circumscribed.

Conclusions – Our results demonstrate the monophyly of each of the relatively species-poor genera *Rosenbergiodendron*, *Sphinctanthus*, and *Tocoyena*, and confirm their close affinity. The serial classification of *Tocoyena* does not reflect the evolutionary history of the genus. The paraphyly of *T. williamsii* with respect to *T. pittieri*, together with their morphological similarities and geographic distributions, support the inclusion of the former in the latter. Our study calls for additional phylogenetic work on *Casasia* and the more species-rich genus *Randia*. While the respective monophyly of both genera is rejected here, future work with a broader representation of *Randia* is needed.

Keywords – *Casasia*; molecular phylogenetics; Neotropics; *Randia*; *Rosenbergiodendron*; *Sphinctanthus*; taxonomy; *Tocoyena*.

INTRODUCTION

The tribe Gardenieae DC. of the coffee family (Rubiaceae) was originally described by De Candolle (1830) to accommodate 33 morphologically diverse genera with large

flowers, many ovulate bilocular ovaries, and indehiscent fruits with a fleshy mesocarp. This broadly delimited Gardenieae was later shown to be highly polyphyletic, including genera currently placed in 13 tribes and three subfamilies of the Rubiaceae. The first infratribal classifications of the tribe

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(Schumann 1891, based on De Candolle's classification) is therefore untenable.

Gardenieae was, however, recognised in all tribal classifications of the subfamily Ixoroideae in the early Rubiaceae classifications of the 20th century (e.g. Verdcourt 1958; Bremekamp 1966), although its circumscription remained unsettled. The most comprehensive taxonomic study of Gardenieae was conducted by Robbrecht & Puff (1986), who proposed new delimitations and a new subtribal classification for the tribe, recognising two subtribes: a) Diplosporiineae, restricted to Africa and encompassing the genera *Argocoffeopsis* Lebrun, *Calycosiphonia* Pierre ex. Robbr., *Cremaspora* Benth., *Diplospora* DC., *Nostolachma* T.Durand, *Sericanthe* Robbr., and *Tricalysia* A.Rich. ex DC., and b) Gardeniineae. This latter subtribe was subdivided into three informal groups: the tetrad group (encompassing the African *Calochone* Keay, *Euclinia* Salisb., *Macrosphyra* Hook.f., *Oligocodon* Keay and *Preussiodora* Keay, and the Neotropical *Casasia* A.Rich. and *Randia* L.), the *Alibertia* group (comprising the Neotropical *Alibertia* A.Rich. ex DC., *Borojoa* Cuatrec., *Cordia* A.Rich. ex DC., *Sphinctanthus* Benth., and *Tocoyena* Aubl.), and the *Aidia* group (including African and Southwest Asian genera).

This subtribal classification of Gardenieae was subsequently adopted by Robbrecht (1988), and widely used until the phylogenetic studies of the subfamily Ixoroideae by Andreasen & Bremer (1996, 2000) revealed the polyphyly of Gardenieae sensu Robbrecht & Puff (1986). Andreasen & Bremer (2000) proposed a narrow circumscription of Gardenieae to include the members of the subtribe Gardeniinae, the genus *Duperrea* Pierre ex Pit. from the tribe Pavetteae, and the genus *Heinsenia* K.Schum. from the tribe Aulacocalyceae. Persson's first molecular phylogenetic study of Gardenieae (Persson 2000) further confirmed the polyphyly of Gardenieae sensu Robbrecht & Puff (1986), with the members of the subtribe Diplosporiinae closely related to the tribe Coffeae and transferred by Davis et al. (2007) to that tribe. Persson (2000) showed that the tetrad group sensu Robbrecht & Puff (1986) should in addition include some genera with pollen grains in monads (viz. *Rosenbergiodendron* Fagerl., *Sphinctanthus*, and *Tocoyena*) and denominated this lineage as the *Randia* clade. The *Randia* clade as defined by Persson (2000) comprised ten genera (*Calochone*, *Casasia*, *Euclinia*, *Macrosphyra*, *Oligocodon*, *Preussiodora*, *Randia*, *Rosenbergiodendron*, *Sphinctanthus*, and *Tocoyena*), a result that was endorsed by Gustafsson & Persson (2002).

Later, Kainulainen & Bremer (2014) and Mouly et al. (2014) showed that the African genus *Pleiocoryne* Rauschert also belongs to the *Randia* clade. Kainulainen & Bremer (2014) further revealed that *Euclinia* was biphyletic and described the new genus *Melanoxerus* Kainul. & B.Bremer to accommodate the Malagasy *Euclinia suavissima* (Homolle ex Cavaco) J.-F.Leroy. Since 2014, the *Randia* clade thus comprises 12 genera, Persson's (2000) 10 genera plus *Pleiocoryne* and *Melanoxerus*. Kainulainen & Bremer (2014) showed that the *Randia* clade comprises two geographically distinct sister groups, an African clade (*Calochone*, *Euclinia*, *Macrosphyra*, *Melanoxerus*, *Oligocodon*, *Pleiocoryne*, and *Preussiodora*) and a Neotropical clade (*Casasia*, *Randia*,

Rosenbergiodendron, *Sphinctanthus*, and *Tocoyena*). However, the informal name "African clade" is confusing, as this lineage also includes the Malagasy genus *Melanoxerus*. In the present study, this lineage is therefore named the Afro-Malagasy clade. In addition, some molecular phylogenetic studies (Gustafsson & Persson 2002; Kainulainen & Bremer 2014) have indicated that the genus *Randia* as delimited by Fagerlind (1943) may be non-monophyletic.

