Botanical Journal of the Linnean Society, 2023, **203**, 253–270
https://doi.org/10.1093/botlinnean/boad023
Advance access publication 4 July 2023
Original Article

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Insights into the morphology and evolution of orbicules in the *Spermacoce* clade (Spermacoceae-Rubiaceae) and implications for systematics

Mariela Nuñez-Florentin[1,2,*], Brecht Verstraete[3], Roberto M. Salas[1,2], Steven Dessein[3]

[1]Instituto de Botánica del Nordeste, CONICET-UNNE, Sargento Cabral 2131, cc. 209, CP. 3400. Corrientes, Argentina
[2]Facultad de Ciencias Exactas, Naturales y Agrimensura, UNNE, Avenida Libertad 5470, C.P. 3400, Corrientes, Argentina
[3]Meise Botanic Garden, Nieuwelaan 38, 1860 Meise, Belgium

*Corresponding author. E-mail address: marielaflorentin22@gmail.com

ABSTRACT

The orbicules are tiny structures of sporopollenin that occur on the interior wall of anthers in several groups of plants, and they are associated with pollen grains and tapetal cells. Although their function remains still unresolved, they have been widely used in systematics. Rubiaceae is one of the most studied families in this aspect. However, scarce information is available about the orbicules in the tribe Spermacoceae, especially in the *Spermacoce* clade. The main objectives of this work were to investigate the occurrence, general morphology, evolution, and systematic utility of the orbicular characters in the *Spermacoce* clade. We investigated the presence/absence, size, abundance, shape, and ornamentation of orbicules in 104 specimens from 84 species in the *Spermacoce* clade using scanning electron microscopy (SEM). On the basis of these features, the ancestral state reconstruction analyses were performed on a custom-made phylogeny by stochastic character mapping. The orbicules are present in 38 (44%) of 84 analysed species, corresponding to 13 genera (56%) of the *Spermacoce* clade. Seven genera have orbicules in all analysed species, eight genera lack orbicules in all analysed species, and six genera show both presence and absence of orbicules. The absence of orbicules was estimated to be the ancestral state for the *Spermacoce* clade and the presence of orbicules evolved several times independently within Clade B. The absence of orbicules in the common ancestor of the *Spermacoce* clade can be seen as one of the independent losses that occurred during the evolutionary history of the Rubiaceae.

Keywords: ancestral state reconstruction; evolutionary trends; phylogenetic analysis; trait evolution; Ubisch bodies

INTRODUCTION

In flowering plants, pollen grains develop within the locules of the anthers and at anthesis, the mature pollen grains are released. On the inner surface of the locule wall, tiny granules may occur that are in close contact with the pollen grains. These granules are called orbicules (Erdtman *et al.* 1961) or Ubisch bodies (Rowley 1962), which develop simultaneously with the growing pollen exine, also composed of sporopollenin.

As a result, the exine ornamentation of the pollen grain and the orbicule wall often show strong similarities (El-Ghazaly and Jensen 1986, Hesse 1986). Studies carried out in Gentianaceae (Vinckier and Smets 2003) and Rubiaceae (Huysmans *et al.* 1997) report a high degree of correlation between orbicule ornamentation and the pollen grain sexine, i.e. the outer, sculptured layer of the exine. Given that the exine ornamentation of pollen grain offers useful characters for systematics, the taxonomic importance of orbicules was also considered for different taxa, such as Apocynaceae (Vinckier and Smets 2002a), Euphorbiaceae (El-Ghazaly and Chaudhary 1993), Gentianaceae (Vinckier and Smets 2003), Loganiaceae (Vinckier and Smets 2002b), Nartheciaceae (Merckx *et al.* 2008), Oxalidaceae (Rosenfeldt and Galati 2005, López and Rosenfeldt 2016), and Rubiaceae (e.g. Huysmans *et al.* 1997, Vinckier *et al.* 2000, Romero *et al.* 2017). According to those studies, orbicule morphology can be used to confirm, justify, or reject taxonomic hypotheses. The most recent studies (Moon 2018) estimate that orbicules are present in 123 of 150 angiosperm families, with at least one species per family investigated. The presence/absence of orbicules is remarkably constant at the family level: only 24

Received 4 January 2023; revised 28 March 2023; accepted 19 May 2023

angiosperm families have both positive and negative observations (Verstraete *et al.* 2014), the Rubiaceae family being one of them.

From a morphological and evolutionary point of view, orbicules of the Rubiaceae family are most intensively studied among angiosperms. The subfamilies Cinchonoideae *s.s.* and Ixoroideae *s.l.* have been widely explored to determinate the presence of orbicules (Huysmans *et al.* 1997, Vinckier *et al.* 2000, Dessein *et al.* 2005) resulting in the description of several orbicules types (Type I 'spiny orbicules', Type II 'microrugulate orbicules', Type III 'smooth orbicules', Type IV 'irregularly folded orbicules', Type V 'granular orbicules', and Type VI 'embedded orbicules'). These types can have taxonomic importance at tribal and generic level: for example, in Ixoroideae *s.l.*, the tribes Coffeeae, Ixoreae, Octotropideae, and Pavetteae are characterized by specific orbicule types (Huysmans *et al.* 1997, Vinckier *et al.* 2000, Verstraete *et al.* 2011). Although there are several studies that have investigated the orbicules of Rubiaceae, information about these structures is scarce within the Rubioideae subfamily, especially in the tribe Spermacoceae.

The tribe Spermacoceae (*sensu* Andersson and Rova (1999)) is the largest herbaceous lineage of Rubiaceae, with ~76 genera and >1000 species concentrated in the (sub)tropics, especially in the Neotropics (Groeninckx *et al.* 2009, Nuñez-Florentin *et al.* 2022). The tribe Spermacoceae *s.s.* (*sensu* Robbrecht 1988, according to the classical definition), or currently known as the *Spermacoce* clade (Kårehed *et al.* 2008), is a derived clade within Rubioideae and historically one of the most taxonomically complex and controversial groups within the family, especially at the generic level (Dessein 2003, Nuñez-Florentin *et al.* 2022). The *Spermacoce* clade is morphologically easily recognized as a group by the following combination of characters: herbaceous plants, presence of raphides, fimbriate stipules, uniovulate ovary locules, and pluri-aperturate pollen grains. In the Americas, the *Spermacoce* clade currently holds 23 genera, taking into account the recently described genus *Paganuccia* R.M.Salas (Nuñez-Florentin *et al.* 2022) and *c.* 250 species (Groeninckx *et al.* 2009).

As mentioned previously, little is known about orbicules in taxa of Rubioideae, especially in the tribe Spermacoceae and *Spermacoce* clade, with limited information about a few taxa scattered in literature. To date, there is only information for *Oldenlandia corymbosa* L., which lacks orbicules (Verstraete *et al.* 2011), while the presence of orbicules has been reported for *Spermacoce filituba* (K.Schum.) Verdc. (Verstraete *et al.* 2011) and *S. riparia* Cham. & Schltdl. (Galati 2003). A comprehensive survey of the morphology and evolution of orbicules in the *Spermacoce* clade could provide additional taxonomic informative characters to understand and untangle the systematics of this complex group.

The present study has the following aims: (i) to investigate the presence and absence of orbicules in 21 out of the 23 genera in the *Spermacoce* clade, covering almost all genera of the group; (ii) to describe the general orbicule morphology, and based on that information determinate which orbicular types are present; (iii) to compare the ornamentation of the orbicules with the sexine of the pollen grains; and (iv) to put the data in a phylogenetic context to investigate the evolution and systematic utility of the orbicular characters in the *Spermacoce* clade.

MATERIAL AND METHODS

Taxon sampling

This study is mainly based on herbarium material, supplemented with fixed material (flowers fixed in FAA, 5 mL formalin, 5 mL acetic acid, and 90 mL 50% ethanol). For morphological analysis, 104 specimens from 84 species in the *Spermacoce* clade and two specimens from the outgroup species *Bouvardia ternifolia* Cav. were selected (Appendix 1). Each genus is represented by at least the type species and three or more other species covering all different infrageneric classifications or subclades. In total, orbicules were surveyed in 21 of the 23 currently recognized genera of the *Spermacoce* clade.

Morphological analysis

Before observing orbicules using scanning electron microscopy (SEM), flowers of herbarium specimens were rehydrated with warm soapy water to simplify dissection. Next, for each specimen, all anthers from a single flower were removed, unless the flower was too small and processed altogether (this was the case for *Micrasepalum* Urb. and *Spermacoce* L.). The material was subsequently dehydrated in an ascending series of acetone, then dried using the critical-point method with CO_2, and mounted afterwards on aluminium stubs with double-sided adhesive tape. To make it easier to observe the orbicules, a longitudinal section of each locule was made using a razor blade and most of the pollen was gently removed using a needle. Stubs were sputter-coated with 20 nm gold-palladium (Denton Vacuum, Desk II, Pleasanton, EUA). Observations were performed at 20 kV with a SEM Jeol LV 5800 at the Electron Microscopy unit at the Universidad Nacional del Nordeste, and with a Carl Zeiss NTS-SUPRA 40 SEM at the Centro de Microscopía Avanzadas, Argentina. All measurements were performed on scanning electron microscopes using ImageJ software (Rasband 2020) for at least 20 orbicules per sample. The morphological terms follow Huysmans *et al.* (1997) and Verstraete *et al.* (2011). The orbicule typology is based on Huysmans *et al.* (1997) and Vinckier *et al.* (2000).

Phylogenetic assessment

From the 85 species used in the morphological analysis, a subset (56 species) was selected based on available genetic data, following the most up-to-date phylogenetic hypothesis for the *Spermacoce* clade from Nuñez-Florentin *et al.* (2022). The phylogenetic tree is based on six molecular markers (nuclear: ITS, ETS, and plastid: *atpB-rbcL*, *petD*, *rps16*, *trnL-trnF*) and comprises 55 ingroup taxa, representing 17 genera. Following previous studies, *Bouvardia ternifolia* was used as the outgroup (Salas *et al.* 2015; Miguel *et al.* 2018, Nuñez-Florentin *et al.* 2022). The Bayesian phylogenetic tree was inferred following the methodology detailed in Nuñez-Florentin *et al.* (2022).

Character coding

Five orbicular characters were studied, following the features previously analysed by Verstraete *et al.* (2011): (C1) presence of orbicules, (C2) size, (C3) abundance, (C4) shape, and (C5) ornamentation. The coding and assignment of states was based on our own analyses carried out from images obtained from the SEM, as previously specified.

