

## Evolution of the outer ovule integument and its systematic significance in Melastomataceae

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Ovule characters have been shown to be informative at higher taxonomic levels and are therefore of interest in angiosperm systematics. In this study, we aimed to describe the evolution of the outer ovule integument thickness in Melastomataceae and to evaluate its systematic and biological significance. This character was investigated in 137 species, and data from the literature were compiled for 22 additional species, totalling 159 taxa. Integument origin was studied in selected species to clarify whether different conditions were associated with different ontogenetic origins. The most recent common ancestor of Melastomataceae probably had an ovule with a two-cell-layered outer integument. A multilayered outer integument evolved independently at least six times in the family. Moreover, reversals to the two-cell-layered plesiomorphic condition have also occurred more than once. The two-cell-layered and multilayered outer integuments have different ontogenetic origins, enhancing the taxonomic usefulness of this character. The thickness of the outer ovule integument is of systematic significance in *Olisbeoideae*, *Rupestrea*, *Melastomateae* and *Miconieae*. The multilayered outer integument may have some adaptive significance for *Miconieae* and *Mouriri*, groups with endozoochoric fleshy fruits. Evidence of a multiplicative testa was observed in a few species with both types of integuments and it may be associated with a massive seed coat found in large seeds.

**ADDITIONAL KEYWORDS:** anatomy – embryology – flower – integument thickness – multilayered outer integument – two-cell-layered outer integument.

### INTRODUCTION

The ovule is the developmental precursor of the seed and, as such, plays an essential role in the complex

and successful reproduction process in spermatophytes (Bouman, 1984; Gasser, Broadhvest & Hauser, 1998; Linkies *et al.*, 2010; Endress, 2011). In angiosperms, important reproductive events such as the female gametophyte and egg cell development, pollen tube attraction and guidance, double fertilization,

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and embryo and endosperm development all occur in the ovule (Fuentes & Vivian-Smith, 2009; Endress, 2011).

The ovule can be morphologically understood as the megasporangium (or nucellus) covered by one or two integuments (Linkies *et al.*, 2010). Gymnosperms typically have ovules with a single integument, whereas the ovules of angiosperms are commonly bitegmic (Gasser *et al.*, 1998; Endress, 2011). After fertilization, these integuments give rise to the seed coat, which in angiosperms comprises a testa and a tegmen, derived from the outer and inner integuments, respectively. The seed coat can be responsible for embryo protection and seed dispersal, and it is a key factor in the regulation of seed germination (Windsor *et al.*, 2000; Kelley & Gasser, 2009).

Despite the remarkable adaptive significance of the seed coat, the thickness (i.e. number of cell layers) of the integuments seems to have been a relatively stable character in angiosperm evolution and therefore to be informative at higher taxonomic levels (Davis, 1966; Tobe, 1989; Endress, 2011). Ovule integuments may consist of two or more cell layers (Bouman, 1984; Endress, 2011). Two-cell-layered integuments are characterized as dermal integuments because they develop exclusively from the dermal layer of the ovule primordium. In contrast, multilayered integuments may be of dermal origin or may derive from dermal and subdermal layers (subdermal integuments) (Bouman, 1984; Shamrov, 2000; Endress, 2011). Dermal or subdermal integuments can become multiplicative and increase in thickness by periclinal divisions during both ovule and seed development (Corner, 1976; Bouman, 1984).

The occurrence of ovules with a two-cell-layered inner integument is one of the embryological characters that define the order Myrtales (Tobe & Raven, 1983a; Tobe, 1989). On the other hand, the thickness of the outer integument is a variable character in the order (Tobe & Raven, 1983a). In Melastomataceae, one of the two largest families in Myrtales (Dahlgren & Thorne, 1984; Conti *et al.*, 1997), the outer ovule integument may consist of two or more cell layers (Ziegler, 1925; Subramanyam, 1942, 1944, 1948, 1951; Etheridge & Herr, 1968; Corner, 1976; Tobe & Raven, 1983a; Medeiros & Morretes, 1996; Cortez & Carmello-Guerreiro, 2008; Caetano *et al.*, 2013; Ribeiro, Oliveira & Silveira, 2015). However, the evolutionary history of this attribute in the family has not previously been studied. Moreover, despite the importance of integument characters for systematics, the scarcity of the data available for the family hampers their use in the delimitation of clades and understanding their relationships and their evolutionary and ecological significance.

Melastomataceae (including Memecylaceae currently treated as subfamily Olisbeoideae) comprises *c.* 5400 species in 170 genera and is one of the larger families of angiosperms, with considerable species richness in the New World (Renner, 1993; Conti *et al.*, 1997; Stone, 2006; MELNet, 2007; Goldenberg *et al.*, 2015). Despite the uncontroversial monophyly of the family, relationships in Melastomataceae are not fully understood and improving their resolution is still a work in progress (Clausing & Renner, 2001; Fritsch *et al.*, 2004; Michelangeli *et al.*, 2004, 2013; Penneys *et al.*, 2010; Goldenberg *et al.*, 2012; Rocha *et al.*, 2016a, b; Veranso-Libalah *et al.*, 2017). Although the latest review for the family based on morphology and anatomy (Renner, 1993) recognized nine tribes, recent studies based on DNA sequence data have proposed the establishment of new ones and considerable changes to the delimitation of others (Clausing & Renner, 2001; Fritsch *et al.*, 2004; Michelangeli *et al.*, 2004, 2011, 2013; Goldenberg *et al.*, 2008, 2012, 2015; Amorim, Goldenberg & Michelangeli, 2009; Penneys *et al.*, 2010; Penneys & Judd, 2011, 2013; Rocha *et al.*, 2016a, b). At least 15 major lineages are currently known in the family, although not all of them are recognized at the tribal level (Goldenberg *et al.*, 2015).

In this sense, knowledge of potentially diagnostic attributes for specific clades of Melastomataceae, such as the thickness of the outer ovule integument, may be of great significance. Therefore, the objective of the present study was to describe the evolution of this character in Melastomataceae, assessing its systematic and biological significance and clarifying whether different conditions (two-cell-layered and multilayered outer integument) are associated with different ontogenetic origins. Additionally, we also investigated the evolutionary association between multilayered outer integument and fleshy fruits.

## MATERIAL AND METHODS

### TAXON SAMPLING

The thickness of the ovule outer integument was examined in 137 species belonging to 14 of 15 major lineages currently recognized in Melastomataceae (*sensu* Goldenberg *et al.*, 2015). The only tribe not sampled in the present study was Astronieae. We also included published data for 22 additional species for a total of 159 taxa (Table 1) (Ziegler, 1925; Subramanyam, 1942, 1944, 1948, 1951; Etheridge & Herr, 1968; Corner, 1976; Medeiros & Morretes, 1996; Caetano, 2010; Caetano *et al.*, 2013; Ribeiro *et al.*, 2015). As outgroups, we added nine species of the CAP clade, a strongly supported lineage sister to

**Table 1.** List of the Melastomataceae species analysed, status of the character observed and literature data; fruit type (dry or fleshy) is also given

Groups	Species*	Character state	Reference†	Fruit type	Reference†	
Outgroups	Crypteroniaceae	<i>Axinandra zeylanica</i>	Two-layered (2–3)	1	Dry	19
		<i>Crypteronia paniculata</i>	Two-layered (2)	2	Dry	19
		<i>Dactylocladus stenostachys</i>	Two-layered (2–3)	3	Dry	19
	Alzateaceae	<i>Alzatea verticillata</i>	Two-layered (2–4)	4	Dry	19
	Penaeeae	<i>Olinia emarginata</i>	Multilayered (3–4)	5	Fleshy	19
		<i>Olinia ventosa</i>	Multilayered (3–5)	5	Fleshy	19
		<i>Penaea mucronata</i>	Two-layered (2)	6	Dry	19
		<i>Rhynchocalyx lawsonioides</i>	Two-layered (2)	7	Dry	19
		<i>Saltera sarcocolla</i>	Two-layered (2)	6	Dry	19
Olisbeoideae		<i>Memecylon heyneanum</i>	Two-layered (2)	8	Fleshy	20
		<i>Memecylon umbellatum</i>	Two-layered (2)	9	Fleshy	9
	<i>Memecylon</i> sp.	Two-layered (2)	9	Fleshy	20	
	<i>Mouriri acutiflora</i>	Multilayered (5–6)	PS	Fleshy	20	
	<i>Mouriri cearensis</i>	Multilayered (5–6)	PS	Fleshy	20	
	<i>Mouriri guianensis</i>	Multilayered (5–6)	PS	Fleshy	20	
	<i>Votomita guianensis</i>	Two-layered (2–4)	PS	Fleshy	20	
Kibessieae	<i>Pternandra azurea</i>	Two-layered (2)	9	Fleshy	21	
	<i>Pternandra coerulescens</i>	Two-layered (2)	PS	Fleshy	21	
Henrietteae	<i>Bellucia grossularioides</i>	Two-layered (2)	PS	Fleshy	22	
	<i>Bellucia mespiloides</i>	Two-layered (2)	PS	Fleshy	22	
	<i>Henriettea ramiflora</i>	Two-layered (2)	PS	Fleshy	22	
	<i>Henriettea saldanhae</i>	Two-layered (2)	PS	Fleshy	22	
	<i>Henriettea succosa</i>	Two-layered (2)	PS	Fleshy	22	
Bertolonieae	<i>Bertonia mosenii</i>	Two-layered (2)	PS	Dry	23	
Blakeae	<i>Blakea trinervia</i>	Two-layered (2)	9	Fleshy	23	
	<i>Blakea multiflora</i>	Two-layered (2)	PS	Fleshy	24	
	<i>Chalybea calyptrata</i>	Two-layered (2)	PS	Fleshy	24	
	<i>Chalybea ecuadorensis</i>	Two-layered (2)	PS	Fleshy	24	
Dissochaeteae	<i>Dissochaeta divaricata</i>	Two-layered (2)	PS	Fleshy	25	
	<i>Oxyspora paniculata</i>	Two-layered (2)	10	Dry	25	
	<i>Sonerila wallichii</i>	Two-layered (2)	11	Dry	26	
Cyphostyleae	<i>Allomaieta grandiflora</i>	Two-layered (2)	PS	Dry	27	
Triolena clade	<i>Triolena amazonica</i>	Two-layered (2)	PS	Dry	28	
	<i>Triolena obliqua</i>	Two-layered (2)	PS	Dry	28	
	<i>Triolena paleacea</i>	Two-layered (2)	PS	Dry	26	
	<i>Behuria glutinosa</i>	Two-layered (2)	PS	Dry	29	
Cambessedesia clade	<i>Cambessedesia espora</i>	Two-layered (2)	PS	Dry	23	
	<i>Dolichoura spiritusanctensis</i>	Two-layered (2)	PS	Dry	29	
	<i>Huberia consimilis</i>	Two-layered (2)	PS	Dry	29	
	<i>Merianthera bullata</i>	Two-layered (2–3)	PS	Dry	29	
	<i>Merianthera parvifolia</i>	Two-layered (2–3)	PS	Dry	29	
Rhexieae	<i>Arthrostemma ciliatum</i>	Two-layered (2)	PS	Dry	30	
	<i>Rhexia mariana</i>	Two-layered (2)	12	Dry	30	
Microlicieae	<i>Chaetostoma armatum</i>	Two-layered (2)	13	Dry	23	
	<i>Microlicia cordata</i>	Two-layered (2)	PS	Dry	31	
	<i>Microlicia euphorbioides</i>	Two-layered (2)	PS	Dry	23	
	<i>Microlicia fasciculata</i>	Two-layered (2)	PS	Dry	23	
	<i>Poteranthera pusilla</i>	Two-layered (2)	PS	Dry	32	
	<i>Rhynchanthera grandiflora</i>	Two-layered (2)	PS	Dry	23	