The Neotropical genus *Tocoyena* has always been classified in Gardenieae, ever since the first classification proposed by De Candolle (1830). The relationship of this genus to the remaining genera of the tribe only became clarified with the study by Persson (2000), whose results indicated that *Tocoyena* is closely related to *Rosenbergiodendron* and *Sphinctanthus* within the *Randia* clade. The close relationship between these three genera was confirmed by later studies (Gustafsson & Persson 2002; Kainulainen & Bremer 2014; Mouly et al. 2014). However, all these studies included only one (to two) representative of *Rosenbergiodendron*, *Sphinctanthus*, and *Tocoyena*, and were therefore not able to test the monophyly of these genera, nor Schumann's (1891) subgeneric division of *Tocoyena* into two series (*T. ser. Acutiflorae* and *T. ser. Obtusiflorae*). The infrageneric classification of *Tocoyena* by Schumann (1891) was based on the shape of the apices of corolla lobes in the floral buds: *T. ser. Acutiflorae* K.Schum. with acute corolla apex in bud and *T. ser. Obtusiflorae* K.Schum. with obtuse corolla apex in bud. *Tocoyena ser. Acutiflorae* was originally circumscribed to include *T. foetida* Poepp., *T. guianensis* K.Schum., and *T. longiflora* (Griseb.) K.Schum. (now *T. cubensis* Britton ex Standl., Standley 1921: 155), while *T. ser. Obtusiflorae* included *T. bullata* (Vell.) Mart. and *T. formosa* (Cham. & Schltdl.) K.Schum. (supplementary file 1). Prado (1987) adopted Schumann's (1891) serial classification but recognised it instead at sectional level: *T. sect. Acutiflorae* K.Schum., comprising seven species, and *T. sect. Tocoyena* (= *T. ser. Obtusiflorae* sensu Schumann 1891), comprising 12 species (supplementary file 1).

More recent studies of *Tocoyena* (i.e. Silberbauer-Gottsberger et al. 1992; Delprete 2008; Gottsberger et al. 2020) endorsed Schumann's (1891) principle for serial classification of *Tocoyena* and proposed new delimitations for some species recognised by Prado (1987). Silberbauer-Gottsberger et al. (1992) included 12 species in "species group A" (i.e. *T. ser. Obtusiflorae* sensu Schumann 1891): *T. brasiliensis* Mart., *T. bullata*, *T. costanensis* Steyerm., *T. formosa*, *T. hispidula* Standl., *T. longiflora* Aubl., *T. obliquinervia* (Standl.) Standl. (now *T. pittieri* (Standl.) Standl., Tropicos 2021), *T. pendulina* Spruce ex Standl., *T. pittieri*, *T. sellowiana* (Cham. & Schltdl.) K.Schum., *T. viscidula* Mart., and *T. williamsii* Standl. *Tocoyena arenicola* Delprete (Delprete 2008) and *T. prostrata* I.Silberb.-Gottsb. & Gottsb. were later added to the group (Gottsberger et al. 2020) (supplementary file 1). These studies do not treat "species group B" (i.e. *T. ser. Acutiflorae* sensu Schumann (1891)) in detail, but Silberbauer-Gottsberger et al. (1992) argue that it includes six species: *T. brevifolia* Steyerm., *T. foetida*, *T. guianensis*, *T. megistantha* Standl., *T. neglecta* N.E.Br. and *T. orinocensis* Standl. & Steyerm. (supplementary file 1). Geographically, *Tocoyena ser.*

Acutiflorae is restricted to the north of the Amazon, whereas *T. ser. Obtusiflorae* has most of its species occurring in the east of the Amazon basin (in savannahs and coastal seashore) (Silberbauer-Gottsberger et al. 1992).

Tocoyena formosa is a morphologically variable species distributed from Surinam to Paraguay (WCSP 2021). The monophyly of this species has yet to be assessed with molecular data. Silberbauer-Gottsberger et al. (1992) conducted a multivariate analysis of vegetative characters of *T. formosa* to investigate variation patterns in the species, and they recognised three varieties: *T. formosa* var. *formosa*, *T. formosa* var. *microdon*, and *T. formosa* var. *pseudobrasiliensis*. The authors argued that *T. formosa* is of hybrid origin with the three varieties being expressions of this (Silberbauer-Gottsberger et al. 1992; Gottsberger et al. 2020). By contrast, Delprete (2008: 986) considered *T. formosa* to be “a plastic species with polymorphic vegetative characters”, and employed the same delimitation proposed by Silberbauer-Gottsberger et al. (1992) but without any subspecific rank recognised. Delprete (2008) argued that while *T. formosa* varies considerably regarding leaf shape and surface morphology, even within populations, floral characters remain constant throughout its (extensive) geographic range.

The main goal of the present study was to produce a molecular phylogeny of the *Randia* clade based on sequence data from two nuclear and two plastid gene regions. The resulting phylogeny was subsequently used to assess: a) the monophyly of *Casasia*, *Rosenbergiodendron*, *Sphinctanthus*, and *Tocoyena*, respectively, and the phylogenetic relationships among the genera of the Neotropical clade of the *Randia* clade; b) the phylogenetic relationships within *Tocoyena* and the monophyly of its subgeneric series *T. ser. Acutiflorae* sensu Schumann (1891) and Silberbauer-Gottsberger et al. (1992), and *T. ser. Obtusiflorae* sensu Schumann (1891) and Gottsberger et al. (2020); and c) the monophyly of the geographically widespread and morphologically variable *T. formosa*. The taxonomic implications of our findings are presented.

MATERIAL AND METHODS

Taxon sampling

Taxa were selected using the *Randia* clade sensu Kainulainen & Bremer (2014) as a framework, with particular emphasis on its Neotropical diversity. For *Tocoyena*, we aimed to have an as dense representation as possible, including species from both the currently recognised subgeneric groups sensu Schumann (1891), Silberbauer-Gottsberger et al. (1992), and Gottsberger et al. (2020) (see also supplementary file 1). We analysed 59 samples, representing 46 species (and six samples undetermined to species level). Taxon names, voucher information, and GenBank accessions are given in supplementary file 2. Taxon authorities follow Tropicos (2021) and distributions are according to the World Checklist of Selected Plant Families (WCSP 2021). Herbarium acronyms follow Index Herbariorum (Thiers continuously updated). Most samples were selected as representatives of the focus of our study, i.e. the Neotropical genera of

the *Randia* clade. For *Casasia* (comprising 10 species, WCSP 2021), four samples representing three species were included. For *Randia* (comprising 112 species, WCSP 2021), 16 samples representing 10 species and four undetermined samples were included. For *Rosenbergiodendron* (comprising 4 species, WCSP 2021), five samples representing three species and one undetermined sample were included. For *Sphinctanthus* (comprising 9 species, WCSP 2021), three samples representing two species and one undetermined were included. For *Tocoyena* (comprising 21 species, WCSP 2021), 20 samples representing 17 species were included.