Estimation of ancestral characters

The ancestral state of the analysed characters was estimated using Bayesian stochastic mapping (SM) (Huelsenbeck *et al.* 2003) in Phytools (Revell 2011) implemented in R (v.1.2.5033; R studio team 2019). Characters were mapped on the combined Bayesian tree (nuclear + plastid combined matrix). The Akaike information criterion (AIC) score was used to select a transitional model among the three available to recover the evolutionary history of the development patterns under investigation: a one-parameter equal rates model (ER), a symmetric model (SYM), and an all-rates different model (ARD). Stochastic character mapping was performed using the make.simmap function implemented in the R package Phytools, running 10 000 simulations. The posterior probability values of each state at each node were summarized on one of the resulting trees.

RESULTS

The presence or absence of orbicules (C1) was analysed in 85 species (including the outgroup), and, when present, variation in size (C2), abundance (C3), shape (C4), and ornamentation (C5) was also observed (Table 1). For each character, three evolutionary models were tested and the most suitable one was selected based on AIC (Table 2). A summary of the orbicule morphology of all of species studied can be found in Table 3. This table also includes a description of the pollen grains sexine (sexine pattern and sexine ornamentation) to compare the ornamentation of orbicules with the sexine of the pollen grains.

Presence

Orbicules are present (C1) in 38 (44%) of the 85 analysed species (Table 3), belonging to 13 (62%) studied genera of the *Spermacoce* clade: *Borreria* G.Mey., *Crusea* Cham. & Schltdl., *Denscantia* E.L.Cabral & Bacigalupo, *Hexasepalum* Bartl. ex DC., *Ernodea* Sw., *Hydrophylax* L.f., *Micrasepalum*, *Paganuccia*, *Planaltina* R.M.Salas & E.L.Cabral, *Psyllocarpus* Mart. & Zucc., *Richardia* L., *Spermacoce*, and *Tessiera* DC. (Figs 1, 2). Variations in orbicule size, density, shape, and ornamentation could be observed (Figs 1–8). The presence of orbicules was also checked for the remaining 46 species (belonging to the other eight genera) in the *Spermacoce* clade but they all lacked orbicules. The outgroup species *Bouvardia ternifolia* also has no orbicules. The inner locule wall shows the characteristic star-shaped endothecium thickenings, and other locule surface structures, but the tapetal membrane is completely smooth without any sporopollenin deposition (Fig. 1J–M).

The occurrence of orbicules in three species—*Borreria alata* (Aubl.) DC., *B. spinosa* Cham. & Schltdl. ex DC., and *Staelia catechosperma* K.Schum.—is difficult to determine. In these species, small structures present in low density and without distinguishable ornamentation are embedded in the tapetal tissue. Possibly, those structures represent artefacts as a result of the drying process of the material. For this reason, we scored orbicules as 'absent'.

When analysing the presence and absence of orbicules at generic level, we found that seven genera have orbicules in all analysed species (i.e. *Crusea*, *Ernodea*, *Hydrophylax*, *Micrasepalum*, *Paganuccia*, *Planaltina*, and *Tessiera*), eight genera lack orbicules in all analysed species (i.e. *Carajasia* R.M.Salas, E.L.Cabral & Dessein, *Diodia* L., *Emmeorhiza* Pohl ex Endl., *Galianthe* Griseb., *Mitracarpus* Zucc., *Schwendenera* K.Schum., *Staelia* Cham. & Schltdl., *Tobagoa* Urb.), and six genera show both presence and absence of orbicules (i.e. *Borreria*, *Denscantia*, *Hexasepalum*, *Psyllocarpus*, *Richardia*, and *Spermacoce*).

Size

The average size of the orbicules (C2) ranges from 0.13 to 2.30 µm. The smallest orbicules were found in both species of *Micrasepalum* (0.13–0.26 µm, Fig. 1H) and in *Paganuccia icatuensis* R.M.Salas (0.13–0.25 µm), whereas the largest appear

Table 1. Characters and characters states of orbicules as coded for the reconstruction of the ancestral character states.

Characters		Character states			
C1	Presence	Absent	Present		
C2	Size	Absent	Small	Medium	Large
C3	Abundance	Absent	Scattered	Abundant	Very abundant
C4	Shape	Absent	Rounded-oblate	Irregular-angular	
C5	Ornamentation	Absent	Psilate-rugose	Microechinate	

Table 2. Comparison of the Akaike information criterion (AIC) scores of different evolution models tested: 'Equal rates' (ER), 'Symmetric rates' (SYM), and 'All-rates different' (ARD). The lowest AIC score is in bold and the corresponding model was selected for the analyses.

Characters		ER	SYM	ARD
C1	Presence	72.57630	72.57630	73.66003
C2	Size	**111.3476**	111.6533	122.1109
C3	Abundance	**112.6341**	118.7946	128.5317
C4	Shape	**106.8540**	110.8215	115.7374
C5	Ornamentation	106.8540	**100.1885**	104.8103

Table 3. Occurrence and morphology of orbicules in the *Spermacoce* clade and outgroup. Abbreviations: a: abundant. eu: eutectate. g: granules. irr-ang: irregular-angular. me: microechinate. msp: microspines. O: orbicules. p: perforate. per-mic. perforate-microreticulate. psi-rug: psilate-rugose. ret: reticulate. s: scattered. rou-obl: rounded-oblate. sp: spines. va: very abundant. Size: min–(average)–max. Symbols: +, present; –, absent and ?, difficult to define orbicule type.

Taxon	Orbicules								Pollen		
	O	Size (μm)	Size	Abundance	Shape	Ornamentation	Fig.	Type	Sexine	Supratectal elements	Source
Borreria alata	-	-	-	-	-	-		-			
Borreria brachystemonoides	+	0.15-(0.22)-0.32	small	a	rou-obl	psi-rug		VI	per-mic	msp	Galati (1988), Pire (1996)
Borreria capitata	-	-	-	-	-	-		-			
Borreria dasycephala	-	-	-	-	-	-		-			
Borreria diacrodonta	+	0.22-(0.32)-0.56	small	s	rou-obl	psi-rug	1A	?	p	msp	Miguel *et al.* (2018)
Borreria diamantinae	+	0.6-(0.7)-0.86	medium	a	rou-obl	psi-rug		V	p	msp	Miguel (2016)
Borreria heteranthera	-	-	-	-	-	-		-			
Borreria latifolia	-	-	-	-	-	-	1J	-			
Borreria orientalis	-	-	-	-	-	-		-			
Borreria remota	+	0.18-(0.3)-0.47	small	s	rou-obl	psi-rug	1B	VI	p	msp	Miguel (2016)
Borreria schumannii	-	-	-	-	-	-		-			
Borreria spinosa	-	-	-	-	-	-		-			
Borreria suaveolens	+	0.15-(0.34)-0.5	small	a	irr-ang	psi-rug		V	per-mic	msp	Miguel (2016)
Borreria tenella	+	0.19-(0.31)-0.44	small	a	irr-ang	psi-rug		V	p	msp	Pire and Cabral (1992), Pire (1996)
Borreria verticillata	+	0.18-(0.34)-0.76	small	s	rou-obl	psi-rug		?	per-mic	msp	Pire (1996), Miguel (2016)
Carajasia cangae	-	-	-	-	-	-		-			
Crusea calocephala	+	0.4-(0.48)-0.6	medium	s	irr-ang	me	1C	I	p	msp	Unpubl. data
Crusea coccinea	+	0.47-(0.60)-0.84	medium	a	irr-ang	me		I	p	msp	(Anderson 1972), Arreguín Sanchez and al. (1995)
Crusea diversifolia	+	0.17-(0.21)-0.26	small	a	irr-ang	psi-rug		I	?	?	-
Crusea hispida	+	0.54-(0.73)-0.92	medium	a	irr-ang	me	1E	I	p	msp	Unpubl. data
Denscantia calcicola	+	0.25-(0.32)-0.43	small	a	irr-ang	psi-rug	1H	VI	p	g	Unpubl. data
Denscantia cymosa	-	-	-	-	-	-		-			
Denscantia macrobracteata	-	-	-	-	-	-		-			
Denscantia monodon	-	-	-	-	-	-		-			
Diodia kuntzei	-	-	-	-	-	-		-			
Diodia saponariifolia	-	-	-	-	-	-		-			
Diodia squamosa	-	-	-	-	-	-		-			
Diodia virginiana	-	-	-	-	-	-		-			
Emmeorhiza umbellata	-	-	-	-	-	-		-			
Ernodea littoralis	+	0.97-(1.31)-1.92	large	a	rou-obl	me	1D	I	p	msp	Dessein (2003)
Galianthe brasiliensis	-	-	-	-	-	-	1L	-			
Galianthe eupatorioides	-	-	-	-	-	-		-			
Galianthe fastigiata	-	-	-	-	-	-		-			
Galianthe grandifolia	-	-	-	-	-	-		-			
Galianthe palustris	-	-	-	-	-	-		-			