Table 1. Continued

Groups	Species*	Character state	Reference†	Fruit type	Reference‡	
Melastomateae	<i>Marcetia</i> alliance					
	<i>Acanthella sprucei</i>	Multilayered (3)	PS	Dry	30	
	<i>Aciotis purpurascens</i>	Multilayered (3)	PS	Dry	30	
	<i>Acisanthera hedyotoidea</i>	Multilayered (3)	PS	Dry	30	
	<i>Acisanthera quadrata</i>	Multilayered (3)	PS	Dry	30	
	<i>Appendicularia thymifolia</i>	Multilayered (3)	PS	Dry	30	
	<i>Comolia microphylla</i>	Multilayered (3)	PS	Dry	30	
	<i>Ernestia glandulosa</i>	Multilayered (3)	PS	Dry	30	
	<i>Macairea radula</i>	Multilayered (3)	PS	Dry	30	
	<i>Marcetia taxifolia</i>	Multilayered (3)	PS	Dry	30	
	<i>Nepsera aquatica</i>	Multilayered (3)	PS	Dry	30	
	<i>Sandemania hoehnei</i>	Multilayered (3)	PS	Dry	30	
	<i>Siphanthera hostmannii</i>	Multilayered (3)	PS	Dry	33	
	Core	<i>Brachyotum ledifolium</i>	Two-layered (2)	PS	Dry	30
		<i>Melastomateae</i>				
		<i>Desmoscelis villosa</i>	Two-layered (2)	PS	Dry	30
		<i>Heterocentron elegans</i>	Two-layered (2)	PS	Dry	30
		<i>Melastoma malabathricum</i>	Two-layered (2)	14	Fleshy	30
		<i>Monochaetum calcaratum</i>	Two-layered (2)	9	Dry	30
		<i>Monochaetum ensiferum</i>	Two-layered (2)	15	Dry	30
	<i>Monochaetum meridense</i>	Two-layered (2)	PS	Dry	30	
	<i>Osbeckia aspera</i>	Two-layered (2)	8	Dry	26	
	<i>Osbeckia brachystemon</i>	Two-layered (2)	8	Dry	26	
	<i>Osbeckia stellata</i>	Two-layered (2)	8	Dry	25	
	<i>Pterolepis glomerata</i>	Two-layered (2)	PS	Dry	30	
	<i>Tibouchina clavata</i>	Two-layered (2)	PS	Dry	30	
	<i>Tibouchina clinopodifolia</i>	Two-layered (2)	PS	Dry	30	
	<i>Tibouchina laevicaulis</i>	Two-layered (2)	PS	Dry	30	
	<i>Tibouchina nodosa</i>	Two-layered (2)	9	Dry	30	
	<i>Tibouchina semidecandra</i>	Two-layered (2)	15	Dry	30	
Merianieae	<i>Axinaea dentata</i>	Two-layered (2–3)	PS	Dry	34	
	<i>Axinaea floribunda</i>	Multilayered (3)	PS	Dry	34	
	<i>Axinaea grandifolia</i>	Two-layered (2–3)	PS	Dry	34	
	<i>Axinaea minutiflora</i>	Two-layered (2–3)	PS	Dry	34	
	<i>Graffenrieda gracilis</i>	Two-layered (2)	PS	Dry	33	
	<i>Graffenrieda harlingii</i>	Two-layered (2)	PS	Dry	28	
	<i>Graffenrieda latifolia</i>	Two-layered (2)	PS	Dry	33	
	<i>Macrocentrum repens</i>	Two-layered (2)	PS	Dry	35	
	<i>Meriania sclerophylla</i>	Multilayered (3)	PS	Dry	33	
	<i>Meriania subumbellata</i>	Multilayered (3)	PS	Dry	33	
	<i>Meriania urceolata</i>	Two-layered (2–3)	PS	Dry	33	
	Miconieae	<i>Eriocnema</i> clade				
		<i>Eriocnema acaulis</i>	Multilayered (3)	PS	Dry	31
		<i>Eriocnema fulva</i>	Multilayered (3)	PS	Dry	31
<i>Physeterostemon thomasii</i>		Two-layered (2)	PS	Dry	36	
<i>Miconia</i> I						
<i>Miconia dodecandra</i>		Multilayered (3)	PS	Fleshy	23	
<i>Miconia superba</i>		Multilayered (3)	PS	Fleshy	35	
<i>Miconia</i> II						
<i>Miconia centrodesma</i>		Multilayered (3)	PS	Fleshy	23	
<i>Leandra</i> + <i>Ossaea</i>						
<i>Leandra reversa</i>		Multilayered (3)	PS	Fleshy	23	
<i>Ossaea capillaris</i>		Multilayered (3)	PS	Fleshy	28	
<i>Tococa</i>						
<i>Tococa guianensis</i>		Multilayered (3)	PS	Fleshy	35	
Caribbean	<i>Charianthus alpinus</i>	Two-layered (2)	PS	Fleshy	37	
	<i>Charianthus nodosus</i>	Two-layered (2)	PS	Fleshy	37	
	<i>Tetrazygia crotonifolia</i>	Multilayered (3)	PS	Fleshy	38	
	<i>Tetrazygia discolor</i>	Two-layered (2)	PS	Fleshy	37	
	<i>Tetrazygia elaeagnoides</i>	Multilayered (3)	PS	Fleshy	37	

Table 1. Continued

Groups	Species*	Character state	Reference†	Fruit type	Reference†	
Monopodial	<i>Clidemia mortoniana</i>	Multilayered (3)	PS	Fleshy	39	
	<i>Clidemia spectabilis</i>	Multilayered (3)	PS	Fleshy	39	
Conostegia	<i>Conostegia bigibbosa</i>	Two-layered (2)	PS	Fleshy	40	
	<i>Conostegia brenesii</i>	Two-layered (2)	PS	Fleshy	40	
	<i>Conostegia consimilis</i>	Two-layered (2)	PS	Fleshy	40	
	<i>Conostegia friedmaniorum</i>	Two-layered (2)	PS	Fleshy	40	
	<i>Conostegia icosandra</i>	Two-layered (2)	PS	Fleshy	40	
	<i>Conostegia lasiopoda</i>	Two-layered (2)	PS	Fleshy	40	
	<i>Conostegia monteagleana</i>	Two-layered (2)	PS	Fleshy	40	
	<i>Conostegia schlimii</i>	Two-layered (2)	PS	Fleshy	40	
	<i>Conostegia speciosa</i>	Two-layered (2)	PS	Fleshy	40	
	<i>Conostegia subcrustulata</i>	Two-layered (2)	PS	Fleshy	40	
	<i>Conostegia xalapensis</i>	Two-layered (2)	PS	Fleshy	40	
	Mecranium, Anaectocalyx and allies	<i>Mecranium acuminatum</i>	Multilayered (3)	PS	Fleshy	41
		<i>Mecranium latifolium</i>	Multilayered (3)	PS	Fleshy	41
	Miconia III	<i>Miconia ciliata</i>	Multilayered (3)	PS	Fleshy	39
<i>Tococa platyphylla</i>		Multilayered (3)	PS	Fleshy	28	
<i>Leandra subseriata</i>		Multilayered (3)	PS	Fleshy	28	
<i>Miconia crocea</i>		Multilayered (3)	PS	Fleshy	28	
<i>Miconia denticulata</i>		Multilayered (3)	PS	Fleshy	28	
<i>Miconia goniostigma</i>		Multilayered (3)	PS	Fleshy	28	
<i>Miconia papillosa</i>		Multilayered (3)	PS	Fleshy	28	
<i>Miconia stenobotrys</i>		Multilayered (3)	PS	Fleshy	42	
Miconia IV ( <i>Miconia discolor</i> subclade)	<i>Miconia budlejoides</i>	Multilayered (3–4)	PS	Fleshy	23	
	<i>Miconia cabucu</i>	Multilayered (6–7)	16	Fleshy	23	
	<i>Miconia capixaba</i>	Multilayered (3–4)	PS	Fleshy	43	
	<i>Miconia castaneiflora</i>	Multilayered (4)	PS	Fleshy	43	
	<i>Miconia chartacea</i>	Multilayered (3–4)	PS	Fleshy	23	
	<i>Miconia cubatanensis</i>	Multilayered (5)	PS	Fleshy	23	
	<i>Miconia discolor</i>	Multilayered (3–4)	PS	Fleshy	23	
	<i>Miconia fasciculata</i>	Multilayered (3)	PS	Fleshy	43	
	<i>Miconia hyemalis</i>	Multilayered (3–4)	PS	Fleshy	43	
	<i>Miconia lymanii</i>	Multilayered (4)	PS	Fleshy	43	
	<i>Miconia pepericarpa</i>	Multilayered (3)	17	Fleshy	43	
	<i>Miconia sclerophylla</i>	Multilayered (4)	PS	Fleshy	43	
	Miconia IV	<i>Miconia albicans</i>	Multilayered (3)	18	Fleshy	23
		<i>Miconia donaeana</i>	Multilayered (3)	PS	Fleshy	43
		<i>Miconia fallax</i>	Multilayered (3)	17	Fleshy	23
<i>Miconia laevigata</i>		Multilayered (3)	PS	Fleshy	28	
<i>Miconia latecrenata</i>		Multilayered (3)	PS	Fleshy	23	
<i>Miconia leucocarpa</i>		Multilayered (3)	PS	Fleshy	23	
<i>Miconia minutiflora</i>		Multilayered (3)	PS	Fleshy	23	
<i>Miconia rubiginosa</i>		Multilayered (3)	PS	Fleshy	23	
<i>Miconia rufescens</i>		Multilayered (3)	PS	Fleshy	33	
<i>Miconia serrulata</i>		Multilayered (3)	PS	Fleshy	28	
<i>Miconia stenostachya</i>	Multilayered (3)	PS	Fleshy	23		

