

Phylogenetic Placement of *Rhopalobrachium fragrans* (Rubiaceae): Evidence from Molecular (*rps16* and *trnT-F*) and Morphological Data

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ABSTRACT. *Rhopalobrachium* is an endemic New Caledonian genus of Rubiaceae with controversial tribal position, because its protologue was apparently based on two distantly related species, *Rhopalobrachium fragrans* and *Rhopalobrachium congestum*. Despite its new circumscription as a monotypic genus (*Rhopalobrachium fragrans*) the tribal placement of *Rhopalobrachium* remained unknown. We investigated, for *Rhopalobrachium fragrans*, all morphological characters currently used for circumscribing tribes in the subfamily Ixoroideae sensu lato. The species has axillary inflorescences, with valvate flower aestivation, drupaceous fruits with a single pendulous ovule per locule, and 3-porate pollen grains. We also performed Bayesian and parsimony analyses based on two chloroplast markers, *rps16* intron and *trnT-F* region, to assess the phylogenetic position of *Rhopalobrachium* within Ixoroideae sensu lato. These analyses produced well-resolved and strongly supported topologies. The results of both the morphological survey and phylogenetic analyses indicated a position of *Rhopalobrachium fragrans* in the tribe Vanguerieae and a close relationship with the Pacific genus *Cyclophyllum*.

KEYWORDS: Chloroplast DNA, *Cyclophyllum*, molecular phylogeny, morphology, palynology, *Rhopalobrachium*, Vanguerieae.

Rhopalobrachium Schltr. & K.Krause is an enigmatic genus of the large cosmopolitan family Rubiaceae (Robbrecht 1988), which was originally described by Schlechter and Krause (1908) to accommodate two New Caledonian species, *R. congestum* Schltr. & K.Krause and *R. fragrans*. These authors did not select the type species of their new genus. In the original publication, *Rhopalobrachium* was described with the following characters: "stipules entire, flowers hermaphrodite, ovary 2-locular with a single pendulous ovule per locule, corolla infundibuliform"; the fruits were unknown. Later, Guillaumin (1930) emended the generic description with fruit characters: "fruits bacciform, 1.5 cm wide, with blackish, persistent calyx lobes, and containing four seeds". The two seeds per locule in *Rhopalobrachium* reported by Guillaumin (1930) were, however, inconsistent with the single ovule per locule cited in the protologue.

Based on fruit morphology (fleshy fruits and pluri-ovulate locules), Tirvengadam (1991) transferred *Rhopalobrachium congestum* to the genus *Pelagodendron* Seem. (Gardenieae). In a later revision of the genus *Aidia* Lour. of Gardenieae, by Ridsdale (1996), this species was treated as *Aidia congesta* (Schltr. & K.Krause) Ridsdale. Since then, *Rhopalobrachium* sensu Tirvengadam (1991) has

been treated as a monotypic genus with an unknown tribal position.

Following Guillaumin's suggestion, Kanehira (1932) transferred a Japanese species *Timonius megacarpum* Kanehira (Guettardeae) with a 2-locular ovary and with each placenta bearing 4–10 ovules to *Rhopalobrachium* (*R. megacarpum* (Kanehira) Kanehira). Kanehira (1935) subsequently transferred it to tribe Gardenieae in the new genus *Trukia* Kanehira. This species was later considered to be a synonym of *T. carolinensis* (Valeton) Kanehira & Hatusima and was more recently recognized by Puttock (1999) as *Atractocarpus carolinensis* (Valeton) C.F.Puttock.

Tirvengadam (1991) formally lectotypified *Rhopalobrachium* choosing *R. fragrans* as lectotype and placed it in the tribe Alberteae following the previous position given for *Rhopalobrachium* sensu Schlechter & Krause (Schlechter and Krause 1908; Krause 1915; Guillaumin 1930, 1948; Puff et al. 1984, with critical remarks). More recently, Robbrecht (1993) considered *Rhopalobrachium* sensu Tirvengadam (1991) to be *incertae sedis*, and no further hypothesis of classification was given.