Table 3. Continued

Taxon	Orbicules								Pollen		
	O	Size (µm)	Size	Abundance	Shape	Ornamentation	Fig.	Type	Sexine	Supratectal elements	Source
Galianthe peruviana	-	-	-	-	-	-		-			
Galianthe spicata	-	-	-	-	-	-		-			
Hexasepalum angustifolium	-	-	-	-	-	-		-			
Hexasepalum apiculatum	-	-	-	-	-	-		-			
Hexasepalum gardneri	+	0.85-(1.25)-1.5	large	a	irr-ang	me	1F	I	p	msp	Cabaña Fader (2013)
Hexasepalum mello-barretoi	+	0.51-(0.74)-1.02	medium	va	rou-obl	psi-rug	1G	V	per-mic	sp	Cabaña Fader (2013)
Hexasepalum radula	-	-	-	-	-	-		-			
Hexasepalum sarmentosum	+	0.52-(0.76)-1.15	medium	a	irr-ang	me		I	p	msp	Cabaña Fader (2013)
Hexasepalum teres	-	-	-	-	-	-		-			
Hexasepalum vaginale	-	-	-	-	-	-		-			
Hexasepalum zappiae	-	-	-	-	-	-		-			
Hydrophylax maritima	+	0.79-(1.6)-2.92	large	va	rou-obl	psi-rug	1A	IIIb	p	msp	Puff (1986), Dessein (2003)
Micrasepalum eritrichoides	+	0.13-(0.19)-0.27	small	va	irr-ang	psi-rug	1H	V	p	msp	Nuñez Florentin *et al.* (2021)
Micrasepalum haitiense	+	0.14-(0.2)-0.26	small	va	irr-ang	psi-rug		V	p	msp	Nuñez Florentin *et al.* (2021)
Mitracarpus hirtus	-	-	-	-	-	-		-			
Mitracarpus megapotamicus	-	-	-	-	-	-		-			
Mitracarpus microspermus	-	-	-	-	-	-	1K	-			
Mitracarpus rigidifolius	-	-	-	-	-	-		-			
Mitracarpus robustus	-	-	-	-	-	-		-			
Paganuccia icatuensis	+	0.13-(0.20)-0.25	small	a	irr-ang	psi-rug		?	per-mic	msp	Nuñez Florentin *et al.* (2022)
Planaltina capitata	+	0.38-(0.66)-0.96	medium	va	irr-ang	me	1G	I	per-mic	msp	Salas and Cabral (2014)
Planaltina lanigera	+	0.33-(0.5)-0.66	medium	a	irr-ang	me		I	per-mic	msp	Salas and Cabral (2014)
Psyllocarpus asparagoides	+	0.34-(0.48)-0.75	small	a	irr-ang	psi-rug		?	eu	msp	Kirkbride (1979), Dessein (2003)
Psyllocarpus cururuensis	-	-	-	-	-	-		-			
Psyllocarpus laricoides	+	0.26-(0.31)-037	small	va	rou-obl	psi-rug	1B	IIIa	eu	msp	Kirkbride (1979)
Psyllocarpus phyllocephalus	+	0.29-(0.36)-0.47	small	va	rou-obl	psi-rug	1I	IIIa	eu	msp	Kirkbride (1979)
Richardia grandiflora	-	-	-	-	-	-		-			
Richardia humistrata	-	-	-	-	-	-		-			
Richardia scabra	-	-	-	-	-	-		-			
Richardia stellaris	+	0.25-(0.34)-0.52	small	a	rou-obl	psi-rug		V	p	sp	Pire (1997)
Schwendenera tetrapyxis	-	-	-	-	-	-		-			
Spermacoce affinis	+	0.39-(0.44)-0.54	small	va	rou-obl	psi-rug	1C	IIIa	p	msp	Florentín *et al.* (2016)
Spermacoce confusa	+	0.48-(0.56)-0.63	medium	a	rou-obl	psi-rug	1D	IIIa	eu	msp	Unpubl. data
Spermacoce dibrachiata	+	1.5-(1.9)-2.3	large	a	irr-ang	me	1E	I	p	msp	Dessein *et al.* (2002)
Spermacoce glabra	+	0.28-(0.45)-0.85	small	a	rou-obl	psi-rug		VI	eu	msp	Nuñez-Florentin *et al.* (2020)
Spermacoce hispida	+	0.78-(1.02)-1.32	large	va	rou-obl	psi-rug		V	per-mic	msp	Unpubl. data
Spermacoce prostrata	+	0.22-(0.31)-0.37	small	a	rou-obl	psi-rug		VI	p	msp	Pire (1996)

Table 3. Continued

Taxon	Orbicules								Pollen		
	O	Size (µm)	Size	Abundance	Shape	Ornamentation	Fig.	Type	Sexine	Supratectal elements	Source
Spermacoce pusilla	-	-	-	-	-	-		-			
Spermacoce riparia	+	0.42-(0.54)-0.74	medium	a	rou-obl	me	1F	I	eu	msp	Nuñez-Florentin *et al.* (2020)
Spermacoce sphaerostigma	+	1.21-(1.47)-1.6 3	large	a	rou-obl	me		I	p	msp	Dessein *et al.* (2002)
Spermacoce tenuior	+	0.36-(0.45)-0.55	small	a	rou-obl	me		I	p	msp	Nuñez-Florentin *et al.* (2020)
Spermacoce tetraqueta	+	0.28-(0.36)-0.53	small	a	rou-obl	psi-rug		V	eu	msp	Unpubl. data
Staelia catechosperma	-	-	-	-	-	-		-			
Staelia herzogii	-	-	-	-	-	-		-			
Staelia thymoides	-	-	-	-	-	-		-			
Staelia virgata	-	-	-	-	-	-		-			
Tessiera hexasepala	+	0.37-(0.62)-0.78	medium	a	irr-ang	psi-rug		?	ret	msp	Salas and Cabral (2014)
Tessiera lithospermoides	+	0.44-(0.79)-1.26	medium	a	irr-ang	psi-rug		?	per-mic	msp	Salas and Cabral (2014)
Tobagoa maleolens	-	-	-	-	-	-	1M	-			
	Outgroup										
Bouvardia ternifolia	-	-	-	-	-	-		-			

in *Spermacoce dibrachiata* Oliv. (1.5–2.3 µm, Fig. 1E). Therefore, the orbicules were grouped into three size classes to optimally represent most of the size variation: 'small' (Fig. 1A, B, H, I), orbicules with a mean size < 0.5 µm (e.g. *Borreria diacrodonta* L.M.Miguel & E.L.Cabral, Fig. 1A); 'medium' (Fig. 1C, F, G), orbicules with a size between 0.5 and 1 µm (e.g. *Hexasepalum mello-barretoi* (Standl.) J.H.Kirkbr. & Delprete, Fig. 1G); and 'large' (Fig. 1D, E), orbicules with a mean size > 1 µm (e.g. *Ernodea littoralis* Sw., Fig. 1D). Most of the analysed species have small orbicules (53%), while in 31 and 16% of the species with orbicules they are medium and large, respectively. All species of *Micrasepalum* and *Psyllocarpus* present small orbicules, the same occurs in *Tessiera* but with medium orbicules. The rest of the genera present variation in orbicules size among the species. For instance, the group called '*Spermacoce* America' is characterized by small and medium orbicules; whereas the '*Spermacoce* Africa + Asia' group presents all large orbicules.

Abundance

The orbicules are randomly dispersed on the inner locule wall and do not follow a particular configuration. Three categories are distinguished taking into account the abundance (C3): scattered (Fig. 1A–C), the orbicules are distributed in low density with the visible locule surface area larger than the surface covered by them (e.g. *Borreria remota* Bacigalupo & E.L.Cabral, Fig. 1C); abundant (Fig. 1D–F), the locule surface is well covered by the orbicules but some portions of the locule surface area still visible, while in others the orbicules are touching each other (e.g. *Spermacoce riparia*, Fig. 1F); and very abundant (Fig. 1G–I), the locule surface area is completely covered by individual orbicules being almost invisible due to their high density (e.g. *Psyllocarpus phyllocephalus* K.Schum., Fig. 1I). Most species analysed have abundant orbicules (66%). Except for *Micrasepalum* (all species with very abundant orbicules) and *Tessiera* (all species with abundant orbicules), the remaining genera exhibit a great variation in abundance of orbicules at infrageneric level e.g. *Crusea*, *Psyllocarpus*, *Spermacoce*, etc.

Shape

Two types of morphology can be defined (C4): rounded-oblate (Fig. 2A–D): orbicules that are rounded, more or less symmetrical in shape, or slightly flattened, with smooth edge (e.g. *Psyllocarpus laricoides* Mart. ex Mart. & Zucc.; Fig. 2B); and irregular-angular (Fig. 2E–H): orbicules that are asymmetrical or have a more or less triangular shape (e.g. *Hexasepalum gardneri* (K.Schum.) J.H.Kirkbr. & Delprete, Fig. 2F). The frequency of rounded-oblate and irregular-angular orbicules is similar, 53 and 47%, respectively. All species within *Crusea*, *Micrasepalum*, *Planaltina*, and *Tessiera*, present irregular-angular orbicules; most of the species of *Spermacoce* have rounded-oblate orbicules, except for *S. dibrachiata*. The remaining genera exhibit a great variation in orbicules shape at infrageneric level e.g. *Psyllocarpus*, *Borreria*.

Ornamentation

Two different character states are recognized according to the ornamentation (C5): psilate-rugose (Fig. 2A–D, H), the orbicules do not have a defined ornamentation pattern, being