Table 1. Continued

Groups	Species*	Character state	Reference†	Fruit type	Reference‡	
<i>Miconia</i> V	<i>Leandra rufescens</i>	Multilayered (3)	PS	Fleshy	35	
	<i>Miconia ceramicarpa</i>	Two-layered (2)	PS	Fleshy	35	
	<i>Miconia chamissois</i>	Multilayered (3)	PS	Fleshy	23	
	<i>Miconia ibaguensis</i>	Multilayered (3)	PS	Fleshy	23	
	<i>Miconia inaequidens</i>	Multilayered (3–4)	PS	Fleshy	23	
	<i>Miconia nervosa</i>	Multilayered (3)	PS	Fleshy	28	
	<i>Miconia paucidens</i>	Multilayered (4)	PS	Fleshy	23	
	<i>Miconia prasina</i>	Multilayered (3)	PS	Fleshy	35	
	<i>Miconia pusilliflora</i>	Multilayered (3–4)	PS	Fleshy	23	
	<i>Miconia rugosa</i>	Multilayered (3–4)	PS	Fleshy	35	
	<i>Clidemia</i>	<i>Clidemia hirta</i>	Multilayered (3)	PS	Fleshy	23
	<i>Leandra</i> s.s. +	<i>Leandra aurea</i>	Multilayered (3)	PS	Fleshy	23
	<i>Pleiochiton</i>	<i>Leandra cardiophylla</i>	Multilayered (3)	8	Fleshy	42
Unplaced species	<i>Leandra melastomoides</i>	Multilayered (3)	PS	Fleshy	23	
	<i>Ossaea amygdaloides</i>	Multilayered (3)	PS	Fleshy	23	
	<i>Ossaea confertiflora</i>	Multilayered (3)	PS	Fleshy	23	
	<i>Pleiochiton ebracteatum</i>	Multilayered (3)	PS	Fleshy	23	
	<i>Phainantha laxiflora</i>	Two-layered (2)	PS	Dry	35	
	<i>Ruprestrea johnwurdackiana</i>	Multilayered (4–5)	PS	Dry	44	

\*Authors for species names can be found in Supporting Information Appendix S1.

†References: 1, Tobe & Raven (1983b); 2, Tobe & Raven (1987a); 3, Tobe & Raven (1987b); 4, Tobe & Raven (1984a); 5, Tobe & Raven (1984b); 6, Tobe & Raven (1984c); 7, Tobe & Raven (1984d); 8, Subramanyam (1942); 9, Corner (1976); 10, Subramanyam (1951); 11, Subramanyam (1944); 12, Etheridge & Herr (1968); 13, Ribeiro *et al.* (2015); 14, Subramanyam (1948); 15, Ziegler (1925); 16, Medeiros & Morretes (1996); 17, Caetano (2010); 18, Caetano *et al.* (2013); 19, Dahlgren & Thorne (1984); 20, Morley (1976); 21, Maxwell (1981); 22, Penneys *et al.* (2010); 23, Martins *et al.* (2009); 24, Penneys & Judd (2013); 25, Jie & Renner (2007); 26, Triana (1871); 27, Michelangeli *et al.* (2011); 28, Wurdack (1980); 29, Goldenberg *et al.* (2012); 30, Michelangeli *et al.* (2013); 31, Cogniaux (1891); 32, Rocha *et al.* (2016b); 33, Wurdack (1973); 34, Cotton, Borchsenius & Balslev (2014); 35, Berry *et al.* (2001); 36, Amorim *et al.* (2009); 37, Michelangeli *et al.* (2004); 38, Judd & Ionta (2013); 39, Almeda (2009); 40, Kriebel (2016); 41, Skean (1993); 42, Michelangeli *et al.* (2008); 43, Caddah (2013); 44, Goldenberg *et al.* (2015); PS, present study.

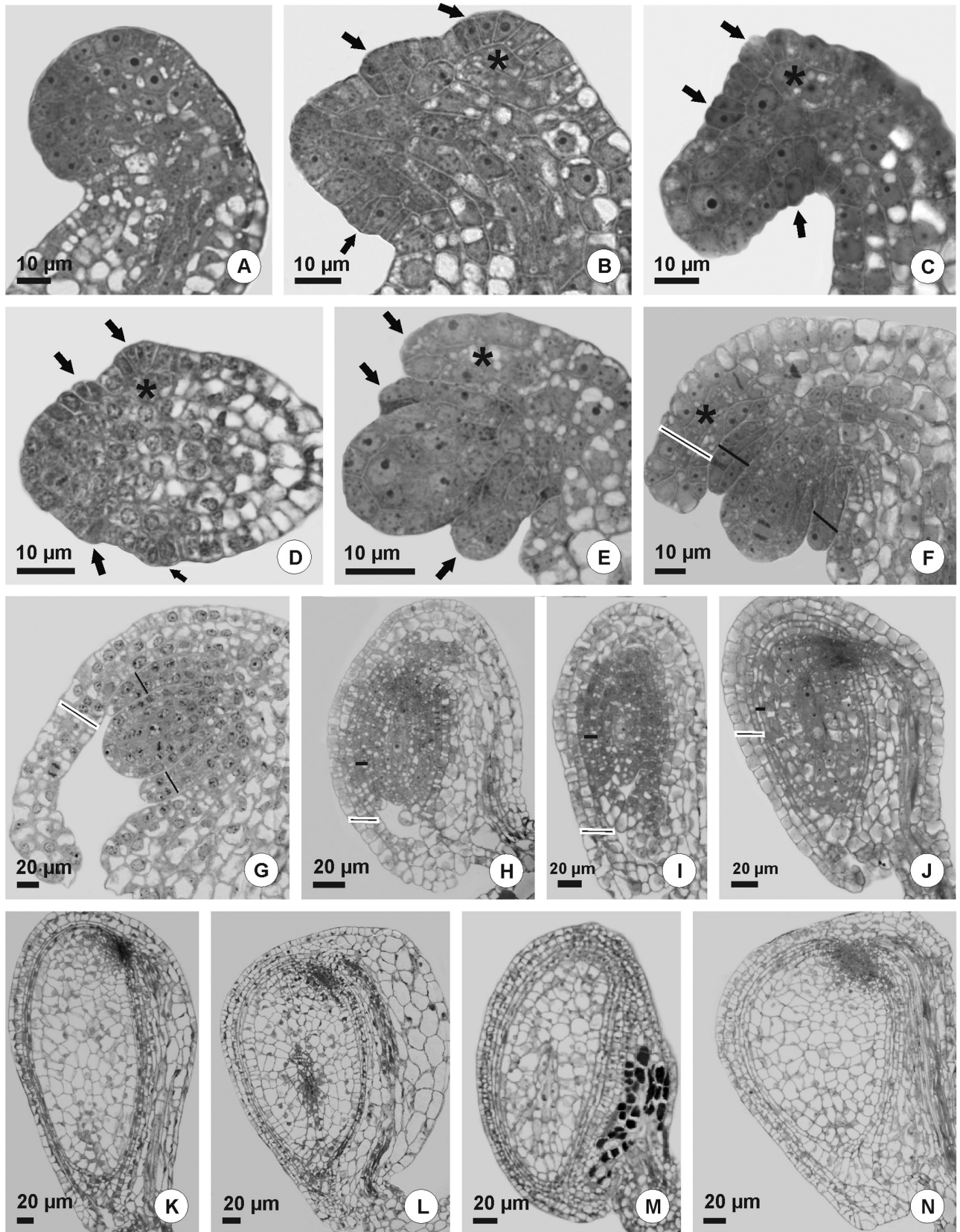
Melastomataceae and formed by Crypteroniaceae, Alzateaceae and Penaeaceae *s.l.* (including Oliniaceae and Rhynchocalycaceae; Conti *et al.*, 2002; APG IV, 2016; Berger *et al.*, 2016). Embryological data for these species were obtained from literature reports (Table 1; Tobe & Raven, 1983b, 1984a, b, c, d, 1987a, b).

#### ANATOMICAL ANALYSIS

We sampled ovules from pre-anthesis buds or anthetic flowers. The samples were obtained from fixed material later preserved in ethanol or from rehydrated herbarium specimens (Appendix 1; Smith & Smith, 1942). The thickness of the outer ovule integument was characterized by counting the number of integument cell layers in transverse and/or longitudinal sections of the median region of the ovule. The micropylar region was not considered for this definition because in this area the integumental cells may divide periclinally, forming additional layers (Subramanyam, 1942, 1948, 1951; Corner, 1976; Medeiros & Morretes, 1996; Caetano *et al.*, 2013; Ribeiro *et al.*, 2015).

To assess the origin of multilayered outer ovule integument, some species were selected for observation of the initiation and development of the integuments (whether dermal or subdermal according to Bouman, 1984): *Acisanthera quadrata* Pers. (Melastomataceae), *Clidemia hirta* (L.) D. Don, *Leandra aurea* (Cham.) Cogn., *Miconia fallax* DC., *M. leucocarpa* DC., *M. pepericarpa* Mart. ex DC. and *Ossaea confertiflora* (DC.) Triana (Miconieae). In these plants, we analysed ovules from flower buds in different developmental phases and from anthetic flowers. The material was fixed in 2% glutaraldehyde and 4% formaldehyde in 0.1 M sodium phosphate buffer, pH 6.8, for 24 h (modified from McDowell & Trump, 1976).

All samples were embedded in resin (Leica Microsystems, Heidelberg, Germany, and Heraeus Kulzer GmbH, Germany) and transversally and longitudinally sectioned with an AO Spencer 820 (GMI Inc., Ramsey, MN, USA) or RM 2245 (Leica) rotary microtome with a steel blade. Sections of c. 5.0 µm were adhered to glass slides and stained with 0.05% Toluidine Blue in 0.1 M sodium phosphate buffer, pH 6.8 (O'Brien, Feder & McCully, 1964). Observations and photomicrographs



were made with a DM 5000 light microscope coupled to a DFC 390 (both Leica Microsystems) digital camera or with a Zeiss Axioplan compound microscope with a Nikon DXM1200C digital camera.

The outer ovule integument was classified as two-cell-layered when formed by two cell layers or variations on this condition (two or three or two to four cell layers in the same ovule) or multilayered when consisting of three or more cell layers (Tobe, 1989; Endress, 2011).