According to its original description (Schlechter & Krause 1908), *Rhopalobrachium fragrans* shares some characteristics of the tribes currently recognized in the subfamily Ixoroideae s.l. (Andreasen

and Bremer 2000; Rova et al. 2002). The goal of the present study is to clarify the tribal position of *Rhopalobrachium* sensu Tirvengadam (1991) within Ixoroideae s.l. and then its relationships within the inferred tribe, using both morphological data and molecular phylogenetics (*rps16* and *trnT-F*).

MATERIALS AND METHODS

Morphology. We investigated selected inflorescence, floral (e.g., shape, ovary organization), pollen, and fruit characters, which are currently used for tribal recognition in Ixoroideae sensu Andreasen & Bremer (2000). Sampling included *R. fragrans* as well as representative taxa from all presently recognized tribes in Ixoroideae s.l. (e.g. species sampled for molecular phylogenetics). The terminology we used followed Robbrecht (1988) and Weberling (1989). Both herbarium and field studies of *R. fragrans* were undertaken by the first author in 2003. The herbarium material was examined at the Brussels Botanical Garden (BR), the Geneva Herbarium (G), the Herbarium of New Caledonia (NOU), and the Paris National Herbarium (P). The determination of the specimens was done by comparisons with the type specimens (isotypes at BR, G, P). Between 5 and 30 specimens where studied per species.

Seventeen species were sampled for palynological observations (Appendix 1). Additional information was taken from the literature (Robbrecht 1988; Andreasen & Bremer 2000; Dessein et al. 2005). The preparation of the pollen grains, sampled from herbarium specimens (P), followed Reitsma (1969). Pollen isolated was dehydrated (glacial acetic acid) and acetolysed (concentrated sulphuric and acetic acid 1:9 for 1–2 min at 95°C). Pollen destined for light microscopy (LM) were mounted in glycerin enclosed by paraffin. The LM pollen collection is deposited at P. Grains observed with scanning electronic microscopes (SEM; JEOL JSM_840 scanning microscope) were suspended in ethanol and air dried on a stub. Before SEM examination, the pollen grains were coated using a JEOL JFC_1200 Fine coater. The terminology we use for pollen descriptions follows Punt et al. (1994).

Molecular Phylogeny. TAXONOMIC SAMPLING. A total of 34 taxa (Appendix 2), representing all major clades (almost all formally recognized as tribes) in Ixoroideae s.l.: Alberteae, *Augusta* group, Bertiereae, Coffeae, Condamineae, Cremasporae, Gardenieae, Ixoreae, Mussaendeae, Octotropideae, Pavetteae, Retiniphyllae, Sabiceae (sensu Bremer & Thulin, 1998), Sipaneeae, and Vanguerieae, were included in the *rps16* analysis to assess the tribal position of *Rhopalobrachium fragrans*. *Chiococca alba* and *Guettarda crispiflora* of subfamily Cinchonoideae and *Luculia gratissima*, the latter positioned basal to the three subfamilies of Rubiaceae (Bremer et al. 1999), were used as outgroup taxa.

According to the first analysis of the *rps16* sequencing data, in which *R. fragrans* was nested within Vanguerieae, a total of 41 Vanguerieae species (Appendix 3), representing most of the recognized genera of the tribe (25 out of 30), were analyzed to further pinpoint the placement of *Rhopalobrachium* using the chloroplast *trnT-F* region. Some rare genera (*Eriosemopsis* Robyns, *Everistia* S.T.Reynolds & R.J.F.Hend., *Perakanthus* Robyns ex Ridl., *Tenmocalyx* Robyns and *Vangueriella* Verdc.) were not included because of lack of sequencable material. Outgroup taxa were chosen from Ixoroideae s.l., following Lantz and Bremer (2005): *Alberta magna* (Alberteae), *Ixora coccinea* (Ixoreae), and *Mussaenda erythrophylla* (Mussaendeae). We were unable to test the monophyly of *Rhopalobrachium* sensu Schlechter and Krause (1908) and sensu Kanehira (1932) due to lack of material for both *R. congestum* and *R. megacarpum*.

DNA EXTRACTION, AMPLIFICATION, SEQUENCING, AND ALIGNMENT. Total DNA was isolated from leaf-material dried in silica-gel or from herbarium specimens following the mini-prep procedure of Saghai-Marouf et al. (1984), as modified by Doyle & Doyle (1987). The extracted DNA was cleaned using Qia-Quick PCR purification kit (Qiagen®). The *rps16* intron was amplified with primer pair *rpsF/rpsR2* (Oxelman et al., 1997). For half of the species, we repeatedly failed to obtain amplification for the whole region using these primers, but we successfully amplified and sequenced the *rps16* with the internal primer pair *rpsF2/rpsR3* (Bremer et al., 2002).