We further included one sample each from three of the African genera of the *Randia* clade (*Calochone*, *Euclinia*, and *Preussiodora*) and the Malagasy genus *Melanoxerus*. Four other genera circumscribed in Gardenieae (*Aulacocalyx* Hook.f., *Catunaregam* Wolf, *Rothmannia* Thunb., and *Schumanniphyton* Harms) were also sampled. As outgroup, we selected representatives of three Ixoroideae genera outside of the tribe Gardenieae (*Alberta* E.Mey. of the tribe Alberteae, *Ixora* L. of the tribe Ixoreae, and *Posoqueria* Aubl. of the tribe Posoquerieae) based on results in Kainulainen et al. (2013) and Wikström et al. (2015). The combined analyses and the plastid analyses were rooted on *Posoqueria*. Nuclear data were missing for *Posoqueria* so the analyses of the nuclear data were rooted on *Ixora* instead.

Whenever possible, we included the type species of the Neotropical genera of the *Randia* clade (i.e. *Casasia calophylla* A.Rich., *Randia aculeata* L., *Rosenbergiodendron formosum* (Jacq.) Fagerl., and *Tocoyena longiflora* Aubl.). No material of the type species of *Sphinctanthus*, *S. rupestris* Benth., was available for DNA analysis.

Molecular laboratory procedures and data assembly

Total DNA was extracted from silica gel or herbarium dried leaves following a CTAB protocol (Doyle & Doyle 1987; Doyle 1991), with subsequent purification using the QIAquick purification kit, according to the manufactory instructions (Qiagen, Hilden, Germany). Part of the nuclear ribosomal DNA region ETS, the low-copy nuclear *Xdh* gene, and the plastid regions *trnT-F* and *petB-petD* were amplified. Primer sequences used for amplification of the regions are described in table 1. Amplification was conducted using standard procedures (see e.g. Kainulainen & Bremer 2014) and the cleaned PCR products were sent to Macrogen Europe (Amsterdam, The Netherlands) for sequencing using the same primers used for amplification. New sequence data were assembled using the Staden package v.2.0.09b (Staden 1996; Staden et al. 2000). Newly produced sequences and those taken from GenBank (accessions are given in supplementary file 2) were aligned using MUSCLE (Edgar 2004), implemented in AliView v.1.18.1 (Larsson 2014).

Phylogenetic analyses

A total of 167 sequences were newly produced for the present study and analysed together with 14 sequences downloaded from GenBank. Phylogenetic analyses were performed on single DNA region datasets, combined datasets of plastid and nuclear regions, respectively, and on a dataset comprising all four regions (nuclear ETS and *Xdh* and plastid *petB-*

Table 1 – Primer information.

Region	Primer	Sequence (5'–3')	Reference
ETS	Erit-F	CTT GTA TGG GTT GGT TGG	Négron-Ortiz & Watson (2002)
	18S-E	GCA GGA TCA ACC AGG TAG CA	Baldwin & Markos (1998)
<i>Xdh</i>	Xdh 481F	CAT TGC TAC GGT CNT CTC AWG	Kainulainen & Bremer (2014)
	Xdh 1170F	GCY GGN ACA CAR ATA AGG AAT	Kainulainen & Bremer (2014)
	Xdh 1247R	TCC TGC AGC CAT CCA AAG A	Kainulainen & Bremer (2014)
	Xdh 1641R	CAT CTT CYT TCA RCA CAA TGT	Kainulainen & Bremer (2014)
<i>trnT-F</i>	trnT-F A1F	ACA GCG ATG CTC TAA CC	Bremer et al. (2002)
	trnT-F IR	CCA ACT CCA TTT GTT AGA AC	Bremer et al. (2002)
	trnT-F CF	CGA AAT CGG TAG ACG CTA CG	Taberlet et al. (1991)
	trnT-F EF	GGT TCA AGT CCC TCT ATC CC	Taberlet et al. (1991)
	trnT-F FR	ATT TGA ACT GGT GAG ACG AG	Taberlet et al. (1991)
<i>petB-petD</i>	1365F	TTG ACY CGT TTT TAT AGT TTA C	Löhne & Borsh (2004)
	738R	AAT TTA GCY CTT AAT ACA GG	Löhne & Borsh (2004)

Table 2 – Information on the DNA regions used in this study. *Each region had its own partition with substitution model as specified for the separate regions.

	ETS	<i>Xdh</i>	<i>petB-petD</i>	<i>trnT-F</i>	Nuclear	Plastid	Total
Number of samples	45	42	43	51	54	55	59
Length (bp)	473	1136	1013	1851	1609	2864	4473
Informative characters	199	108	33	87	307	120	427
Substitution model	GTR+ Γ	GTR+ Γ	HKY+I+ Γ	GTR+I+ Γ	*	*	*

petD and *trnT-F*), using Markov chain Monte Carlo methods (Yang & Rannala 1997; Larget & Simon 1999; Mau et al. 1999) as implemented in MrBayes v.3.2.6 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). Models of nucleotide substitution were selected for each dataset based on the Akaike information criterion (AIC), using MrModeltest (Nylander 2004). The best-fitting models are specified in table 2. MrBayes was run for 10^8 generations, sampling every 10 000 generations. The first 25% of the trees and parameters were discarded as burnin before Bayesian posterior probability values were calculated. Analytical convergence was verified using Tracer v.1.7.1 (Rambaut & Drummond 2007).

We also performed maximum likelihood analyses on all datasets using RAxML-HPC v.8.0 (Stamatakis 2014). Statistical support for the nodes was assessed based on 1000 bootstrap replicates. All analyses were run on the CIPRES Science Gateway v.3.3 (Miller et al. 2010). Based on information in the literature (Alfaro et al. 2003; Erixon et al. 2003), we have chosen to consider a Bayesian posterior probability (PP) ≥ 0.95 and/or a maximum likelihood bootstrap support (BS) ≥ 85 as indications of strong statistical support.

RESULTS

The combined dataset (nuclear and plastid data) contained 4473 base pairs (bp), of which 473 from nuclear ribosomal ETS, 1136 from the nuclear low-copy *Xdh*, 1013 from *petB-petD*, and 1851 from *trnT-F* (table 2). Of these 4473 bp, 427 (ca 10%) were phylogenetically informative (table 2). There are no incongruences between the results from the Bayesian analyses and maximum likelihood analyses. We show (and discuss) results based on the Bayesian analysis but mention the support values from both analyses (fig. 1). Results from the respective analyses of nuclear and plastid data are provided as supplementary files 3 and 4.