#### PHYLOGENETIC RECONSTRUCTION

Tree inference and divergence time estimation were performed under a Bayesian framework implemented in BEAST 1.8.0 (Drummond *et al.*, 2012). Molecular data for the species of interest were gathered from the NCBI database (GenBank), where the most common markers available across our sampled taxa were selected. The molecular data set includes six plastid and two nuclear markers, the plastome being represented by three intergenic spacers (*accD-psaI*, *psbK-psbI* and *trnS-trnG*), two protein-coding genes (*ndhF* and *rbcL*) and one intron (*rpl16*), and nuclear DNA being represented by two ribosomal spacers (nrETS and nrITS). GenBank accessions for all sequences included in this analysis are available in [Supporting Information Appendix S2](#). Phylogenetic inference was performed on 160 terminals, including nine species from the Crypteroniaceae–Alzateaceae–Penaecaceae (CAP) clade treated as the outgroup (Conti *et al.*, 2002; Berger *et al.*, 2016). Individual loci were aligned with MAFFT 7.3 (Katoh & Standley, 2013) using the G-INS-i strategy. The best DNA partitioning scheme and models were estimated with PartitionFinder2 (Lanfear *et al.*, 2012) under the Bayesian information criterion (BIC). The best scheme resulted in five partitions (*accD-psaI*, *ndhF* plus *rpl16*; nrETS; nrITS; *psbK-psbI* plus *trnS-trnG*; *rbcL*). The GTR+G model was recovered as the best fit for all partitions. The molecular clock prior was set to the lognormal uncorrelated model and the tree prior was set to the birth-death model. Fossil and secondary calibration constraints were placed in three nodes: (1) Melastomataceae crown (prior = lognormal, mean 1.5, SD 1, offset 56), based on the Palaeocene leaf fossil *Melastomites montanensis* R.W.Brown (Brown, 1962); (2) *Rhexia* Gronov. + *Arthrostemma* Pav. ex D.Don crown (prior = lognormal, mean 1.5, SD 1, offset 23), based on Miocene seed fossils (Collinson & Pingen, 1992); and (3) CAP clade crown (prior normal, mean 52.7, SD 6), based on the estimated age recovered in a wider

analysis of Myrtales (Berger *et al.*, 2016). We performed three independent runs of 30 million generations each, sampling every 1000 generations, and the stable posterior distributions of the independent runs were combined with LogCombiner v.1.8.0. Convergence was assessed using Tracer v.1.6 (Rambaut *et al.*, 2014), and runs were considered satisfactory with effective sample size (ESS) values greater than 200. A maximum clade credibility tree was generated with TreeAnnotator v.1.8.0. Overall, both topology and divergence times were congruent with recently published hypotheses (Penneys & Judd, 2005, 2013; Stone, 2006; Goldenberg *et al.*, 2008, 2015; Penneys *et al.*, 2010; Caddah, 2013; Michelangeli *et al.*, 2013; Kriebel, Michelangeli & Kelly, 2015; Meirelles, 2015; Berger *et al.*, 2016; Reginato, 2016; Rocha *et al.*, 2016a).

#### ANCESTRAL STATE RECONSTRUCTION AND CORRELATED EVOLUTION

Ancestral state reconstruction of the outer integument thickness in Melastomataceae was inferred using stochastic character mapping. This character was treated as discrete and unordered, i.e. all transitions among the states of the character can occur at equal probabilities. The test for the correlated evolution of the outer integument thickness and fruit type in this family was performed using Pagel's model for binary characters (Pagel, 1994; Pagel & Meade, 2006). The fruit type considered (dry or fleshy) was based on literature (see Table 1). All analyses were carried out using the packages ape (Paradis, Claude & Strimmer, 2004) and phytools (Revell, 2012) in the R 3.2.5 environment (<http://www.r-project.org/>). To visualize phenotypic evolution on the trees, we mapped character histories across phylogenetic trees using a colour scheme (Revell, 2013).

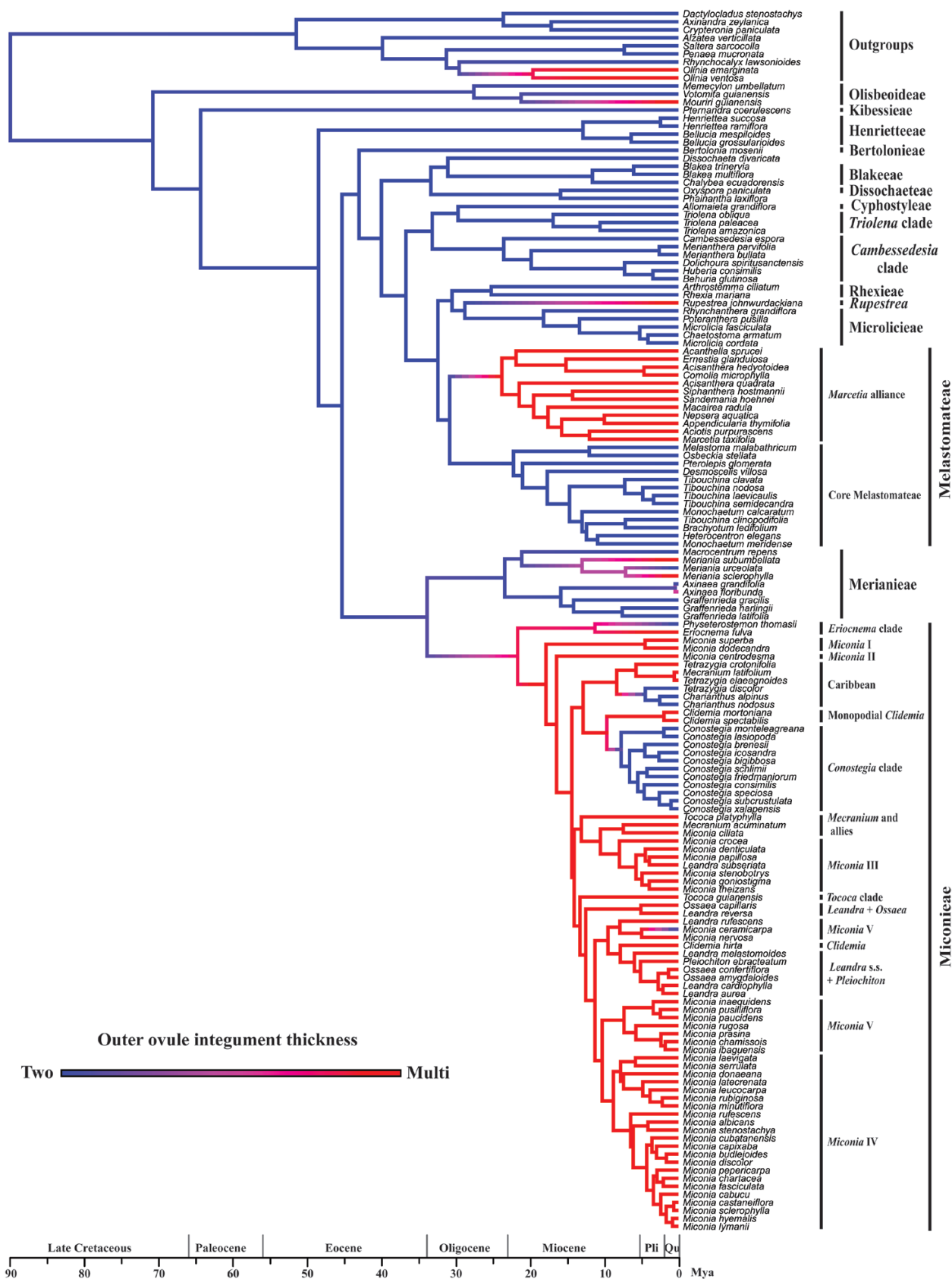
## RESULTS

#### INTEGUMENT INITIATION AND DEVELOPMENT

The multilayered outer integument was derived from both dermal and subdermal layers in *Acisanthera quadrata*, *Clidemia hirta*, *Leandra aurea*, *Miconia fallax*, *M. leucocarpa*, *M. pepericarpa* and *Ossaea confertiflora*. In all seven studied species, the initiation and development of the integuments occur simultaneously by means of periclinal divisions of epidermal cells located at the base of the ovule primordium, characterizing it as a subdermal integument (Fig. 1A–E).

**Figure 1.** Longitudinal sections showing the initiation (indicated by arrows) and development of the integuments in Melastomataceae species with a multilayered outer ovule integument. A–B, *Leandra aurea*. C, *Clidemia hirta*. D, *Miconia pepericarpa*. E, *Ossaea confertiflora*. F, *L. aurea*. G–H, *M. fallax*. I, *L. aurea*. J, *C. hirta*. K, *L. aurea*. L, *Miconia leucocarpa*. M, *M. pepericarpa*. N, *M. fallax*.





The outer integument grows more rapidly than the inner one and extends beyond the apex of the nucellus first (Fig. 1F–H). However, when the megagametophyte starts its development, both integuments already cover the entire nucellus, forming a ‘zig-zag’ micropyle (Fig. 1I, J). Starting from this stage, the integuments extend, accompanying the growth of the rest of the ovule until its maturity (Fig. 1K–N).

#### OUTER OVULE INTEGUMENT THICKNESS

In ten of the 14 major lineages sampled, the species had only ovules with a two-cell-layered outer integument (Table 1; Fig. 2): Kibessieae (Fig. 3A), Henrietteae (Fig. 3B, C), Bertolonieae (Fig. 3D), Blakeeae (Fig. 3E), Dissochaeteae, Cyphostyleae (Fig. 3F), *Triolena* Naudin clade (Fig. 4A), *Cambessedesia* DC. clade (Fig. 4B–D), Rhexieae (Fig. 4E) and Microlicieae (Fig. 4F).

In the *Cambessedesia* clade, although the outer integuments of *Merianthera bullata* R. Goldenb., Fraga & A. P. Fontana and *M. parvifolia* R. Goldenb., Fraga & A. P. Fontana are mostly two-cell-layered, punctual periclinal divisions led to the formation of a third cell layer in some regions of the ovule and indicate a multiplicative integument in *Merianthera* Kuhl. (Fig. 4C, D).

Ovules with a multilayered outer integument occur in some species of the remaining groups: Olisbeoideae, Melastomateae, Merianieae and Miconieae (Table 1; Fig. 2). *Rupestrea johnwurdackiana* (Baumgratz & D’El Rei Souza) Michelang., Almeda, & R. Goldenb., not yet formally placed in any tribe in the family, also has a multilayered outer integument with four to five cell layers, possibly because of a multiplicative origin (Table 1; Figs 2, 5A, B).

In Olisbeoideae, the outer ovule integument is two-cell-layered in *Memecylon* L. and in *Votomita guianensis* Aubl. (Fig. 5C). In the latter taxon, this integument is multiplicative and its cells undergo periclinal divisions, forming up to four cell layers in the chalazal region (Fig. 5C). On the other hand, in all species of *Mouriri* Aubl. sampled the outer integument is multilayered, with four to six cell layers (Fig. 5D).

In Melastomateae, the outer integument is two-cell-layered in species of core Melastomateae (Fig. 5E, F). It is multilayered with three cell layers in species of the *Marcetia* DC. alliance (Figs 5G, 6A, B).