The entire *trnT-F* region (including the *trnL* gene) of all newly investigated specimens was amplified in two parts. The *trnT-trnL* segment was amplified with the primer pair A1/I (Razafimandimison and Bremer 2002; Bremer et al. 2002) and the *trnL-trnL-trnF* region with the pair C/F (Taberlet et al. 1991). Sequencing reactions were prepared using the two external primer pairs A1/I and C/F. Two internal primers D/E (Taberlet et al. 1991) were added to produce complete sequences of the entire regions of *trnL-F*, with at least partial overlap (from 10 to 25%).

The sequences were assembled using Staden Package v 1.6.0 beta-test (Staden 1996) and Sequencher 3.1.1 and edited manually. All sequences (Appendices 2–3) were aligned manually with Se-Al v1.0al (Sequence Alignment Editor Version 1.0 alpha 1; Rambaut 1996). The gap locations were considered unambiguous when only one logical sequence alignment was possible due to the conservation of both gap length and nucleotide motifs adjacent to the 5' and 3' boundaries of the gap. Unambiguous and informative insertions and deletions (indels) were then coded as additional characters by using 0 and 1 symbols for deletions and insertions, respectively (Swofford 1993). There was 13.8% missing data in the *rps16* matrix and 6.1% in the *trnT-F* matrix. The matrices are available in TreeBASE (study number S1765).

PHYLOGENETIC ANALYSES. Parsimony analyses were performed with PAUP* 4.0b10 (Swofford 2002) using the following settings: heuristic search, tree-bisection-reconnection (TBR) branch swapping, with 100 replicates of random stepwise addition, MULTREES option on. Characters were given equal weights, gaps were treated as missing data, and phylogenetically informative indels were coded as an additional partition. The Consistency Index (CI; Kluge and Farris 1969) and the Retention Index (RI; Farris 1989) were calculated to estimate the level of homoplasy. The bootstrap re-sampling method (BS; Felsenstein 1985), using 10000 replicates, MULTREES option off, nearest neighbour interchanges (NNI) branch swapping, and five random addition sequences, was used as a measure of the relative support for the identified clades. Groups receiving a bootstrap support over 95% were regarded as well supported (Felsenstein 1985).

Bayesian analyses were performed with Mr. Bayes 3.0b4 (Huelsenbeck and Ronquist 2001). The Bayesian approach evaluates the posterior probability (PP) of a tree given the character matrix, i.e. the probability that the tree is correct. The MrModeltest 2.0 (Nylander 2004) was used for choosing the model of nucleotide substitution that best fit the data. A partitioned Bayesian analysis was conducted to account for the *rps16* sequence regions and the coded gap data. The selected model was General Time Reversible (Yang 1994) with among-site substitution rate heterogeneity described by a gamma distribution and all sites constrained to be variable (GTR + Γ) using the Akaike information criterion (Akaike 1973). The gap-coded data were analyzed with a binary model. A partitioned Bayesian analysis was then performed with *trnT-F*, including standard binary characters from gap-coding.

TABLE 1. Summary of characters in Ixoroideae tribes compiled from our morphological and palynological study of *Rhopalobranchium fragrans* and the available literature.