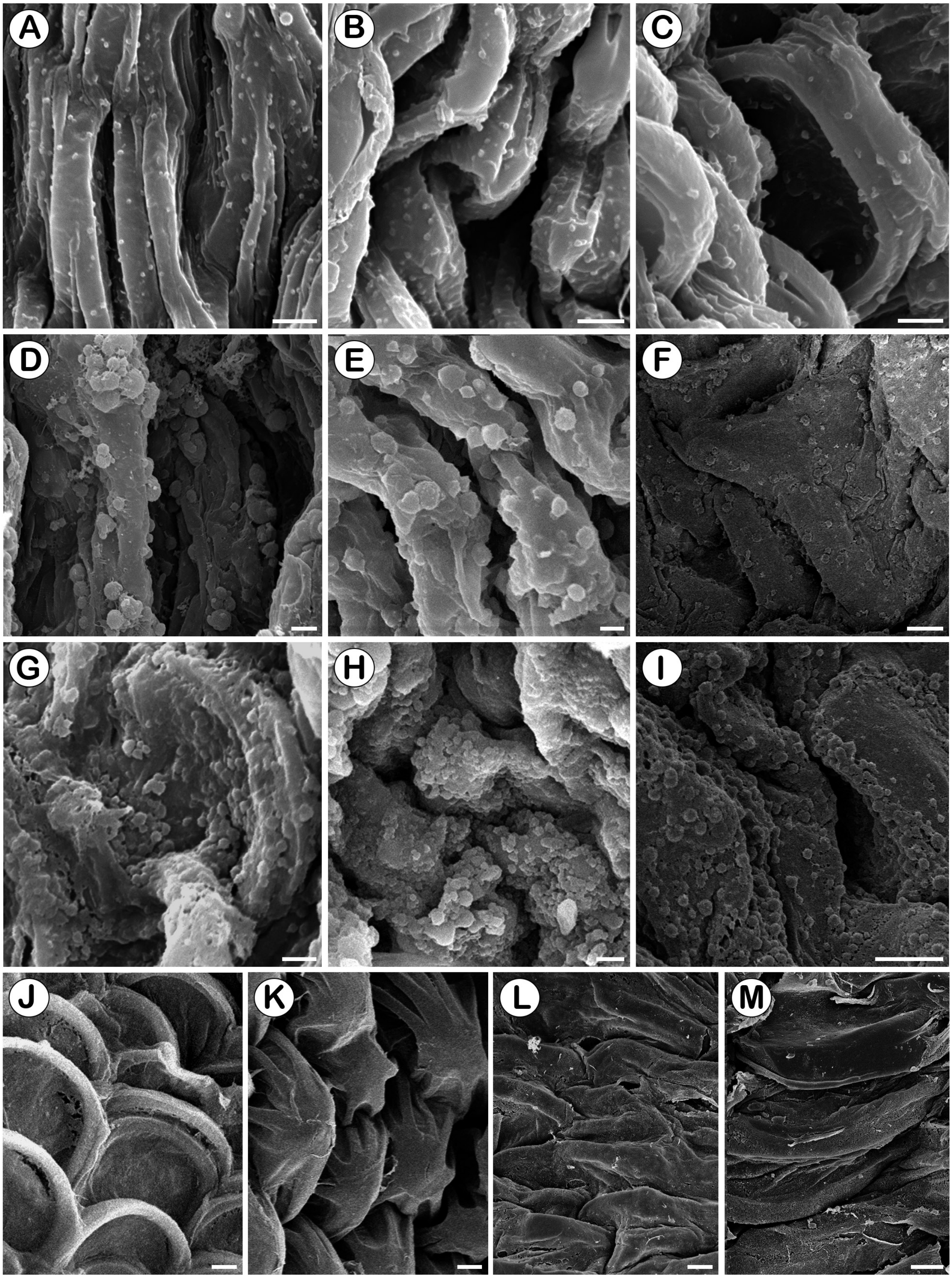

Figure 1. SEM pictures of orbicules in the *Spermacoce* clade showing presence/absence and variation in abundance and size. A–C, Orbicules scattered. D–F, Orbicules abundant. G–I, Orbicules very abundant. J–L, Orbicules absent. A, *Borreria diacrodonta*, small orbicules. B, *B. remota*, small orbicules. C, *Crusea calocephala*, medium orbicules. D, *Ernodea littoralis*, large orbicules. E, *Spermacoce dibrachiata*, large orbicules. F, *Spermacoce riparia*, medium orbicules. G, *Hexasepalum mello-barretoi*, medium orbicules. H, *Micrasepalum eritrichoides*, small orbicules. I, *Psyllocarpus phyllocephalus*, small orbicules. J, *Borreria latifolia*, note the ridges of the endothecium that form small loops. K, *Mitracarpus microspermus*¸ note the ridges of the endothecium. L, *Galianthe brasiliensis*, note the ridges of the endothecium. M, *Tobagoa maleolens*, note the ridges of the endothecium. Scale bars: 2 μm.

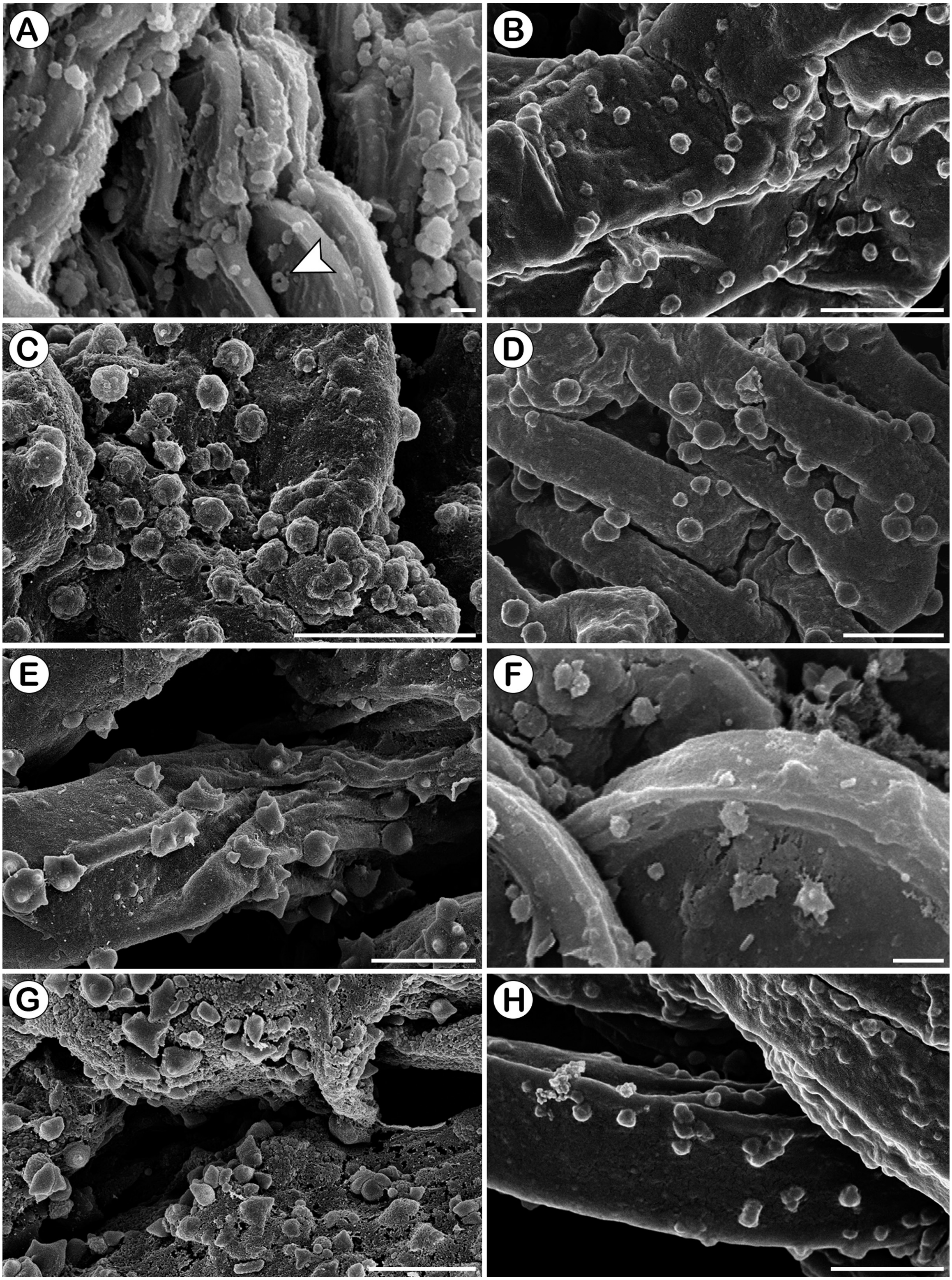

Figure 2. SEM pictures of orbicules in the *Spermacoce* clade showing variation in shape and ornamentation. A–D, Rounded-oblate orbicules. E–H, Irregular-angular orbicules. A, *Hydrophylax maritima*, psilate-rugose orbicule. Orbicules with central perforation were also observed (white arrow). B, *Psyllocarpus laricoides*, psilate-rugose orbicule. C, *Spermacoce affinis*, psilate-rugose orbicule. D, *Spermacoce confusa*, psilate-rugose orbicule. E, *Crusea hispida*, microechinate orbicule. F, *Hexasepalum gardneri*, microechinate orbicule. G, *Planaltina capitata*, microechinate orbicule. H, *Denscantia calcicola*, psilate-rugose orbicule. Scale bars: 2 µm.

principally smooth in appearance (e.g. *Spermacoce confusa* Rendle, Fig. 2D); and microechinate (Fig. 2E–G), when the orbicule wall presents one to several microspines on its surface (e.g. *Crusea hispida* (Mill.) B.L.Rob., Fig. 2E). A central perforation may be present in *Hydrophylax maritima* L. (Fig. 2A). Psilate-rugose orbicules dominate in the *Spermacoce* clade

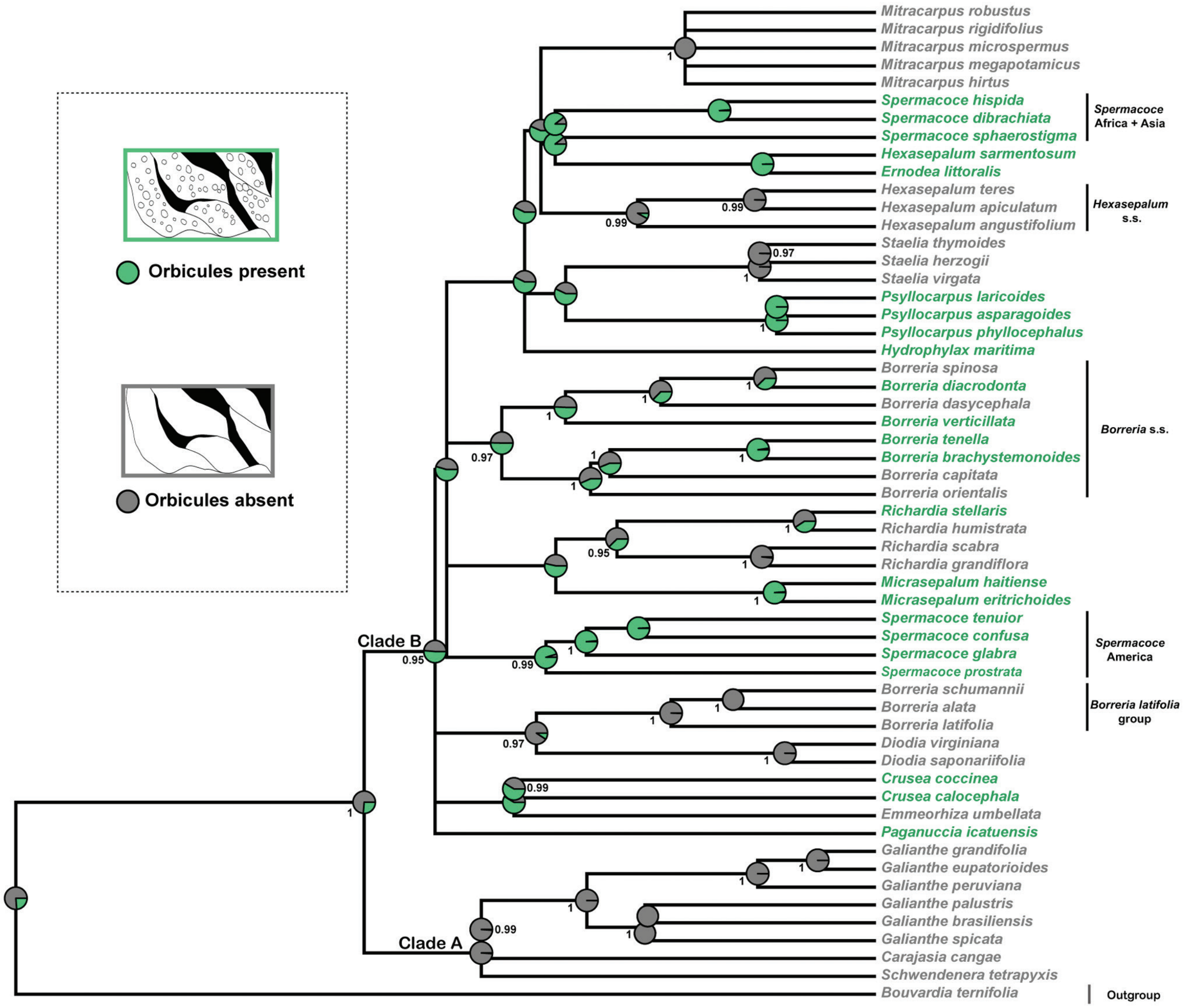

Figure 3. Ancestral character state estimates of presence and absence of orbicules (C1) by Bayesian stochastic mapping, based on the 50% majority-rule consensus tree obtained from the Bayesian analysis of the concatenated molecular datasets (ITS + ETS + *atpB-rbcL* + *petD* + *rps16* + *trnL-trnF*). Only probability values PP > 0.95 are shown at the nodes. Pie charts at the nodes indicate posterior ancestral state probabilities from the stochastic mapping and tip label colours represent tip states. Vertical lines indicate informal groups recognized within the *Spermacoce* clade.

occurring in 68% of the species with orbicules. Some genera, such as *Borreria*, *Micrasepalum*, and *Psyllocarpus*, are characterized by the presence of psilate-rugose orbicules; whereas *Spermacoce*, including both groups—*Spermacoce* America and *Spermacoe* Africa + Asia—present a great variation in orbicules ornamentation among their species.