In Merianieae, a two-cell-layered outer integument was observed in *Graffenrieda gracilis* (Triana) L. O. Williams, *G. harlingii* Wurdack (Fig. 6C), *G. latifolia* (Naudin) Triana and *Macrocentrum repens* (Gleason) Wurdack. An originally two-cell-layered

integument, although with a third cell layer formed in some regions, occurs in *Axinaea dentata* E. Cotton (Fig. 6D), *A. grandifolia* (Naudin) Triana, *A. minutiflora* E. Cotton and *Meriania urceolata* Triana (Fig. 6E). In *Axinaea floribunda* (Naudin) Triana, *Meriania sclerophylla* (Naudin) Triana (Fig. 6F) and *M. subumbellata* Cogn., the outer integument is multilayered, with three cell layers.

Most species of Miconieae have ovules with multilayered outer integuments (Table 1) (Fig. 7A–C). The thickness of this integument ranges from three to seven cell layers, but three layers were most frequent (Table 1) (Fig. 7A). More than three cell layers were observed only in species of the *Miconia* Ruiz & Pav. IV clade (Fig. 7B) and in the *Miconia* V grade (Fig. 7C) (Table 1), with evidence of a multiplicative integument. The two-cell-layered condition was observed only in the *Eriocnema* Naudin and *Conostegia* D. Don clades and in the Caribbean and *Miconia* V grades. In the *Eriocnema* clade, the outer integument is two-cell-layered in *Physeterostemon thomasi* Amorim, Michelangeli & Goldenb. (Fig. 7D), but multilayered with three cell layers in *Eriocnema acaulis* Triana (Fig. 7E) and *E. fulva* Naudin. All species belonging to the *Conostegia* clade have ovules with a two-cell-layered outer integument (Fig. 7F, G). In the Caribbean grade, *Charianthus alpinus* (Sw.) R. A. Howard, *C. nodosus* (Desr.) Triana (Fig. 7H) and *Tetrazygia discolor* (L.) DC. (Fig. 7I) have two-cell-layered outer integuments, whereas in *Tetrazygia crotonifolia* (Desr.) DC. (Fig. 7J) and *T. elaeagnoides* (Sw.) DC. it is multilayered, with three cell layers. In the *Miconia* V grade, a two-cell-layered outer integument was observed only in *M. ceramicarpa* (DC.) Cogn.

#### ANCESTRAL STATE RECONSTRUCTION

Stochastic mapping analyses indicate the most recent common ancestor of Melastomataceae as probably having an ovule with a two-cell-layered outer integument. Ovules with a multilayered outer integument evolved independently at least six times: (1) in the ancestor of *Mouriri*; (2) in the ancestor of *Rupestrea* R. Goldenb., Almeda & Michelang.; (3) in the ancestor of the *Marcetia* alliance; (4) in the ancestor of a clade in Merianieae; (5) in *Axinaea floribunda*; and (6) in the ancestor of Miconieae. However, multiple reversals from multilayered to two-cell-layered integuments apparently also occurred independently in Merianieae in *Meriania urceolata* and in Miconieae in *Physeterostemon* R. Goldenb. &

**Figure 2.** Stochastic mapping of outer ovule integument thickness onto the resulting phylogenetic tree of Melastomataceae from the Bayesian analysis using six plastid and two nuclear markers. Red corresponds to the multilayered status, and blue to the two-cell-layered status. Changes in colour along the branches indicate transitions between characters states. Mya, million years ago; Pli, Pliocene; Qa, Quaternary.

Amorim, in a small clade in the Caribbean grade, in the *Conostegia* clade and in *Miconia ceramicarpa* (Fig. 2). A test for correlated evolution showed that outer ovule integument thickness is not significantly correlated with fruit type in the family (Fig. 8; likelihood ratio: 2.1037;  $P = 0.35$ ).

## DISCUSSION

### DIFFERENT ONTOGENETIC PATHWAYS GAVE ORIGIN TO THE OUTER INTEGUMENT IN MELASTOMATACEAE

Multilayered ovule integuments may originate from the dermal layer and become thicker by periclinal division of epidermal cells (dermal integuments), or from both the dermal and the subdermal layers (subdermal integuments) (Bouman, 1984; Endress, 2011). At least in Miconieae and in the *Marcetia* alliance (Melastomataceae), the multilayered outer integument is classified as 'subdermal' because it derives from the dermal and subdermal layers of the ovule primordium (Bouman, 1984; Shamrov, 2000). This pattern contrasts with the dermal origin typically observed in species with a two-cell-layered outer integument (Bouman, 1984; Endress, 2011), a condition that is found in most Melastomataceae. Although dermal and subdermal integuments can become multiplicative and produce multilayered integuments, the different ontogenetic patterns observed support the distinct nature of these two character states (two-cell-layered and multilayered outer integument) and their use in the delimitation of clades in Melastomataceae.

### OUTER OVULE INTEGUMENT THICKNESS AS A TAXONOMIC MARKER FOR MELASTOMATACEAE CLADES

During the evolutionary history of Melastomataceae, the thickness of the outer ovule integument has apparently remained relatively stable, enabling us to recognize many clades in the family. Ovules with a two-cell-layered outer integument are probably a plesiomorphic condition in Melastomataceae and occur in most of the analysed lineages. However, ovules with a multilayered outer integument evolved independently in *Mouriri*, *Rupestrea*, the *Marcetia* alliance, Merianieae and Miconieae. Moreover, reversals to the plesiomorphic two-cell-layered integument condition have also occurred more than once.

In Olisbeoideae, *Mouriri* differs from *Memecylon* and *Votomita* Aubl. by having a multilayered outer integument. Molecular data support each of these genera as monophyletic (as well as three other genera in the subfamily) and show that the Neotropical genera *Mouriri* and *Votomita* are sister groups (Stone, 2006, 2014). Additional data on the outer integument thickness of *Lijndenia* Zoll. & Moritzi, *Spathandra* Guill. & Perr. and *Warneckea* Gilg are necessary to

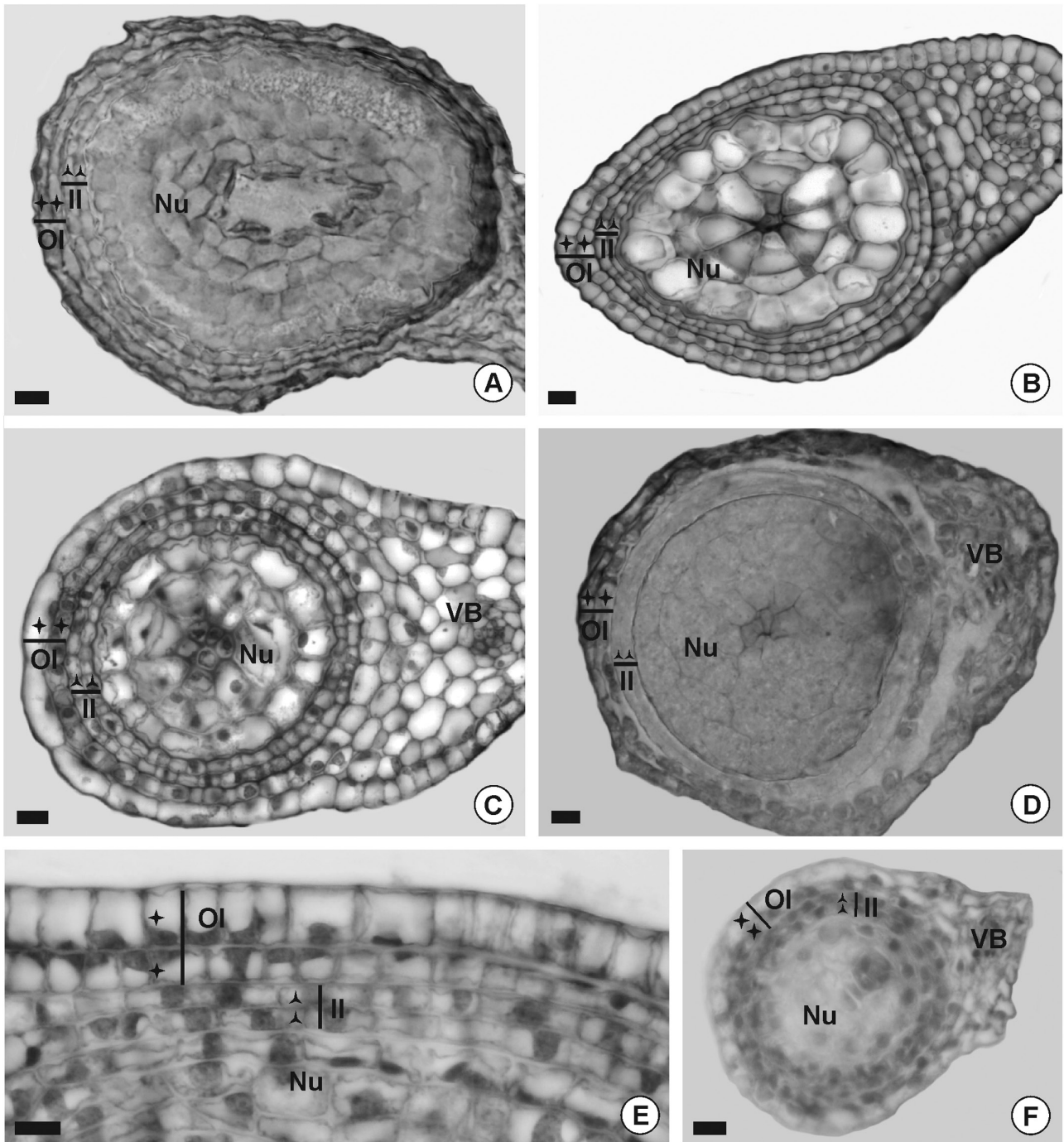
establish whether the thick outer integument found in Olisbeoideae is a single condition observed in *Mouriri* or if it is homoplastic. Regardless, this character state could be used to separate *Mouriri* from *Votomita* from an embryological standpoint.

The presence of a multilayered and considerably thick outer integument characterizes and delimits *Rupestrea*. This genus of two species was positioned as sister to Microlicieae in our analysis, although recently placed with low support as sister to the Microlicieae + Melastomataceae + Rhexieae clade (Goldenberg *et al.*, 2015).

In Melastomataceae, the three-layered outer integument is a synapomorphy for the *Marcetia* alliance as species in core Melastomataceae and the closely associated tribes Rhexieae and Microlicieae have two cell layers, although *Rupestrea* has a multilayered integument. Microlicieae, Rhexieae, the *Marcetia* alliance and core Melastomataceae form a large monophyletic group and share stamens with elongated pedoconnectives and capsular fruits, but their relationships are still being debated (Michelangeli *et al.*, 2013; Rocha *et al.*, 2016a). So far, the *Marcetia* alliance has been characterized by tetramerous flowers (with some exceptions), a tetralocular ovary (or reduced to two or three locules), absence of crown hairs on the ovary apex, and by cochleate, ovate or lacrimiform seeds (Michelangeli *et al.*, 2013; Rocha *et al.*, 2016a).