Results of the combined analysis of nuclear and plastid data

The *Randia* clade (PP/BS = 1/95) (fig. 1) comprised the Afro-Malagasy clade (0.98/84) sister to the Neotropical clade (1/95). Within the Neotropical clade, a well-supported *Randia armata* subclade (1/100) was sister to remaining species (0.54/-), within which the *Randia-Casasia* subclade (1/98) was sister to the *Rosenbergiodendron* subclade (1/99). *Randia* as delimited by Fagerlind (1943) was not monophyletic.

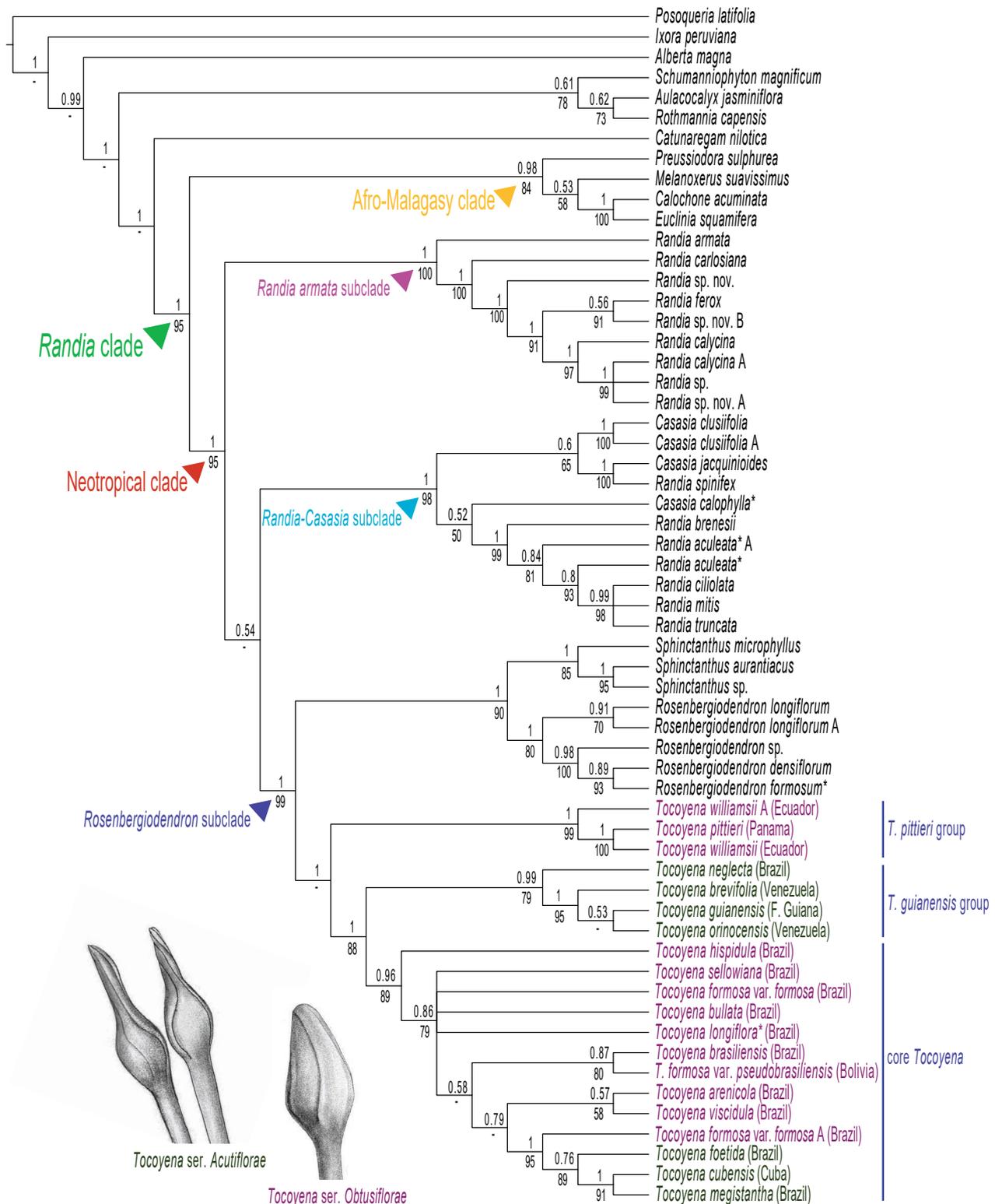


Figure 1 – Cladogram resulting from the Bayesian analysis of nuclear and plastid data (ETS, *Xdh*, *petB-petD*, and *trnT-F*) with support values (PP above and BS below the branches). BS < 50% are indicated by a hyphen (-). Taxa marked with an asterisk (*) denote type species of the genera in the Neotropical clade of the *Randia* clade. Clade names in colour are discussed in the text. The species included in the current (non-monophyletic) subgeneric classification of *Tocoyena* (i.e. *T.* ser. *Acutiflorae* and *T.* ser. *Obtusiflorae* sensu Schumann 1891, Silberbauer-Gottsberger et al. 1992, and Gottsberger et al. 2020) are indicated in green and purple, respectively, together with drawings that show the botanical basis for the serial division (following Schumann 1891). The countries from which the samples of *Tocoyena* were collected are indicated in parenthesis after the taxon name. Illustrations by Natanael Nascimento.

In the *Randia armata* subclade (fig. 1), *R. armata* (Sw.) DC. was sister to remaining sampled species (1/100), and the next diverging species was *R. carlosiana* K.Krause. The subclade also comprised *R. calycina* Cham., *R. ferox* (Cham. & Schltl.) DC., and several presumably new, yet undescribed, species. Results in the *Randia armata* subclade were generally well resolved and supported.

In the *Randia-Casasia* subclade (fig. 1), neither *Casasia* nor *Randia* was monophyletic. A clade (0.6/65) comprising *Casasia clusiifolia* (Jacq.) Urb. (1/100) plus the sisters *Casasia jacquinioides* (Griseb.) Standl. and *Randia spinifex* (Roem. & Schult.) Standl. (1/100) was sister to the remaining species of the subclade (0.52/50), which comprised the type species of *Casasia* (i.e. *C. calophylla*) and *Randia* (i.e. *R. aculeata*), as well as *Randia brenesii* Standl., *R. ciliolata* C.Wright, *R. mitis* L., and *R. truncata* Greenm. & C.H.Thomps.