Orbicule types

On the basis of the morphological characters analysed, especially shape and ornamentation, the orbicule types (Huysmans *et al*. 1997, Vinckier *et al*. 2000) that can be distinguished in the *Spermacoce* clade are as follows (Table 3):

Type I spiny orbicules: orbicules up to 3 μm in diameter, not embedded in the tapetal membrane, which are characterized by having few to numerous microspines, resulting in their often irregular shape. Perforations may occur, but they have not been observed in this type of orbicules. This type includes 13 species, for example: *Crusea calocephala* DC. (Fig. 1C), *C. coccinea* DC., *Hexasepalum gardneri* (Fig. 2F), *Spermacoce dibrachiata* (Fig. 1E), and *Planaltina capitata* (K.Schum.) R.M.Salas & E.L.Cabral (Fig. 2G).

Type III smooth orbicules: orbicules <1.4 μm in diameter, psilate orbicular wall, rarely embedded in the tapetal membrane. Aggregates of two or more orbicules can occur. Within this type, the subtype type IIIa can be found in some species, such as *Psyllocarpus laricoides* (Fig. 2B) and *Spermacoce affinis* (DC.) R.M.Salas (Fig. 2C), characterized by rounded, oblate orbicules. *Hydrophylax maritima* (Fig. 2A) is an example of subtype IIIb in which orbicules are similar to subtype IIIa but more circular and flattened with a central perforation.

Type V granular orbicules: orbicules are more or less flattened and irregular, not embedded in tapetal membrane. Nine species have this type, such as *Borreria tenella* (Kunth) Cham. & Schltdl., *Micrasepalum eritrichoides* (C.Wright ex Griseb.) Urb. (Fig. 1H), and *Spermacoce tetraquetra* A.Rich.

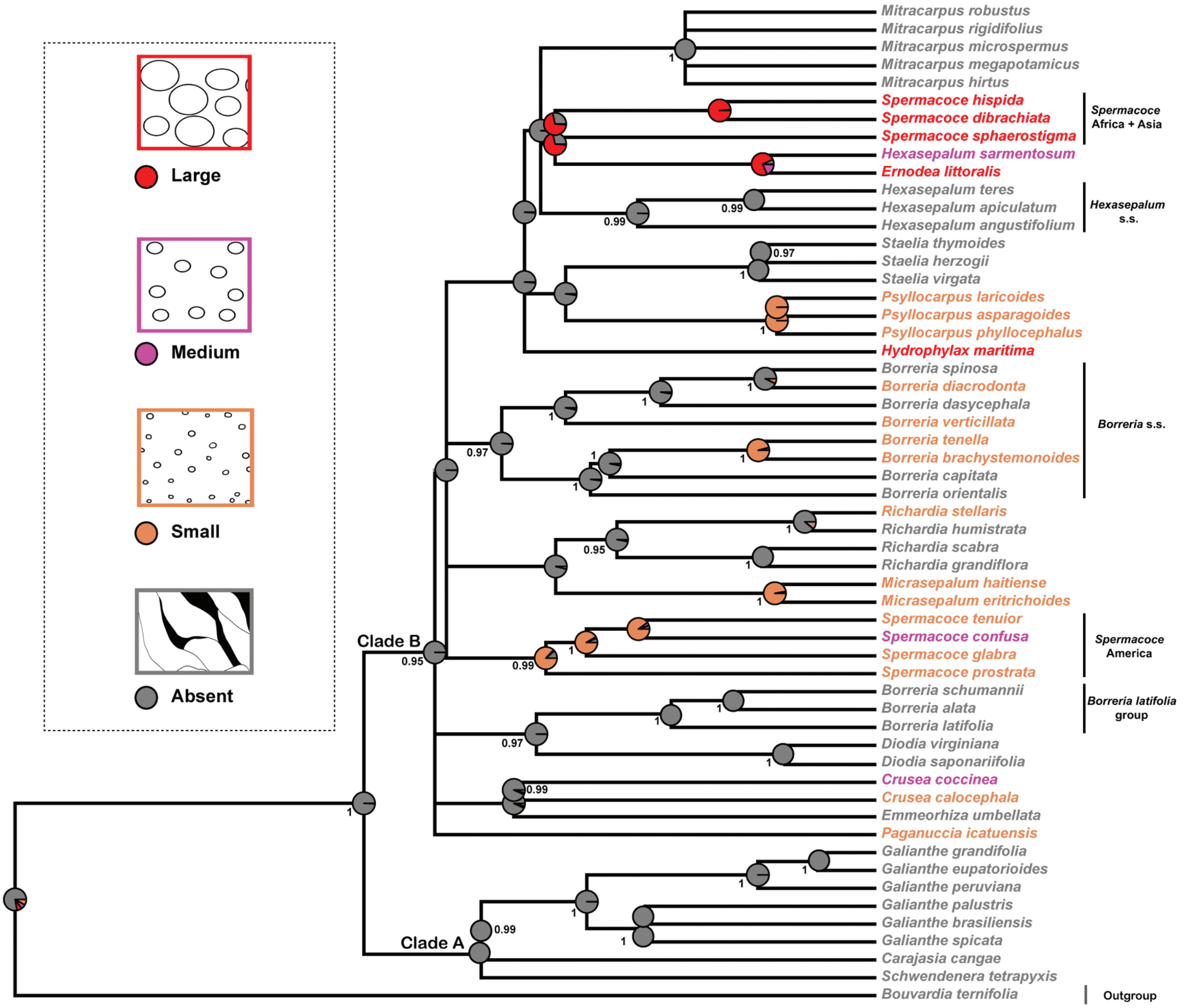

Figure 4. Ancestral character state estimates of size of orbicules (C2) by Bayesian stochastic mapping, based on the 50% majority-rule consensus tree obtained from the Bayesian analysis of the concatenated molecular datasets (ITS + ETS + *atpB- rbcL* + *petD* + *rps16* + *trnL-trnF*). Only probability values PP > 0.95 are shown at the nodes. Pie charts at the nodes indicate posterior ancestral state probabilities from the stochastic mapping and tip label colours represent tip states. Vertical lines indicate informal groups recognized within the *Spermacoce* clade.

Type VI embedded orbicules: orbicules small (0.5–1.6 µm), more or less flattened, embedded in the tapetal membrane, regular or irregular in shape, rough-surfaced often with sporopollenin granules. This group contains *Borreria brachystemonoides* Cham. & Schltdl., *B. remota* (Fig. 1B), *Denscantia calcicola* R.M.Salas & E.L.Cabral (Fig. 2H), *Spermacoce prostrata* Aubl., and *S. glabra* Michx.

Some species, such as *Borreria verticillata* (L.) G.Mey., *Paganuccia icatuensis*, *Psyllocarpus asparagoides* Mart. ex Mart. & Zucc., among others, are difficult to categorize in one of these types due to their intermediate character states.

Relationship between orbicule ornamentation–pollen sexine

In those species that have orbicules, we investigated the sexine of the pollen grain (Table 3). The sexine pattern and ornamentation in relation to orbicule ornamentation is shown in Figure 7. Of all species with orbicules, *c.* 31.5% (12 species) have microechinate orbicules and these species have pollen grains with microspines as supratectal elements, but with differences in their sexine patterns: most of them have a perforate sexine (e.g. *Crusea* species, *Hexasepalum gardneri*, *Spermacoce dibrachiata*, *S. tenuior*), but a perforate-microreticulate sexine (*Planaltina capitata* and *P. lanigera* (DC.) R.M.Salas & E.L.Cabral) is also present, as well as an eutectate sexine (*Spermacoce riparia*).

Species with psilate-rugose orbicules (26 species) can, however, also have pollen grains with microspines as supratectal elements (*c.* 22 species, e.g. *Borreria tenella*, *Psyllocarpus* species, *Tessiera* species), meaning that the relationship between microspines on pollen grains and microechinate orbicules is not absolute.

Species with psilate-rugose orbicules can also present spines and granules as supratectal elements in the pollen grain ornamentation (spines: *Hexasepalum mello-barretoi*, *Richardia stellaris* (Cham. & Schltdl.) Steud.; granules: *Denscantia calcicola*).