The considerable variation in the outer ovule integument thickness and the absence of a broad sampling in Merianieae limit the utility of our data for systematic conclusions at this time. In this tribe, although the shape of the calyx and stamens has been traditionally used in taxonomy, these floral characters have proved to be poorly informative for the circumscription of the more diverse genera (Mendoza-Cifuentes & Fernández-Alonso, 2010, 2012). In this respect, embryological characters such as the one tested here can provide important information for clade delimitation. The variation detected in Merianieae indicates that this character should be considered and more extensively investigated in future systematic studies on the tribe.

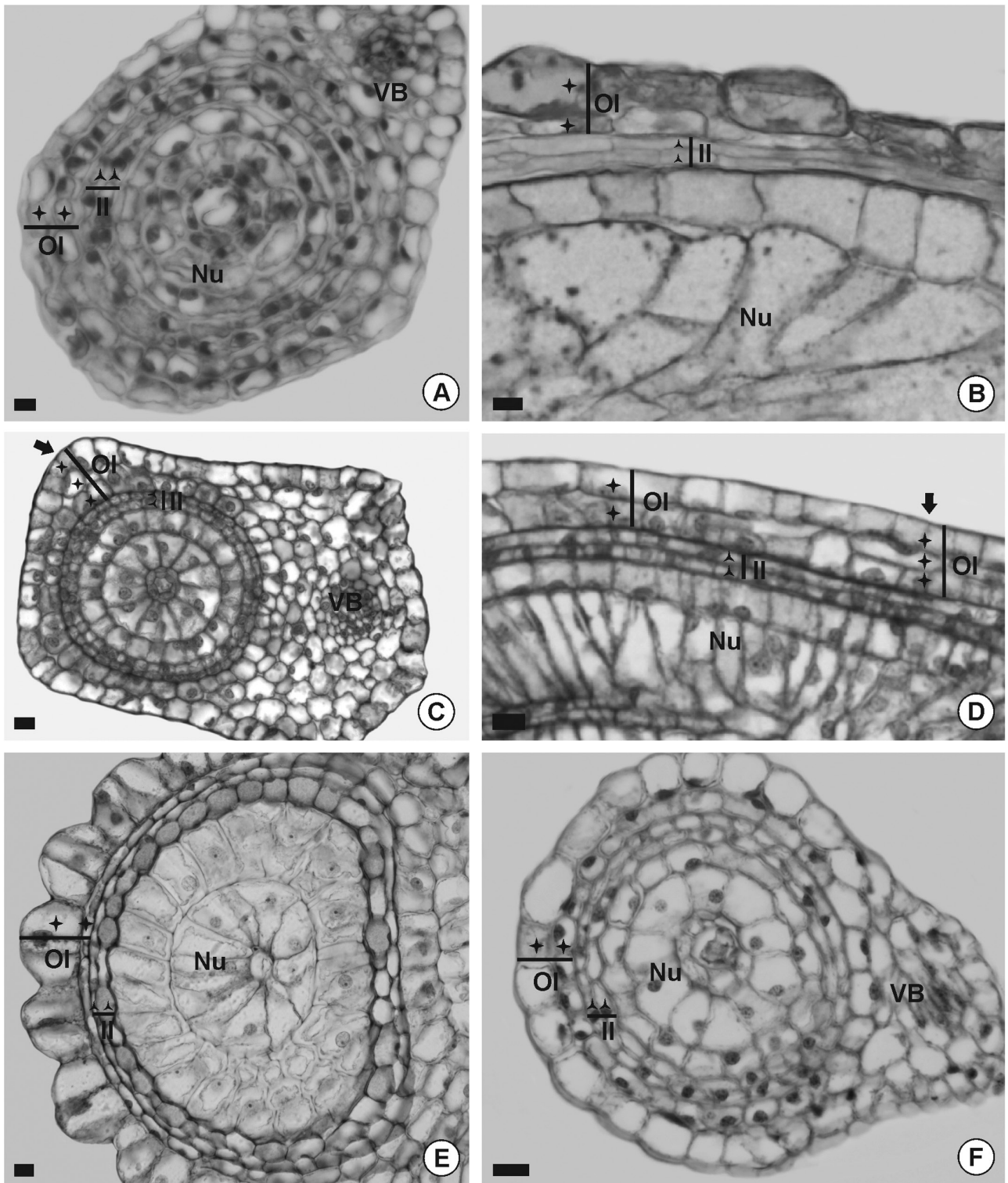
Finally, in Miconieae, despite the considerably frequent multilayered condition, ovules with a two-cell-layered outer integument delimit the genus *Physeterostemon* and two clades. Molecular data indicate that *Physeterostemon* is a well-supported monophyletic genus sister to *Eriocnema*, the two forming a clade sister to the remaining Miconieae (Amorim *et al.*, 2009; Goldenberg *et al.*, 2015). *Physeterostemon* also differs from *Eriocnema* by its inferior ovary (Cogniaux, 1891; Fritsch *et al.*, 2004; Goldenberg & Amorim, 2006; Amorim *et al.*, 2009; Amorim, Jardim & Goldenberg, 2014). Both *Eriocnema* and *Physeterostemon* have capsular fruits, different from the fleshy fruits found in other Miconieae (Amorim *et al.*, 2009).



**Figure 3.** Transverse (A–D and F) and longitudinal (E) sections showing ovules of Melastomataceae species belonging to tribes that exhibit exclusively a two-cell-layered outer integument. A, *Pternandra coerulescens* (Kibessieae). B, *Bellucia grossularioides* (Henrietteae). C, *Henriettea ramiflora* (Henrietteae). D, *Bertolonia mosenii* (Bertolonieae). E, *Chalybea ecuadorensis* (Blakeeae). F, *Allomaieta grandiflora* (Cyphostyleae). Outer integument cell layers are indicated by four-point stars and inner integument cell layers by three-point stars. Nu, nucellus; OI, outer integument; II, inner integument; VB, vascular bundle. Scale bars = 10  $\mu\text{m}$ .

In the Caribbean grade, the clade with *Charianthus* D. Don and *Tetrazygia discolor* differs from the remaining species of *Tetrazygia* Rich. ex DC. by

having a two-cell-layered outer integument. This clade stands out from the grade because of its biogeography, consisting of endemic species of the Lesser Antilles



(Michelangeli *et al.*, 2008), but the relationships in the group remain undefined (Penneys & Judd, 2005; Goldenberg *et al.*, 2008).

The two-cell-layered condition of the outer ovule integument can be seen as a synapomorphy for the *Conostegia* clade because its sister group, the monopodial *Clidemia* D. Don clade, has a multilayered outer integument. The *Conostegia* clade, which included species of *Clidemia*, *Conostegia* and *Miconia* (currently recognized as *Conostegia*) (Kriebel, 2016), is well supported by molecular analyses, but thus far no morphological or anatomical character has been found to diagnose the group (Kriebel *et al.*, 2015).

#### RELATIONSHIP BETWEEN THE MULTILAYERED OUTER INTEGUMENT AND FLESHY FRUITS IN MELASTOMATACEAE

Although we hypothesized that the outer ovule integument thickness would be correlated with fruit type, the results failed to achieve significance. Nonetheless, ancestral state reconstructions show a pattern in which a multilayered outer integument tends to occur more frequently among clades with fleshy fruits, such as the Miconieae and *Mouriri* (Fig. 8). Although a lack of significance in this kind of analysis might be related to the small number of changes in character states over the phylogeny (Felsenstein, 1985), the results may also indicate a more complex scenario for the evolution of this anatomical character.

Fleshy fruits in Melastomataceae are mainly dispersed by endozoochory by birds, but also by other animals such as rats, bats, monkeys and fishes (Magnusson & Sanaiotti, 1987; Renner, 1989; Stiles & Rosselli, 1993; Ellison *et al.*, 1993; Galetti & Stotz, 1996; de Figueiredo & Longatti, 1997; Garcia, Rezende & Aguiar, 2000; Lapenta & Procópio-de-Oliveira, 2008; Maruyama, *et al.*, 2013). Since the seed testa is the mechanical layer responsible for the protection of the embryo in the family (Corner, 1976; Cortez & Carmello-Guerreiro, 2008), the presence of more layers in the outer ovule integument may correspond to more sclerified layers in the seed coat, which in turn could make this seed coat thicker and more rigid. In *Miconia albicans* (Sw.) Triana, for