In the *Rosenbergiodendron* subclade (fig. 1), *Sphinctanthus* (1/85) and *Rosenbergiodendron* (1/80) formed a monophyletic group (1/90) that was sister to *Tocoyena* (1/-). *Tocoyena* was in turn resolved in three main monophyletic groups: one comprising *T. pittieri* and *T. williamsii* (1/99) (hereafter the *Tocoyena pittieri* group), a second comprising *T. neglecta*, *T. brevifolia*, *T. guianensis*, and *T. orinocensis* (0.99/79) (hereafter the *Tocoyena guianensis* group), and a third group (0.96/89) containing the remaining sampled taxa of *Tocoyena* (hereafter the core *Tocoyena*). Within the *T. pittieri* group, *T. williamsii* was shown to be paraphyletic with respect to *T. pittieri*. Within the *T. guianensis* group, *T. neglecta* was resolved as sister to a clade formed by *T. brevifolia*, *T. guianensis*, and *T. orinocensis* (1/95). Within the core *Tocoyena*, the sampled specimens of *T. formosa* did not form a clade. The two samples representing *T. formosa* var. *formosa* were not resolved monophyletic and the sample representing *T. formosa* var. *pseudobrasiliensis* was sister to *T. brasiliensis*. In addition, neither *Tocoyena* ser. *Acutiflorae* nor *T. ser. Obtusiflorae* was monophyletic (fig. 1). The former was resolved as biphyletic and was nested within the latter.

Results of the analysis of nuclear data

In the analysis of the combined nuclear data (supplementary file 3), the *Randia* clade was supported (1/96). The Afro-Malagasy clade was collapsed with *Preussiodora* being sister to the remaining species of the *Randia* clade (0.67/58). Within the Neotropical clade (0.99/84), the *Randia-Casasia* subclade (1/96) was sister to a clade formed by the remaining sampled species (0.65/57), within which the *Randia armata* subclade (1/99) and the *Rosenbergiodendron* subclade (1/75) were sisters. This result contrasts the results of the combined dataset, where the *Randia armata* subclade was sister to remaining species of the Neotropical clade (fig. 1), but none of these alternative topologies were strongly supported. *Randia carlosiana* was included in the *Randia armata* subclade with strong support. Within the *Rosenbergiodendron* subclade, the *Tocoyena pittieri* group (comprising *T. pittieri* and *T. williamsii*) (1/93) was sister to remaining species (0.73/59). *Rosenbergiodendron* (1/97) and *Sphinctanthus* (1/91) were sisters (1/100), and this latter

group was sister to a clade formed by the remaining sampled species of *Tocoyena* (1/97).

Results of the analysis of plastid data

In the analysis of plastid data (supplementary file 4), the *Randia* clade was supported (1/92). The Afro-Malagasy clade was collapsed and relationships within the Neotropical clade (0.69/59) were poorly resolved. The relationships among the three subclades of the Neotropical clade were not resolved either. In addition, the *Randia armata* subclade was collapsed, with *Randia armata* having an unresolved position in the Neotropical clade. In contrast to the results of the nuclear data (supplementary file 3) and the combined data (fig. 1), *Randia carlosiana* was included in a (poorly supported) clade (0.52/-) that also included the taxa of the *Randia-Casasia* subclade. Relationships within the *Rosenbergiodendron* subclade (1/65) were poorly resolved as well.

DISCUSSION

The *Randia* clade, first identified by Persson (2000), now encompasses 12 genera (*Calochone*, *Casasia*, *Euclinia*, *Macrosphyra*, *Melanoxerus*, *Oligocodon*, *Pleiocoryne*, *Preussiodora*, *Randia*, *Rosenbergiodendron*, *Sphinctanthus*, and *Tocoyena*) from mainland Africa, Madagascar, and the Neotropics (Persson 2000; Kainulainen & Bremer 2014; Mouly et al. 2014). Results in Kainulainen & Bremer (2014) indicated that the *Randia* clade comprises an Afro-Malagasy clade and a Neotropical clade and our results support that (fig. 1). The members of the Afro-Malagasy clade are mostly scandent (Kainulainen & Bremer 2014, and references therein to floristic studies) and have pollen grains in tetrads (Keay 1958; Robbrecht & Puff 1986; Persson 1993).

The Neotropical clade

The Neotropical clade of the *Randia* clade is morphologically variable and thus difficult to define, but in contrast to the Afro-Malagasy clade, most species are shrubs or small trees (Kainulainen & Bremer 2014). Most taxa have, similar to those of the Afro-Malagasy clade, pollen grains in tetrads (Persson 1993, but see below on the *Rosenbergiodendron* clade and the genus *Randia*). The Neotropical clade comprises three major and well-supported lineages: the *Randia armata* subclade, the *Randia-Casasia* subclade, and the *Rosenbergiodendron* subclade (fig. 1). These groups were also indicated by Kainulainen & Bremer (2014), although with a smaller sampling. Our analyses do not provide a clear result regarding their interrelationships (fig. 1; supplementary files 3 and 4), and this phylogenetic question has proven to be difficult to answer in earlier studies as well. Their interrelationships are unresolved in older studies (Persson 2000; Gustafsson & Persson 2002) and poorly supported in more recent work (Kainulainen & Bremer 2014). Comparison with Mouly et al. (2014) is not possible because their study had a much broader aim (the Gardenieae complex), and the few representatives of the Neotropical clade included in their work do not form a monophyletic group.

However, and in line with indications in previous work (Gustafsson & Persson 2002; Kainulainen & Bremer 2014), our results show that the species of *Randia* as currently circumscribed (i.e. sensu Fagerlind 1943) fall into two subclades, with species of *Randia* included in the *Randia-Casasia* subclade being more closely related to *Casasia* than to the species of *Randia* in the *Randia armata* subclade (fig. 1). Pollen morphology provides interesting indications that species of *Randia* may have pollen in monads or tetrads (Persson 1993), but the distribution of these character states has not been studied within the entire genus and needs more research. The *Randia armata* and *Randia-Casasia* subclades (fig. 1) correspond, respectively, to the South American and the Central American *Randia* clades of Gustafsson & Persson (2002), but we have avoided these attributes because they are misleading. While species diversity in the *Randia armata* and *Randia-Casasia* subclades is centred in South America and Central America, respectively, several members of both subclades are not restricted to either of these regions.