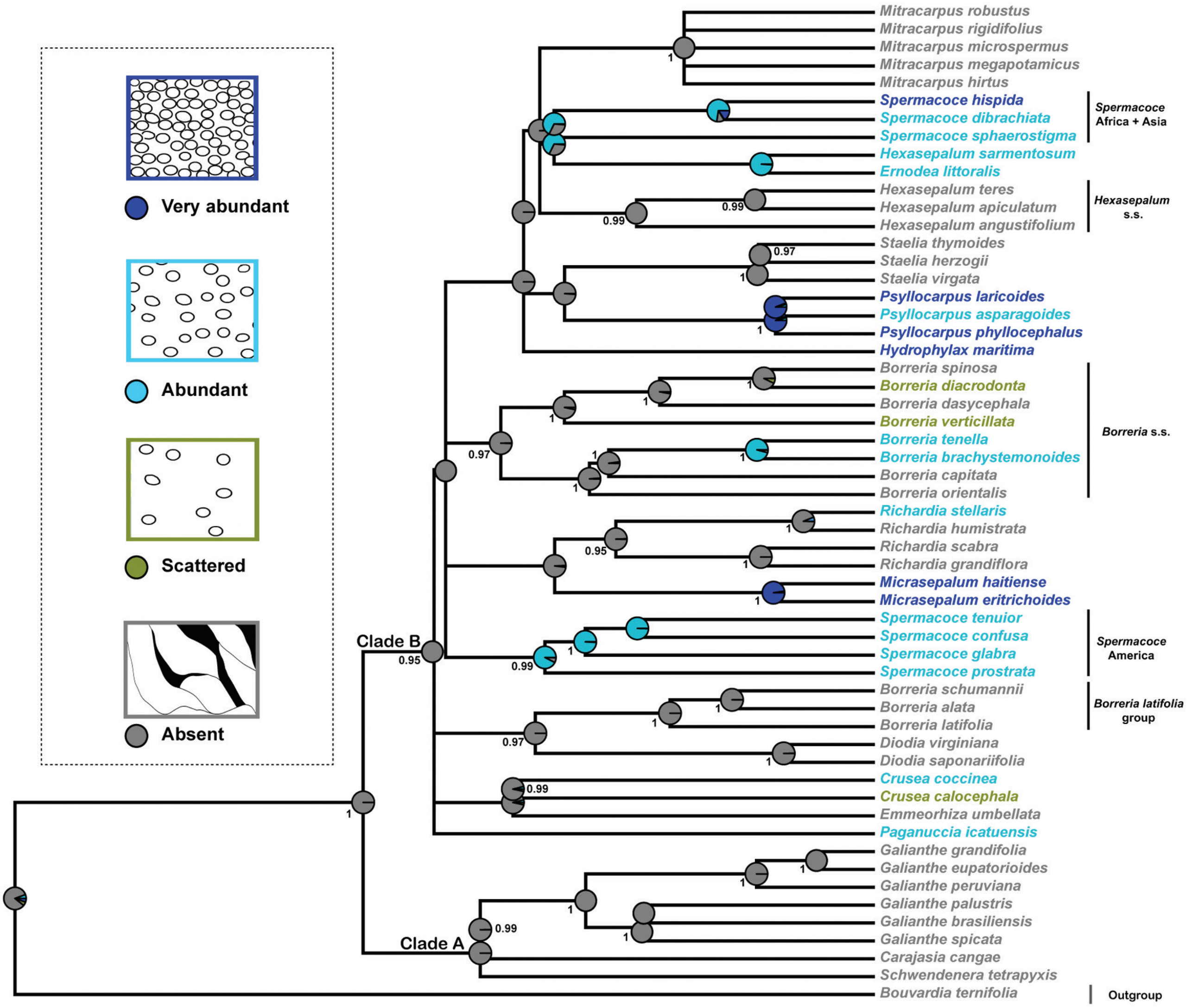

Figure 5. Ancestral character state estimates of abundance of orbicules (C3) by Bayesian stochastic mapping, based on the 50% majority-rule consensus tree obtained from the Bayesian analysis of the concatenated molecular datasets (ITS + ETS + *atpB- rbcL* + *petD* + *rps16* + *trnL-trnF*). Only probability values PP > 0.95 are shown at the nodes. Pie charts at tree nodes indicate posterior ancestral state probabilities from the stochastic mapping and tip label colours represent tip states. Vertical lines indicate informal groups recognized within the *Spermacoce* clade.

Evolution of orbicule characters

The absence of orbicules was estimated to be ancestral state for the *Spermacoce* clade (Fig. 3) and the presence of orbicules evolved several times independently within Clade B. Clade A is characterized by the absence of orbicules.

Concerning the size of orbicules, when orbicules are present, they are mostly small (Fig. 4). In the subclade '*Spermacoce* America', the common ancestor and most of the species are estimated to have small orbicules, but there is a shift to medium orbicules in *Spermacoce confusa*. The common ancestor of *Hexasepalum sarmentosum* (Sw.) Delprete & J.H. Kirkbr. and *Ernodea littoralis* is estimated to have large orbicules with a shift to medium orbicules in *H. sarmentosum*. Within the subclade '*Borreria s.s*'., small orbicules appeared at least three times independently.

The very abundant orbicules (Fig. 5) evolved at least five times independently in *Hydrophylax maritima*, the common ancestor of *Micrasepalum*, the common ancestor of *Psyllocarpus*, and in *Spermacoce hispida* L. Within *Psyllocarpus*, there has been a shift to abundant orbicules in *P. asparagoides*. The common ancestor of the group '*Spermacoce* Africa + Asia' is estimated to have abundant orbicules, with a transition to very abundant orbicules in *S. hispida*. Within the subclade '*Borreria s.s*'., the common ancestor of *B. brachystemonoides* and *B. tenella* (Kunth) Cham. & Schltdl. is estimated to have abundant orbicules, while the scattered orbicules evolved independently at least two times, in *B. diacrodonta* and in *B. verticillata*.

Regarding the shape of orbicules (Fig. 6), the irregular-angular and rounded-oblate orbicules evolved several times independently through the evolutionary history of the *Spermacoce* clade. The common ancestor of the *Micrasepalum* species is estimated to have irregular-angular orbicules with a 100% of probability, while the common ancestor of the subclade '*Spermacoce* America' is estimated to have rounded-oblate orbicules with 95% probability. A similar situation occurs in the common ancestor of the *Psyllocarpus* species, but there is a shift to irregular-angular orbicules in *P. asparagoides*.

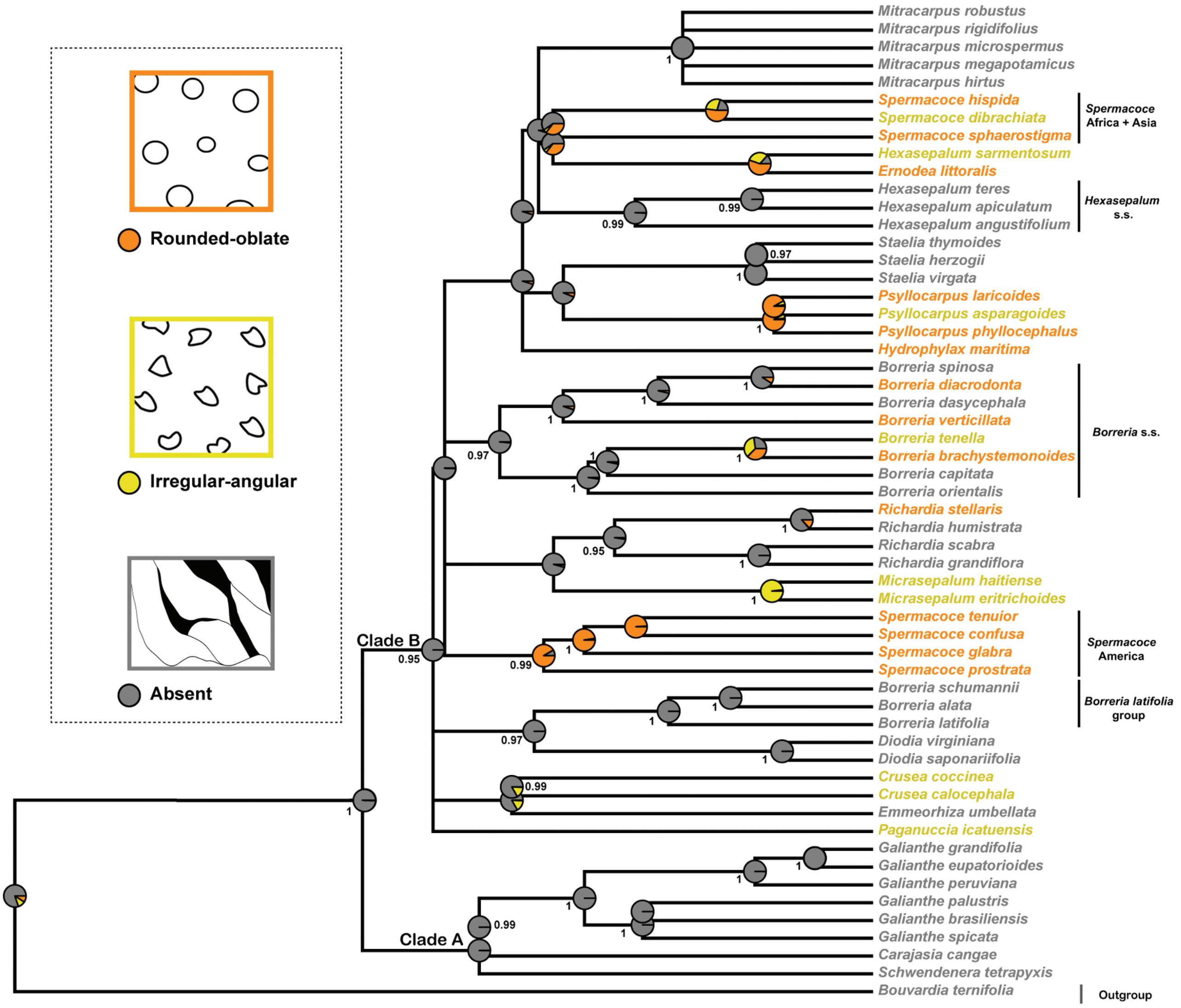

Figure 6. Ancestral character state estimates of shape of orbicules (C4) by Bayesian stochastic mapping, based on the 50% majority-rule consensus tree obtained from the Bayesian analysis of the concatenated molecular datasets (ITS + ETS + *atpB- rbcL* + *petD* + *rps16* + *trnL-trnF*). Only probability values PP > 0.95 are shown at the nodes. Pie charts at the nodes indicate posterior ancestral state probabilities from the stochastic mapping and tip label colours represent tip states. Vertical lines indicate informal groups recognized within the *Spermacoce* clade.

When orbicules are present, they are mostly psilate-rugose in ornamentation (Fig. 7). Microechinate orbicules evolved at least four times independently, in *Crusea calocephala*, *C. coccinea*, *Spermacoce tenuior* L., and the common ancestor of *Ernodea littoralis*, *Hexasepalum sarmentosum*, and the group '*Spermacoce* Africa + Asia'. Within the latter group, there is a shift to psilate-rugose orbicules in *Spermacoce hispida*. On the contrary, the common ancestor of the subclade '*Spermacoce* America' is estimated to have psilate-rugose orbicules, with a shift to microechinate orbicules in *Spermacoce tenuior*.

DISCUSSION

The idea by Huysmans *et al.* (2003) that all herbaceous Rubiaceae might lack orbicules is in conflict with the observation of orbicules in some Spermacoceae species. The Spermacoceae tribe is the largest herbaceous lineage of Rubiaceae and one of the derived clades in the Rubioideae subfamily, but it is one of the less investigated groups with respect to the presence and morphology of orbicules. The present study analysed the presence and morphology of orbicules in 84 species of the *Spermacoce* clade, of which 38 species (representing 13 genera) were found to have orbicules (Table 3).