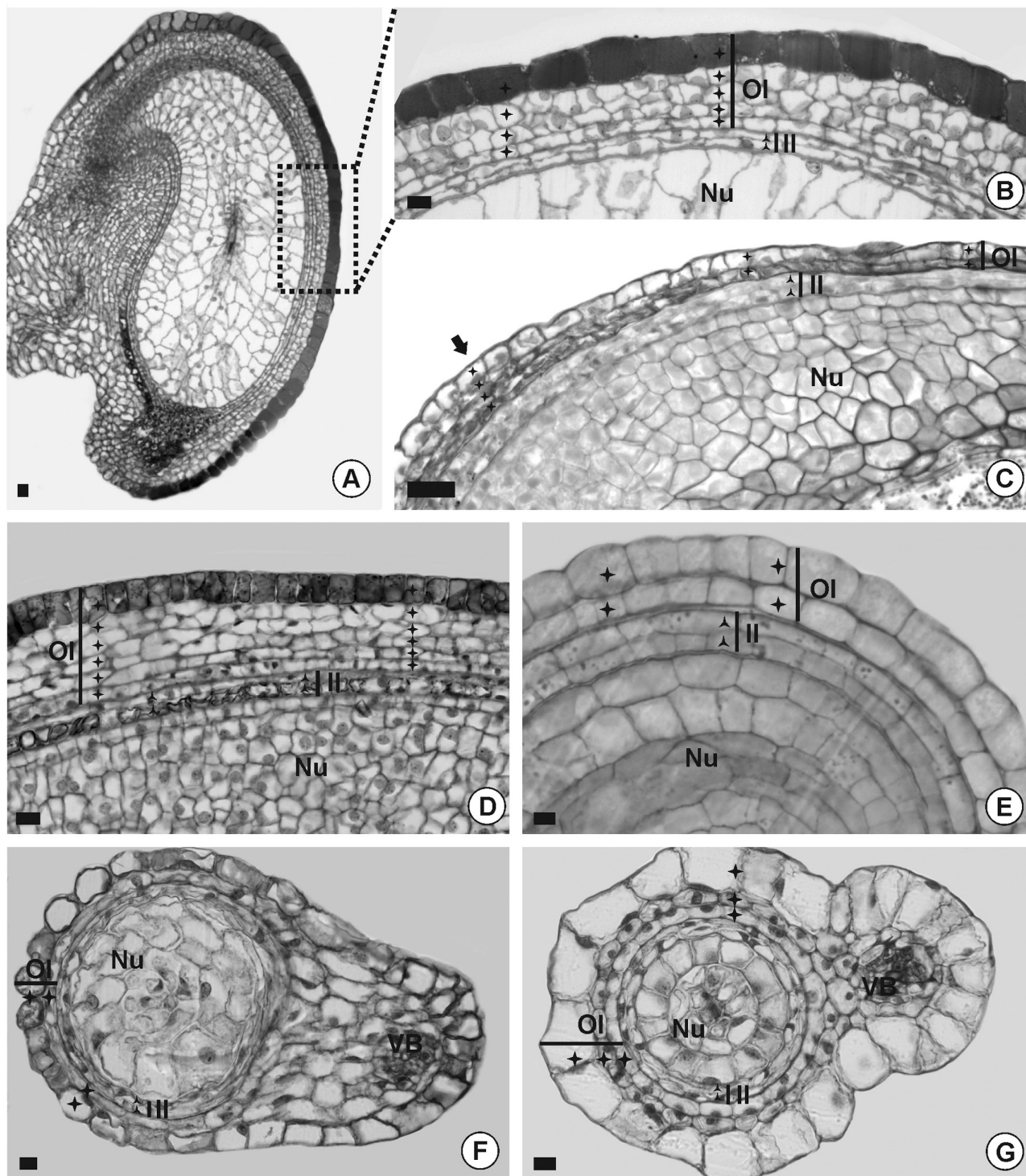
example, the three-layered outer ovule integument gives rise to the testa, which consists of an exotesta of sclerified palisade cells covering the meso- and endotesta with cuboidal sclerified cells (Cortez & Carmello-Guerreiro, 2008). Endozoochory has been frequently related to a resistant seed coat (Baskin & Baskin, 2014), responsible for the protection of the embryo and of other seed components during passage through the digestive tract of animals (Boesewinkel & Bouman, 1984; Mohamed-Yasseen *et al.*, 1994). Thus, the presence of an additional layer in the outer ovule integument may have been an important evolutionary step, particularly in Miconieae and *Mouriri*. Apart from these clades, endozoochoric fleshy fruits have also evolved independently in Blakeeae, Dissochaeteae and Henrietteae (Renner, 1989; Stiles & Rosselli, 1993; Clausen, Meyer & Renner, 2000; Penneys *et al.*, 2010), all tribes with a two-cell-layered outer ovule integument, proving that the multilayered condition is not strictly associated with fleshy fruits. In addition to the multilayered outer integument, the presence of a multiplicative integument can also lead to thicker seed coats, as reported for *Memecylon* (Olisbeoideae) (Corner, 1976). In *Blakea trinervia* L. (Blakeeae), the seed coat is apparently non-multiplicative (Corner, 1976), but we do not know whether this pattern is repeated through the entire tribe. There is no anatomical information on the seed coat structure for Dissochaeteae and Henrietteae.

The relationship between ovules with a multilayered outer integument and fleshy fruits seems to occur in the sister group of Melastomataceae. Crypteroniaceae, Alzateaceae and Penaeaceae mostly have ovules with a two-cell-layered outer integument and capsular fruits (Meijer, 1972; Tobe & Raven, 1983b, 1984a, c, d, 1987a, b; Conti *et al.*, 2002). The only exception is *Olinia* Thunb. (Penaeaceae), with multilayered outer integument and fleshy fruits (Rao & Dahlgren, 1969; Tobe & Raven, 1984b; Conti *et al.*, 2002).

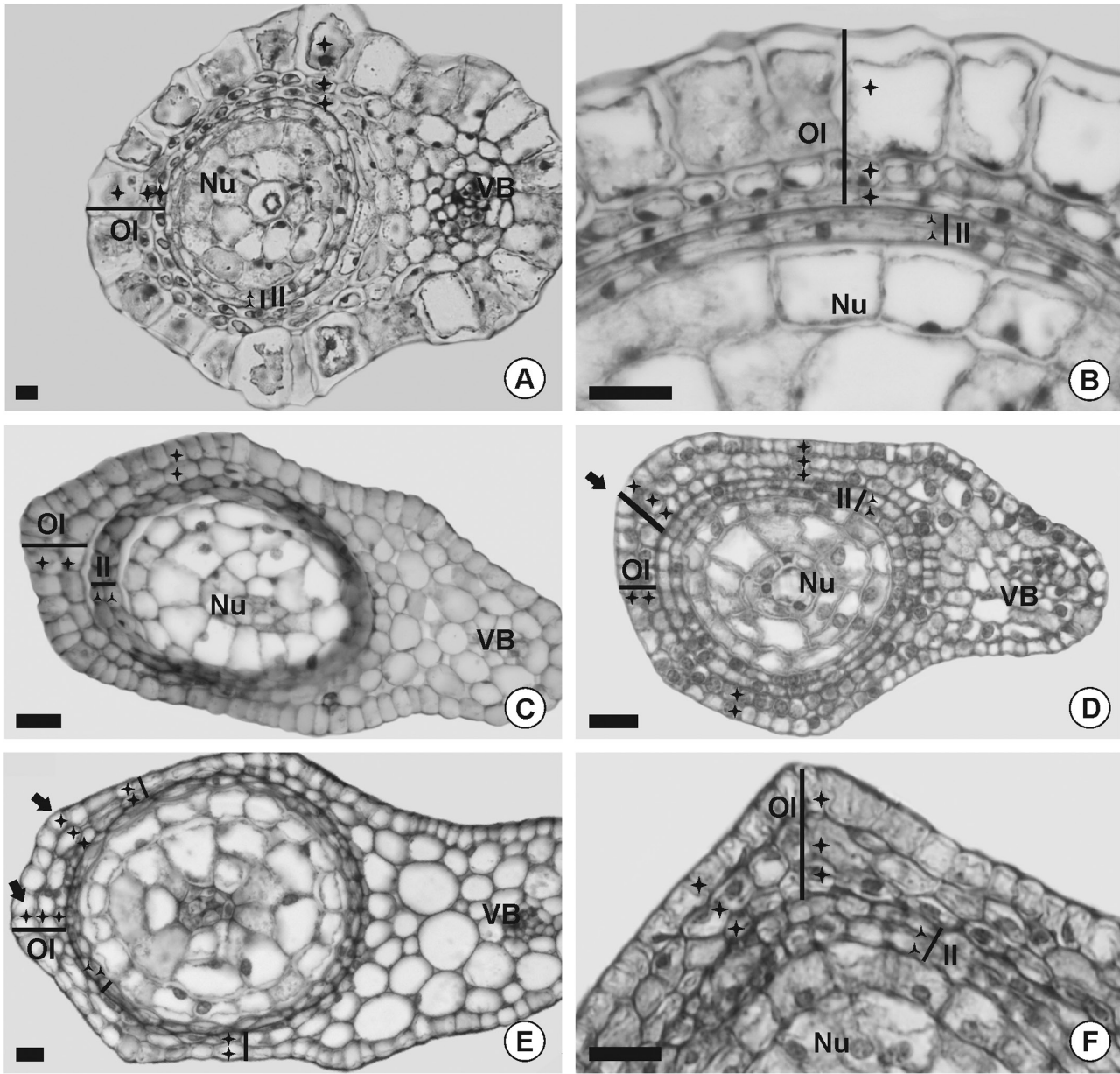
#### MULTIPLICATIVE OUTER INTEGUMENT AND LARGE SEEDS IN MELASTOMATACEAE

Indications of a multiplicative integument were observed in *Votomita* (Olisbeoideae), in species of the

**Figure 4.** Transverse (A, C and E–F) and longitudinal (B, D) sections showing ovules of Melastomataceae species belonging to tribes or clades that exhibit exclusively a two-cell-layered outer integument. A, *Triolena obliqua* (*Triolena* clade). B, *Cambessedesia espora* (*Cambessedesia* clade). C, *Merianthera bullata* (*Cambessedesia* clade). D, *Merianthera parvifolia* (*Cambessedesia* clade). In C and D, punctual periclinal divisions may give rise to a third layer in certain regions of the outer integument (arrows). E, *Arthrostemma ciliatum* (Rhexieae). F, *Microlicia cordata* (Microlicieae). Outer integument cell layers are indicated by four-point stars and inner integument cell layers by three-point stars. Nu, nucellus; OI, outer integument; II, inner integument; VB, vascular bundle. Scale bars = 10 µm.



**Figure 5.** Longitudinal (A–E) and transverse (F–G) sections showing ovules of *Rupestrea* and Melastomataceae species belonging to tribes that exhibit variation in outer integument thickness. A–B, multilayered integument consisting of four to five cell layers in *Rupestrea johnwurdackiana*. C, two-cell-layered integument of *Votomita guianensis* (Olisbeoideae) with a thicker calazal region (arrow). D, multilayered integument of *Mouriri cearensis* (Olisbeoideae). Two-cell-layered integument in species of core Melastomataceae (Melastomataceae). E, *Heterocentron elegans*; F, *Tibouchina clavata*; G, multilayered integument with three cell layers in *Aciotis purpurascens* (Marcetia alliance, Melastomataceae). Outer integument cell layers are indicated by four-point stars and inner integument cell layers by three-point stars. Nu, nucellus; OI, outer integument; II, inner integument; VB, vascular bundle. Scale bars = 10 µm.