The *Randia armata* subclade – The *Randia armata* subclade (fig. 1) has been indicated in previous work (Gustafsson & Persson 2002; Kainulainen & Bremer 2014), although the different sampling in our study prevents complete comparison. It is furthermore clear from our results as well as from those of Gustafsson & Persson (2002) that there is undescribed species diversity in the subclade. One potentially interesting, albeit unsupported, result is the conflicting position of *Randia carlosiana* from Ecuador. It is with strong support included in the *Randia armata* subclade based on the total evidence analysis (fig. 1) and the nuclear data (supplementary file 3). Based on the plastid data alone, however, it clusters with the species of the (unsupported) *Randia-Casasia* subclade (supplementary file 4).

The *Randia-Casasia* subclade – Our results provide strong support for non-monophyly of the genera *Casasia* and *Randia* (fig. 1). In Gustafsson & Persson (2002), *Casasia* was resolved as monophyletic when morphological characters were included in the analysis, but the authors discussed the lack of statistical support for this result. Lorence (1986) and Lorence & Dwyer (1987) discussed the morphological similarities of *Casasia* and *Randia*, and questioned whether the current generic status of *Casasia* could be maintained. Here, we find that the sampled species of *Casasia* fall within two clades (fig. 1), and both these clades include species of *Randia* as well. One of the clades includes (besides species of *Casasia*) *Randia spinifex*, a species not included in Gustafsson & Persson (2002). The *Randia-Casasia* subclade includes additional species of *Randia*, species with mostly (but not exclusively) Central American distributions. The type species of both genera (*Randia aculeata* and *Casasia calophylla*) belong to the *Randia-Casasia* subclade (fig. 1).

The *Rosenbergiodendron* subclade – Many studies have shown that the three genera *Tocoyena*, *Sphinctanthus*, and *Rosenbergiodendron* (fig. 2) form a clade (e.g. Persson 2000) with the latter two being sisters (Gustafsson & Persson 2002; Kainulainen & Bremer 2014; Mouly et al. 2014). These results were, however, based only on one sample from each of these genera (two from each genus in Gustafsson & Persson 2002). Here, using a greatly extended sampling of the *Rosenbergiodendron* subclade, we demonstrate the

monophyly of each of the genera *Tocoyena*, *Sphinctanthus*, and *Rosenbergiodendron*, and confirm that the former is sister to the latter two. Again, pollen morphology appears to reflect evolutionary relationships in the entire *Randia* clade; while most taxa in the *Randia* clade have pollen in tetrads, the species of the *Rosenbergiodendron* subclade have pollen in monads (Persson 1993; Gustafsson 1998).

Rosenbergiodendron is characterized by having short shoots with single terminal white flowers (fig. 2A) and fruits that are striped in green and pale/white at early developmental stages (fig. 2B) (Gustafsson 1998). *Sphinctanthus* is distinct by its creamy-white or yellow to brightly orange flowers (fig. 2C–D) and by having an annulus (ring of hairs) near the base of the corolla (Steyermark 1981; Delprete & Persson 2013). The genus *Tocoyena* (fig. 2E–G) has white to yellowish flowers with long corolla tubes and radiantly expanded lobes (fig. 2F) and corolla apices varying from acute to rounded, and globose to subglobose fruits (Silberbauer-Gottsberger et al. 1992; Delprete 2008).

Phylogenetic relationships within *Tocoyena* – Our results show that *Tocoyena* can be subdivided into three lineages: the *Tocoyena pittieri* group, the *Tocoyena guianensis* group, and the core *Tocoyena* (fig. 1). None of these groups has any obvious morphological synapomorphies.

Within the *T. pittieri* group, *T. williamsii* is paraphyletic with respect to *T. pittieri*, implying that their current delimitations are untenable. These species have large and glabrous leaves, turning black when dried, triangular, deciduous stipules, inflorescences thyriform bearing flowers up to 9 cm long, and 7 cm long berry fruits. We argue that the parphyly of *T. williamsii*, coupled with its morphological resemblance to *T. pittieri*, justifies its inclusion in the latter, which has priority over the former. The *T. pittieri* group is known from northwestern and western tropical South America (Colombia, Ecuador, and Peru) and Central America (Panama, Costa Rica, and Honduras), with *T. williamsii* also present in Bolivia and western Brazil. This group is sister to the remaining species of the genus with strong statistical support (fig. 1). It should be noted though, that the analysis based on nuclear data places the *T. pittieri* group as sister to the entire remaining *Rosenbergiodendron* subclade (albeit with poor statistical support).

The species in the *T. guianensis* group occur primarily in the Amazonian savannahs (*T. brevifolia*, *T. neglecta*, and *T. orinocensis*) and one (*T. guianensis*) in savannahs as well as in humid vegetation.

Phylogenetic relationships in the core *Tocoyena* are mostly poorly supported and need more research. This group has a wide distribution in tropical South America (ranging from Paraguay to Venezuela), with a single species on Cuba (*T. cubensis*), but has its centre of species diversity in Brazil. It has been questioned whether *T. cubensis* belongs in *Tocoyena* or perhaps in *Casasia* instead (Dwyer 1968: 449; Silberbauer-Gottsberger et al. 1992: 159), but our results clearly confirm its inclusion in *Tocoyena*; *Tocoyena* would be paraphyletic unless *T. cubensis* is included in the genus. It is also interesting to note that the sister relationship between *T. arenicola* and *T. viscidula*, although poorly supported, corroborates Delprete's (2008) interpretation based on