Morphology of the orbicules in the Spermacoce clade

The presence of orbicules has been confirmed for 38 species of the *Spermacoce* clade. A summary of the orbicular morphological variation studied in a phylogenetic context is given in Figure 8. In most species, the orbicules are small, abundant, rounded-oblate in shape, and have a psilate-rugose ornamentation. For some genera, the orbicular characters are similar for all species. *Crusea* species have irregular-angular orbicules of medium size and with microechinate ornamentation, except for *C. diversifolia* W.R.Anderson, which is the only species with small, psilate-rugose orbicules. In addition, the species of *Psyllocarpus* have small, rounded-oblate (or slightly

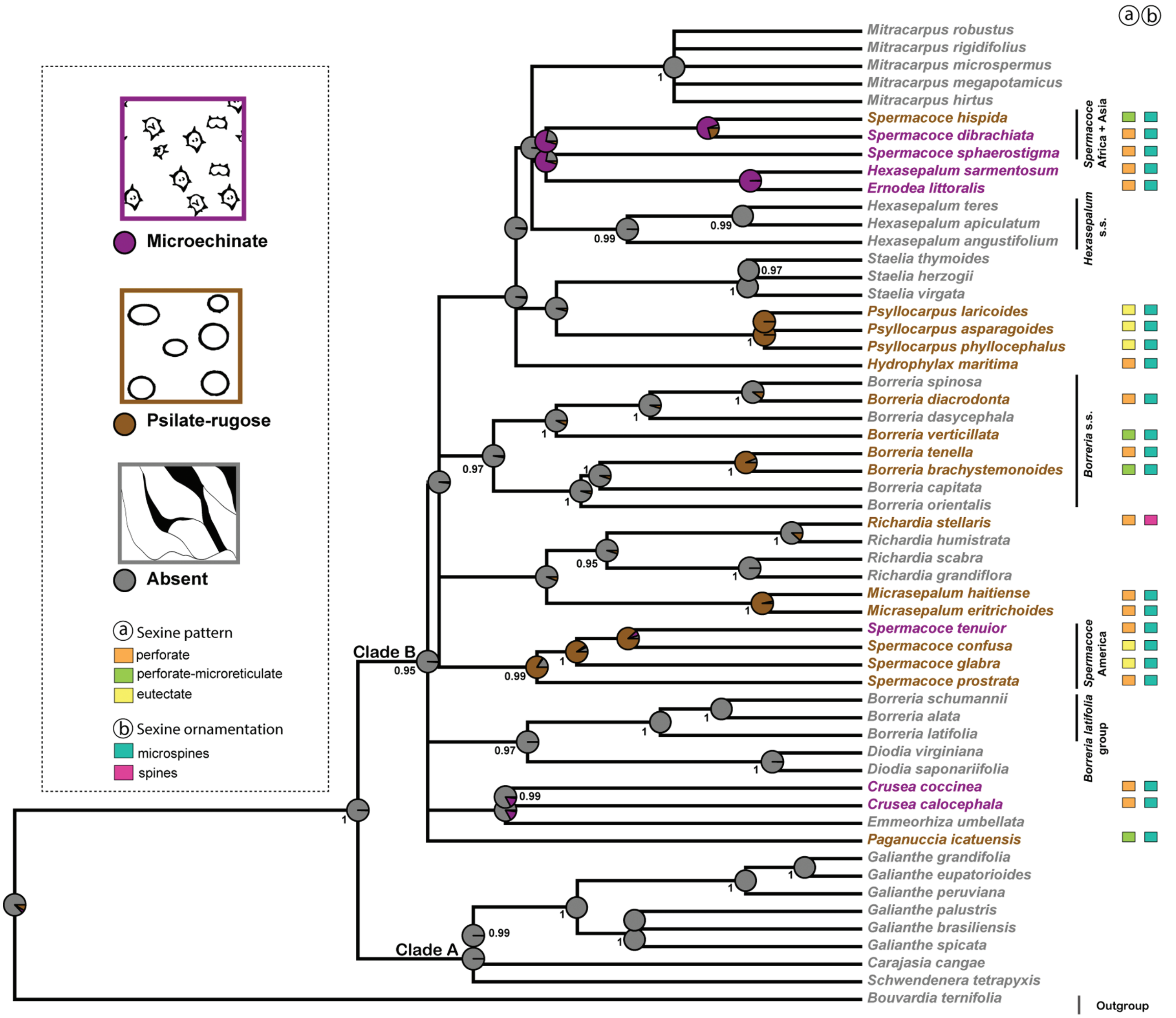

Figure 7. Ancestral character states estimates of ornamentation of orbicules (C5) by Bayesian stochastic mapping, and relationship with the sexine pattern (a) and ornamentation (b) of the pollen grains. The analysis is based on the 50% majority-rule consensus tree obtained from the Bayesian analysis of the concatenated molecular datasets (ITS + ETS + *atpB- rbcL* + *petD* + *rps16* + *trnL-trnF*). Only probability values PP > 0.95 are shown at the nodes. Pie charts at the nodes indicate posterior ancestral state probabilities from the stochastic mapping and tip label colours represent tip states. Vertical lines indicate informal groups recognized within the *Spermacoce* clade.

irregular-angular in *P. asparagoides*), and psilate-rugose orbicules. However, there are other genera such as *Borreria* or *Spermacoce*, which present a large variation in the morphological orbicular characters (Fig. 8).

Huysmans *et al.* (1997) were the first to define four types of orbicule for Rubiaceae, mainly on the basis of the shape and ornamentation of orbicules from Cinchonoideae taxa. Later, Vinckier *et al.* (2000), based on transmission electron microscopy (TEM) analysis of Ixoroideae species, defined two more types and two subtypes within Type III. Thus, six orbicular types are described for the family (Dessein *et al.* 2005). This typology was applied to other families in Gentianales by Vinckier and Smets (2002a, b, c, 2003) and Verellen *et al.* (2004). As highlighted by Vinckier *et al.* (2000), not all orbicules can be classified into these types, and a broader study at a higher taxonomic level is probably required. Taking this into account, we have still decided to classify the orbicules observed in the *Spermacoce* clade into these types to summarize the morphological variation within the group.

On the basis of the shape and ornamentation found in this survey, the orbicule types present in the *Spermacoce* clade are: Type I 'spiny orbicules' (although Huysmans *et al.* (1997) described Type I as orbicules that are 1–3 μm in size but we here nevertheless include *Hexasepalum gardneri*, *Planaltina capitata*, and *Spermacoce dibrachiata* despite being smaller than 1 μm); Type III 'smooth orbicules' (both subtypes); Type V 'granular orbicules'; and Type VI 'embedded orbicules'. Some genera are characterized by just one orbicule type e.g. *Crusea* (Type I), *Micrasepalum* (Type V), while there are other genera such as *Spermacoce*, including subclade *Spermacoce* America and the group *Spermacoce* Africa + Asia, and *Borreria* s.s. that present more than one orbicule type (Fig. 8). A further study involving TEM will be useful to complement the

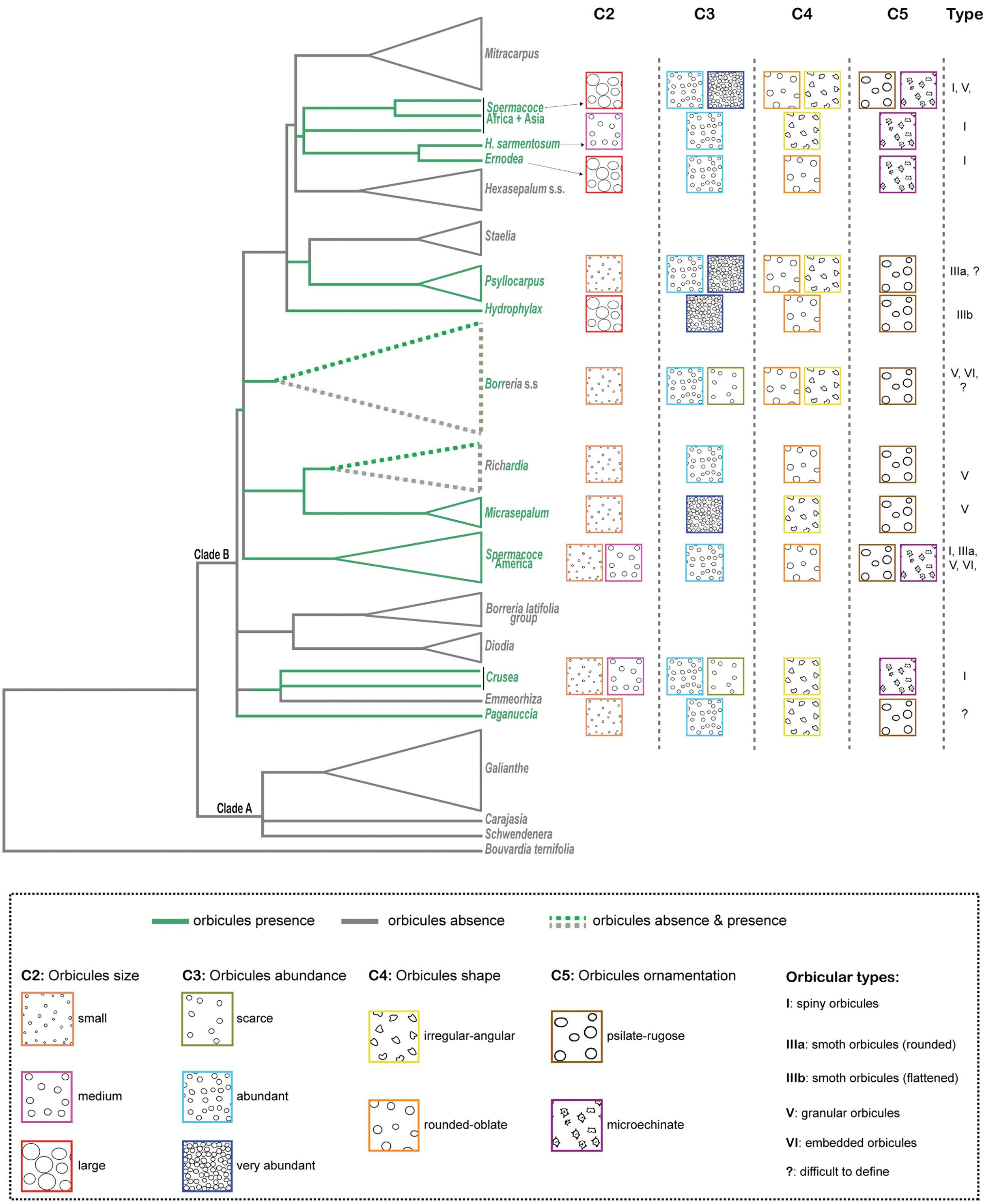

Figure 8. Summary of the orbicular characters examined mapped onto a simplified version of the *Spermacoce* clade phylogeny used elsewhere in this paper.

description of the here analysed species. However, according to the findings of this study, the definition according to the existing orbicule types seems to not be helpful when covering the variation present in the *Spermacoce* clade, and do not have a phylogenetic correlation with it.