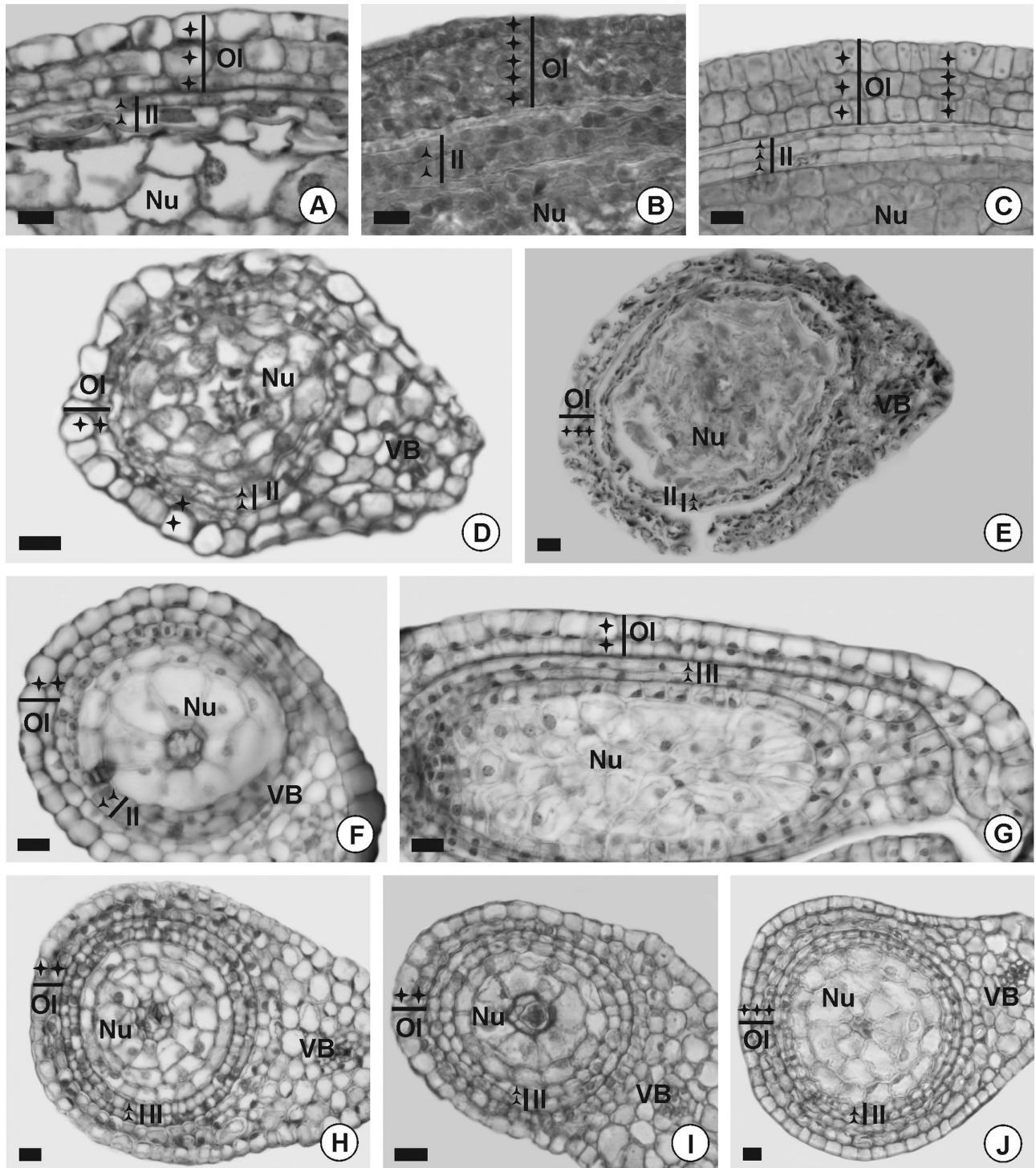
**Figure 6.** Transverse (A and C–F) and longitudinal (B) sections showing ovules of Melastomataceae species belonging to tribes that exhibit variation in outer integument thickness. Multilayered integument with three cell layers in species of the *Marcetia* alliance, Melastomateae: A, *Ernestia glandulosa*; B, *Nepsera aquatica*. Outer integument thickness in Merianieae: C, *Graffenrieda harlingii*; D, *Axinaea dentata*; E, *Meriania urceolata*; F, *Meriania sclerophylla*. Outer integument cell layers are indicated by four-point stars and inner integument cell layers by three-point stars. Nu, nucellus; OI, outer integument; II, inner integument; VB, vascular bundle. Scale bars = 10  $\mu$ m.

*Miconia discolor* subclade and of the *Miconia* V grade (Miconieae), in *Merianthera* (*Cambessedesia* clade) and in *Rupestrea*. In general, ovules that become large seeds have multiplicative integuments, which give rise to thicker and massive seed coats (Corner, 1976; Boesewinkel & Bouman, 1995). We presume that this relationship also exists in different lineages of

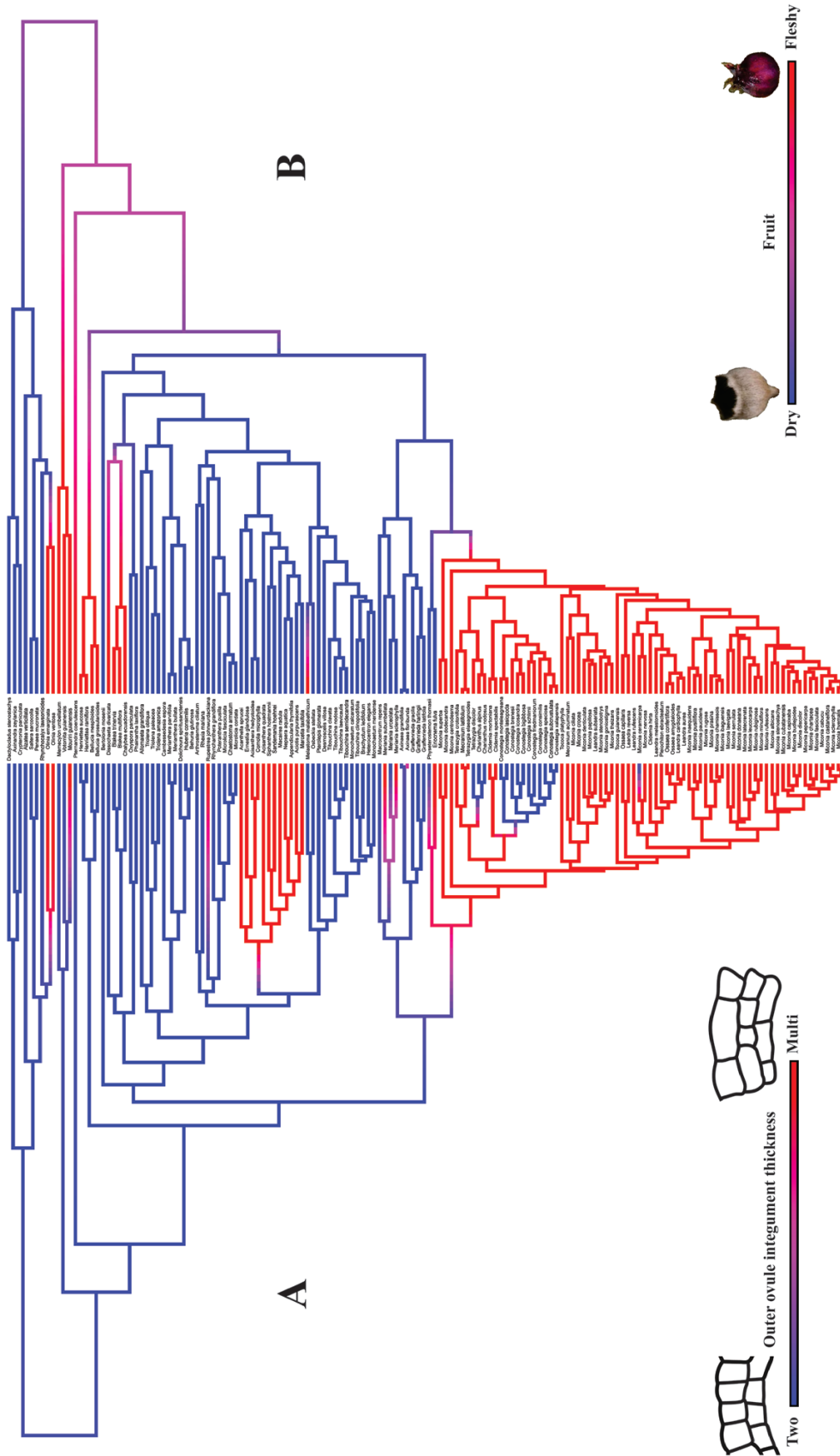
Melastomataceae, independent of the initial condition, as it was found in species with multilayered and two-cell-layered outer ovule integuments.

Olisbeoideae are known to have few ovules that will develop into one or several large seeds (Morley, 1976, 1999; Bremer, 1981; Clausen & Renner, 2001). In this subfamily, in addition to the multiplicative





**Figure 7.** Longitudinal (A–C and G) and transverse (D–F and H–J) sections showing ovules of Melastomataceae species belonging to the Miconieae tribe. A, multilayered integument with three cell layers in *Clidemia hirta*. B, multilayered integument with five cell layers in *Miconia cubatanensis*. C, multilayered integument with three to four cell layers in *Miconia inaequidens*. D, two-cell-layered integument in *Physeterostemon thomasi*. E, multilayered integument with three cell layers in *Eriocnema acaulis*. Two-cell-layered integument in species of the *Conostegia* clade: F, *Conostegia lasiopoda*; G, *Conostegia subcrustulata*. H, two-cell-layered integument in *Charianthus nodosus*. I, two-cell-layered integument in *Tetrazygia discolor*. J, multilayered integument with three cell layers in *Tetrazygia crotonifolia*. Outer integument cell layers are indicated by four-point stars and inner integument cell layers by three-point stars. Nu, nucellus; OI, outer integument; II, inner integument; VB, vascular bundle. Scale bars = 10 µm.



**Figure 8.** Mirror composite trees showing the stochastic mapping of outer ovule integument thickness (A) and fruit type (B) onto the resulting phylogenetic tree of Melastomataceae from the Bayesian analysis using molecular markers. Tree A (outer ovule integument thickness): blue corresponds to the two-cell-layered status, and red to the multilayered status. Tree B (fruit type): blue corresponds to the dry status, and red to the fleshy status.

integument detected in *Votomita*, the same structure has been reported for *Mouriri* and *Memecylon* (Corner, 1976).

In Miconieae, a multiplicative integument consisting of more than three cell layers is a recurrent condition exclusively detected in species belonging to the *Miconia discolor* subclade (*Miconia* IV clade) and the *Miconia* V grade. The *Miconia discolor* subclade and some species of the *Miconia* V grade are notable for their fruits with few large seeds (Goldenberg, 1999; Martins *et al.*, 2009; Caddah, 2013; Ocampo & Almeda, 2013; Ocampo, Michelangeli & Almeda, 2014), which differ from fruits with numerous minuscule seeds observed in most other species of Miconieae (Baumgratz, 1985; Groenendijk, Bouman & Cleef, 1996; Bécquer, Michelangeli & Borsch, 2014; Ocampo *et al.*, 2014). A similar relationship between multiplicative integument and seed size occurs in *Rupestrea*, with a few large seeds in the ovary (Goldenberg *et al.*, 2015).

### CONCLUSIONS

We studied the evolution of the outer ovule integument thickness in Melastomataceae, reporting this condition in a large number of species. In addition, we showed that two-cell-layered and multilayered integuments have different ontogenetic origins. The results indicate that the outer ovule integument thickness is relatively stable in the family, but the variations observed in Olisbeoideae, *Rupestrea*, Melastomateae and Miconieae are of systematic value for the groups. Finally, there is a tendency of association between the multilayered integument and fleshy fruits in Miconieae and *Mouriri*, and between multiplicative integument and large seeds in a few members of the family.

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#### SUPPORTING INFORMATION

**Appendix S1.** List of the species of Melastomataceae analysed in this study, information source for its systematic position, data source and herbarium and voucher data.

**Appendix S2.** GenBank accession numbers for sequences included in the phylogenetic analyses based on plastid and nuclear markers.