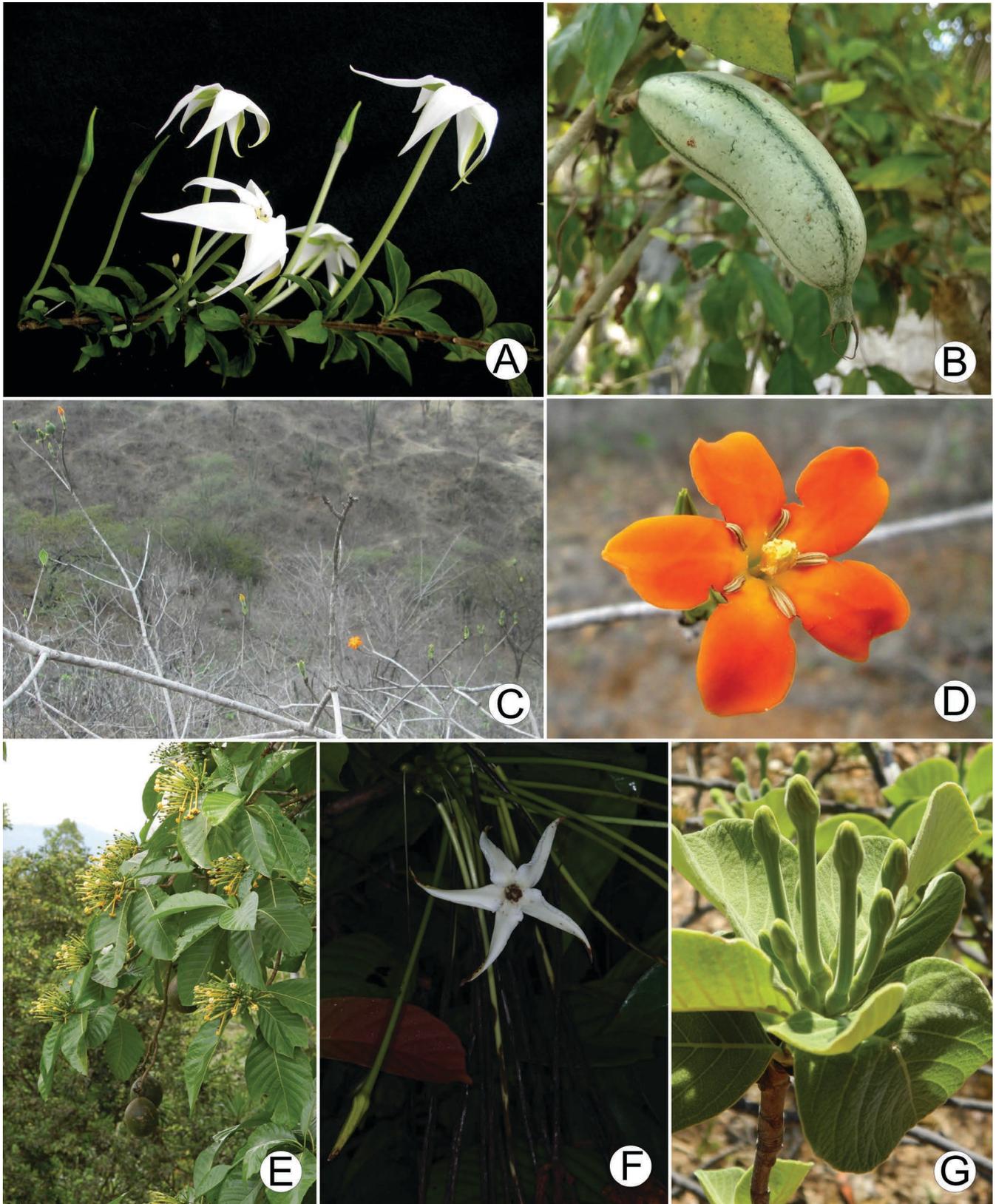


Figure 2 – Selected species of the *Rosenbergiodendron* clade. **A–B.** *Rosenbergiodendron formosum*, flowers (lateral view) and fruit. **C–D.** *Sphinctanthus aurantiacus*, habit and flower. **E.** *Tocoyena pittieri* (*T. pittieri* group), inflorescences, flowers, and fruits. **F.** *Tocoyena guianensis* (*T. guianensis* group) inflorescence and flower. **G.** *Tocoyena formosa* (core *Tocoyena*), inflorescence and flower buds, lateral view. Photographs by André Benedito (A), Eduardo Salazar (B), Jorge Zapata (C–D), Barry Hammel (E), Hervé Galliffet (F), and Rodrigo Borges (G).

morphology, of a close affinity between them. These species, which occur in white sandy vegetation in the Brazilian Cerrado, have collectors that copiously secrete blastocolla (Robbrecht 1988) on the internal surface of the stipules and small corollas. In addition, the here included samples of *T. formosa* were not resolved as monophyletic. We included two of the three varieties of *T. formosa* recognised in recent classificational work (Silberbauer-Gottsberger et al. 1992; Gottsberger et al. 2020). The two samples of *T. formosa* var. *formosa* (both from Brazil) did not group together, and *T. formosa* var. *pseudobrasiliensis* from Bolivia is sister to *T. brasiliensis*. This implies that the circumscription of the geographically widespread and morphologically variable *T. formosa* needs to be re-assessed, preferably based on molecular as well as morphological data from a large sample of specimens from the entire distribution area of the species.

Subgeneric classification of *Tocoyena* – The subgeneric series of *Tocoyena* as delimited by Schumann (1891) were based on the shape of the apices of corolla lobes in the floral buds (acute for *T. ser. Acutiflorae* and obtuse for *T. ser. Obtusiflorae*). Prado (1987), Silberbauer-Gottsberger et al. (1992), and Gottsberger et al. (2020) build on Schumann's (1891) classification and use the same character states to assign additional species to these two subgeneric groups. The utilization of the shape of the floral buds as a basis for the subdivision of *Tocoyena* is, however, not supported by our results (fig. 1). *Tocoyena ser. Acutiflorae* seems to be biphyletic and is nested within *T. ser. Obtusiflorae*. In other words, *T. ser. Acutiflorae* and *T. ser. Obtusiflorae* are mutually paraphyletic, and are thus not tenable.

Synonymization of *Tocoyena williamsii*

Tocoyena pittieri (Standl.) Standl. (Standley 1933: 151) (fig. 2E) – *Posoqueria pittieri* Standl. (Standley 1928: 167) – Type: PANAMA • Trinidad river, canal zone, collected near the hydrographic station on the Trinidad river; 17 May 1914; *H. Pittier 6635*; holotype: US[0037457]; isotypes: F[V0070145F], G[00436794], P[P03804097].