Correlation between orbicule ornamentation and pollen sexine

There can be a remarkable similarity between the ornamentation of the orbicules and that of the pollen grains (El-Ghazaly and Jensen 1986, Hesse 1986). Huysmans *et al.* (1997) observed that orbicules with spines (Type I 'spiny orbicules') always have pollen grains with microspines as supratectal elements, but for the other types of orbicules no similar resemblance was observed. On the other hand, Vinckier *et al.* (2000) concluded that the relationship between pollen exine and orbicule ornamentation does not exist between the species in their sampling of Ixoroideae; however, it was observed that species characterized by pollen grains with perforated or microreticulate sexine often have orbicules with perforations. Outside the Rubiaceae family, Vinckier and Smets (2003) reported a high correlation between the pollen exine and the orbicular surface in Gentianaceae taxa; not only between spiny orbicules (Type I) and microspiny pollen grains, but also in irregularly folded orbicules (Type IV) and striated or striated-microreticulate pollen grains.

In general, in the present study, species with microechinate orbicules always have pollen grains with microspines as supratectal elements, but with some differences in the sexine pattern (perforate, perforate-microreticulate, or eutectate). However, species with psilate-rugose orbicules also present pollen grains with microspines, even with spines and granules, and not with psilate pollen grains as is expected. Therefore, we agree with Huysmans *et al.* (1997), that 'spiny orbicules' are always correlated with pollen grains with microspines, but for the other types of orbicules, no similar resemblance is observed between orbicule ornamentation and pollen grain exine.

Furthermore, orbicules ornamentation has been linked to pollination syndromes. Galati *et al.* (2010, 2019) studying several species that present different modes of pollination concluded that anemophilous species generally present microechinate orbicules, as do dipterophilous ones. Melitophilous species often have smooth-surfaced orbicules, while psychophiles often have irregular orbicules with invaginations. Although this theory is interesting and proposes a new perspective, it cannot be investigated in the studied species of the *Spermacoce* clade, since knowledge about the pollination biology as well as about the reproductive biology in this group is scarce (Machado and Loiola 2000, Souza *et al.* 2007, Cruz and Martins 2014, Nicora Chequín 2018). However, it would be valuable to study the link between orbicule characters and the pollination syndrome in other groups of Rubiaceae where the pollination biology is better understood.

Evolution of the orbicules in the Spermacoce clade

Verstraete *et al.* (2011) studied the evolution and phylogenetic signal of orbicules at the Rubiaceae family level, and confirmed that these structures are a common feature within the family and that more derived taxa tend to lack orbicules. Furthermore, they concluded that the presence of orbicules is the plesiomorphic character state within Rubiaceae and that orbicules appeared to have been independently lost at least nine times within the family, with two subsequent reversals (both within the Rubioideae subfamily).

The analysis carried out in this paper found that the absence of orbicules is the ancestral state of the *Spermacoce* clade. The *Spermacoce* clade is a derived clade of the Spermacoceae tribe within the Rubioideae subfamily, and this finding is therefore not surprising. The absence of orbicules in the common ancestor of the *Spermacoce* clade can be seen as one of the independent losses that occurred during the evolutionary history of the Rubiaceae family. The fact that orbicules reappear several times in different taxa within this group can then be seen as reversals to the ancestral state of the family of having orbicules.

The entire Clade A, comprising *Galianthe*, *Carajasia*, and *Schwendenera*, is characterized by the total absence of orbicules and only in Clade B, containing the rest of the *Spermacoce* clade taxa, there are reversals to the presence of orbicules.

Systematic distribution of orbicules and their systematic utility in the Spermacoce clade

While investigating orbicules within flowering plants, Verstraete *et al.* (2014) concluded that the distribution of orbicules is generally consistent at the generic level. The observation of both presence and absence within a single genus is very rare and it is only found in eight genera in angiosperms, for example in *Coptosapelta* Korth., a member of the Rubiaceae family. Verstraete *et al.* (2011) concluded that Rubioideae is the subfamily with the largest number of negative observations, with orbicules absent in the tribes Rubieae and Palicoureeae, present in Psychotrieae, and a more heterogeneous situation for Spermacoceae, with most genera lacking orbicules except for *Spermacoce* (i.e. *S. riparia*). The present study confirms the heterogeneous situation in Spermacoceae and identifies more variation at generic level than expected.

From the 23 currently recognized genera for the *Spermacoce* clade, eight genera lack orbicules in all species investigated, while seven genera are found to have orbicules in all the species studied for the first time. However, six genera present positive and negative observations. Therefore, *Borreria*, *Denscantia*, *Hexasepalum*, *Psyllocarpus*, *Richardia*, and *Spermacoce* join *Coptosapelta* in the list of deviant genera, as named in Verstraete *et al.* (2014) as those genera that present intergeneric variation for presence/absence of orbicules. It is useful to note that the genera for which both presence and absence of orbicules has been confirmed are also those genera that are traditionally known to have taxonomic conflicts and are polyphyletic, such as *Borreria*, *Spermacoce*, and *Hexasepalum* (Miguel and Cabral 2013, Cabaña Fader *et al.* 2016; Florentín *et al.*, 2016; Miguel *et al.* 2018). Despite the fact that there are monophyletic genera characterized by the complete absence of orbicules (e.g. *Galianthe*, *Mitracarpus*) or by their presence (e.g. *Crusea*, *Planaltina*), we cannot claim consistency of orbicule presence at generic level within the *Spermacoce* clade, due to the six deviant genera that present variation in orbicules presence/absence.

The taxonomic utility of orbicules characters has been investigated for several families and genera of angiosperms (e.g. *Euphorbia* L.-Euphorbiaceae, El-Ghazaly and Chaudhary 1993; tribe Hillieae-Rubiaceae, D'Hondt *et al.* 2004; *Coptosapelta*-Rubiaceae, Verellen *et al.* 2004; *Oxalis* L.-Oxalidaceae, López and Rosenfeld 2016; tribe Spiraeeae-Rosaceae, Song *et al.* 2017). There is variation in orbicule distribution in the *Spermacoce* clade, as there are genera that completely lack orbicules, while others were found to have orbicules, and in certain genera there is even a mix of both presence and absence (Fig. 8). This variation might be useful to establish some systematic relevance for this character in the *Spermacoce* clade.

The entire Clade A, comprising *Galianthe*, *Carajasia*, and *Schwendenera*, is characterized by the total absence of orbicules. Although the phylogenetic position of *Tobagoa maleolens* Urb. is still unknown, it is related to these taxa from a morphological point of view (Salas *et al.*, 2015). The absence of orbicules in this taxon (Fig. 1M) indicates its possible relationship with Clade A, along with the presence of heterostylous flowers and pollen with double reticulum (Dessein 2003).

Dessein (2003) and Cabaña Fader *et al.* (2016) proposed the recognition of two different groups within *Hexasepalum* (priority name for *Diodella* Small) based on molecular and morphological data (pollen, embryo, and fruits): the '*Diodella teres* group', corresponds to *Hexasepalum s.s.* (delimitation according to Cabaña Fader *et al.* 2016) and comprises *H. angustifolium* Bartl. ex DC., *H. teres* (Walter) J.H.Kirkbr., *H. apiculatum* (Willd.) Delprete & J.H.Kirkbr., etc.; and the informally known group '*Diodella sarmentosa* group' or *Hexasepalum s.l.* Despite the fact that the phylogenetic relationships within *Hexasepalum* are still incompletely known, orbicule characters could shed light on the taxonomy of this group. According to this survey, all studied species of *Hexasepalum s.s.* lack orbicules, whereas *Hexasepalum s.l.*, represented in this study by *H. sarmentosum*, *H. gardneri*, and *H. mello-barretoi*, have orbicules. The investigation of more species of this genus for orbicules might be able to confirm this distinction.

Another example in which the presence of orbicules might be useful for the taxonomic delineation of taxa is in *Psyllocarpus*. Following the taxonomic revision of Kirkbride (1979), *Psyllocarpus* is classified into two sections based on morphology and geographic distribution: *Psyllocarpus* sect. *Psyllocarpus*, comprising five species (*P. asparagoides*, *P. goiasensis* J.H.Kirkbr., *P. laricoides*, *P. phyllocephalus* and *P. schwakei* K.Schum.), and *Psyllocarpus* sect. *Amazonica* J.H.Kirkbr., comprising three species (*P. campinorum* (Krause) J.H.Kirkbr., *P. cururuensis* J.H.Kirkbr., and *P. psyllocarpoides* (Sucre) J.H.Kirkbr.). In the present analysis (Table 3), three representatives of *P. sect. Psyllocarpus* were analysed, all of which are positive for orbicules; whereas *P. cururuensis*, a representative of *P.* sect. *Amazonica*, lacks orbicules. A further study within other species of the latter section is needed, but the results of the present study may indicate a difference between these two sections.

Denscantia is a genus described by Cabral and Bacigalupo (2001a, b) based on species with scandent habit. Years later, Salas and Cabral (2012) extended the genus concept by describing a new species (*D. calcicola*). All species share the same habit and seed morphology, but there are differences in habitat, pollen, and some fruit and flower characteristics between *D. calcicola* and the other taxa of the genus. None of the species of *Denscantia* have never been included in a molecular phylogenetic analysis, and therefore the affinities between them and with other genera are uncertain. However, the present survey adds another difference between *D. calcicola* and the rest of the species of the group, because this species is the only one that has orbicules (Fig. 2H).

CONCLUSION

This is the first survey of the orbicules in the *Spermacoce* clade, integrating morphology and phylogeny. It adds orbicule data for 85 Rubiaceae species (including the outgroup *Bouvardia ternifolia*), of which 38 species present orbicules and 47 lack them. Orbicules in this group are a more common feature than expected, since their absence in the common ancestor of the group is considered as one of the losses that occur through the evolutionary history of the Rubiaceae. The observed orbicules are reversals to the ancestral state. Despite the fact that orbicules can have some taxonomic usefulness in some genera, it cannot be generalized to the whole *Spermacoce* clade. The orbicules are not constant at generic level; there are at least six genera with presence and absence of orbicules in the same genus, and with a great variation in morphology. Therefore, to elucidate the phylogenetic potential of orbicules at the tribal level, further studies will be necessary to investigate the occurrence of orbicules and their characters in the entire tribe Spermacoceae, and with TEM analyses to complement the morphological descriptions made so far.

ACKNOWLEDGEMENTS

MNF and RMS thank the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) for the grants awarded that supported this work (PICT-2016-3517). MNF is grateful to Javier Florentín, Marina Judkevich, and Domingos Cardoso for their valuable suggestions.

FUNDING

Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET) doctoral grant supported this work. This study was also partially funded by the Agencia Nacional de Promoción Científica y Técnica (FONCYT PICT-2016-3517) and the Universidad Nacional del Nordeste (PI A009-17 and PI 16P001 grants)

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY

The data underlying this article are available in the article and in its online supplementary material. Further information is available from the corresponding author.

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