Tocoyena williamsii Standl. (Standley 1931: 349), **syn. nov.** – Type: PERU • Iquitos, Dept. Loreto; 120 m; 1 Apr. 1930; *L. Williams 8097*; holotype: F[V0041140F]; isotypes: F[V0041139F], G[00436792], K[K000424424], S[S05-848], US[00037475].

Tocoyena obliquinervia (Standl.) Standl. (Standley 1933: 152) – *Posoqueria obliquinervia* Standl. (Standley 1928: 167) – Type: COSTA RICA • Forests of Rio Naranjo; 200–250 m; Mar. 1893; *A. Tonduz 9528*; holotype: US[0037456].

Tocoyena cuatrecasii Steyerl. (Steyermark 1964: 113) – Type: COLOMBIA • Sucre, Cordillera oriental, Quebrada de la Calaña; 1000–1100 m; 6 Apr. 1940; fl.; *J. Cuatrecasas 9198*; holotype: US[00037462]; isotypes: COL[COL000004702, COL000004703].

CONCLUSIONS

Deep divergences in the Neotropical clade of the *Randia* clade have proven difficult to resolve. The relationships among its three major subclades are still unclear and there

are weak (unsupported) indications of possible cytonuclear discordance that should be further investigated in the future. Kainulainen & Bremer (2014) discuss that the reason for the difficulties in resolving relationships in the Neotropical *Randia* clade may be a paucity of sequence divergence in the group, possibly as a consequence of rapid (perhaps recent) radiation. If so, the remedy may be simple, i.e. to add massive amounts of molecular data produced by new sequencing techniques. To date, all studies of the *Randia* clade have been based on no more than a handful of molecular markers.

It is, however, now clear that the *Randia* clade comprises an Afro-Malagasy clade sister to a Neotropical clade, which in turn comprises three major subclades, the *Randia armata* subclade, the *Randia-Casasia* subclade, and the *Rosenbergiodendron* subclade. The *Randia armata* subclade appears, interestingly, to include yet undescribed species richness (also indicated in earlier work). Future field work in tropical South America may very well reveal additional currently unknown species diversity in this clade. The *Randia-Casasia* subclade comprises species of *Randia* and *Casasia* and the nomenclatural types of both genera. The monophyly of *Rosenbergiodendron*, *Sphinctanthus*, and *Tocoyena*, and the non-monophyly of *Casasia* and *Randia* are supported. The close affinity of the former three genera to each other is confirmed, with *Tocoyena* being sister to *Rosenbergiodendron* + *Sphinctanthus*. Species of *Tocoyena* fall into three major lineages, the *Tocoyena pittieri* group, the *Tocoyena guianensis* group, and the core *Tocoyena*. The former two lineages are sisters. These phylogenetic results are not congruent with the current subgeneric series *T. ser. Acutiflorae* and *T. ser. Obtusiflorae* as delimited by Schumann (1891), Silberbauer-Gottsberger et al. (1992), and Gottsberger et al. (2020). The paraphyly of *T. williamsii* with respect to *T. pittieri*, coupled with their morphological resemblance and similar geographic distribution, justify merging the latter in the former. Neither *T. formosa*, nor the variety *T. formosa* var. *formosa*, is monophyletic, and *Tocoyena formosa* var. *pseudobrasiliensis* is sister to another species (*T. brasiliensis*). The delimitation of *T. formosa* and its intraspecific variation should thus be further investigated.

Finally, the non-monophyly of *Randia*, clearly demonstrated in our results and indicated (but unsupported) in previous work with adequate sampling (Gustafsson & Persson 2002), calls for a taxonomic revision. One solution could be to employ a broader delimitation of *Randia* that includes all members of the Neotropical clade. This choice would, however, render *Randia* highly heterogeneous in morphology. Another option is to restrict *Randia* to only include (all) the members of the clade that contains the type species (*Casasia calophylla* and *Randia aculeata*), i.e. the *Randia-Casasia* clade (*Randia* L. has taxonomic priority over *Casasia* A.Rich.). The species of the *Randia armata* subclade would then need a new generic name. We find this scenario the better choice because the generic status of *Rosenbergiodendron*, *Sphinctanthus*, and *Tocoyena* could then be maintained. In addition, it renders *Randia sensu stricto* (= the *Randia-Casasia* subclade, fig. 1) as well as the new genus (= the *Randia armata* subclade, fig. 1) monophyletic and more morphologically and geographically homogeneous compared to a broadly circumscribed *Randia*. Gustafsson &

Persson (2002: 672) discussed the matter and stated that “no historical names of this group of species are available for revival”. However, one possibility worth exploring could be to resurrect the genus *Basanacantha* Hook.f. (Hooker 1873), although additional research is needed. For example, none of the originally described species of *Basanacantha* (i.e. *B. dioica* (H.Karst.) Hook.f., *B. hebecarpa* (Benth.) Hook.f., and *B. monantha* (Benth.) Hook.f.; Hooker 1873: 82–83) has been sequenced or otherwise investigated for phylogenetic purposes. For now, we continue to maintain Fagerlind’s (1943) circumscription of *Randia*, pending further studies.

SUPPLEMENTARY FILES

Supplementary file 1 – Summary of subgeneric classifications of *Tocoyena* (Gardenieae, Rubiaceae), selected information relevant for the present study.

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Supplementary file 2 – Taxon sampling, voucher information, and GenBank accessions.

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Supplementary file 3 – Cladogram resulting from the Bayesian analysis of nuclear data (ETS and *Xdh*) with support values (PP above and BS below the branches). BS < 50% are indicated by a hyphen (-). Taxa marked with an asterisk (*) denote type species of the genera in the Neotropical clade of the *Randia* clade. Clade names in colour are discussed in the text. Phylogram on the bottom left with indication of the major clades (in colour) and outgroup removed.

<https://doi.org/10.5091/plecevo.2021.1889.2575>

Supplementary file 4 – Cladogram resulting from the Bayesian analysis of plastid data (*petB-petD* and *trnT-F*) with support values (PP above and BS below the branches). BS < 50% are indicated by a hyphen (-). Taxa marked with an asterisk (*) denote type species of the genera in the Neotropical clade of the *Randia* clade. Clade names in colour are discussed in the text. Phylogram on the bottom left with indication of the major clades (in colour) and outgroup removed.

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