| Lineage | Inflorescence | Aestivation | Stigma | Nb. ovule/locule | Seed | Fruit | Pollen |
|-------------------------|-----------------------|-------------|---------------------------------------|------------------|-----------|------------|------------------------------|
| Alberteae | Terminal | Contorted | Rounded bifid | 1 | Pendulous | Dry | 3-colporate |
| <i>Augusta</i> group | Terminal | Contorted | Linear bifid | Many | Adaxial | Dry | 3-colporate |
| Bertiereae | Terminal | Contorted | Linear bifid | Many | Adaxial | Fleshy | 3-colporate |
| Coffeae | Axillary | Contorted | Linear bifid | 1 | Adaxial | Fleshy | 3-colporate |
| Condamineae | Terminal | Valvate | Variable | Many | Adaxial | Dry | 3-colporate |
| Cremsporeae | Axillary | Contorted | Linear bifid | 1 | Adaxial | Fleshy | 3-colporate |
| Gardenieae | Terminal/ Axillary | Contorted | Linear bifid | 1 to many | Variable | Fleshy | 3-colporate/ 3-po(ro)rate |
| <i>Greenea</i> group | Terminal | Contorted | Linear/Globose | Many | Adaxial | Dry | 3-colporate |
| Ixoreae | Terminal | Contorted | Linear bifid | 1 | Adaxial | Fleshy | 3-colporate |
| Mussaendeae | Terminal | Valvate | Linear bifid | Many | Adaxial | Fleshy/Dry | 3-colporate |
| Octotropideae | Axillary | Contorted | Linear bifid | 1 to many | Variable | Fleshy | 3-colporate |
| Pavetteae | Terminal | Contorted | Linear bifid | 1 to many | Adaxial | Fleshy | 3-colporate |
| Retiniphyllae | Terminal/Axillary | Contorted | Globose 5-merous | 2 | Adaxial | Fleshy | 3-colporate |
| Sabiceae | Axillary | Valvate | Linear 5-merous | Many | Adaxial | Fleshy | 3-colporate |
| Sipaneae | Terminal/Axillary | Contorted | Globose bifid | Many | Adaxial | Fleshy | 3-colporate |
| Vanguerieae | Axillary | Valvate | Stylar head complex/ Rounded bifid | 1 | Pendulous | Fleshy | 3-po(ro)rate |
| <i>Rhopalobranchium</i> | Axillary | Valvate | Rounded bifid | 1 | Pendulous | Fleshy | 3-pororate |

For *trnT-F* sequence data, three partitions were used to represent the different parts of the marker, the *trnT-L* spacer, the coding *trnL* and the *trnL-F* spacer. The selected model were GTR + Γ with variant sites for the *trnT-trnL* spacer and the same model with invariant sites (+I) for *trnL* and *trnL-trnF*. The gap-coded matrix was analyzed as a fourth partition by assignment of a binary model. All analyses were conducted with four independent Markov chains run for 2 millions metropolis-coupled MCMC generations, with trees sampled every 1,000 generations, and burn-in after 500,000 generations as detected by plotting the log likelihood scores against generation number. The analyses were replicated four times using different random starting trees to assess the convergence of the likelihood values and clade posterior probabilities (Huelsenbeck et al. 2002). The trees of the four independent analyses were used to compute the consensus tree.

RESULTS

The morphological, palynological, and phylogenetic approaches gave numerous new results for *Rhopalobranchium fragrans* characteristics and relationships with other species.

Morphology. The plants of *Rhopalobranchium fragrans* are functionally dioecious shrubs. They have opposite leaves with entire interpetiolar stipules. The inflorescences are axillary, paired at nodes, and poorly flowered. The bud aestivation is true valvate and the corollas are hypocrateriform (tube cylindrical and slender, with perpendicular lobes). The stigmas are pollen presenters, with slightly bifid and convex at base. The ovaries are two locular and contain a single pendulous ovule per locule. The fruits are drupaceous, with seeds protected by pyrenes. The main characters generally used in combination for tribal recognition are summarized in Table 1, for the Ixoroideae tribes.

The Table 1 clearly shows that *R. fragrans* is morphologically similar to Vanguerieae.

Pollen Morphology. The pollen of *Rhopalobranchium fragrans* is 3-zono-porate, bearing elliptic pores and a foveo-reticulate tectum, with larger luminae in polar axes. Grains are around $45\text{--}50 \times 30\text{--}35 \mu\text{m}$. The pollen of the Vanguerieae representatives is 3-zono-po(ro)rate, with circular to elliptic pores. The tectal ornamentation varies from slightly punctate to foveo-reticulate and is slightly differential from aperture to polar axes in *Cyclophyllum* and *Pyrostria* representatives. In contrast, it is uniformly reticulate with deep muri in *Psydrax odorata* and *P. paradoxa*. Grains are around $35\text{--}50 \times 25\text{--}35 \mu\text{m}$. The pollen of tribes Alberteae, Coffeae, and Ixoreae is, contrarily to former taxa, 3-zono-colporate, with smooth to rugulose and punctate to foveolate tectum. Grains are of a small size, $15\text{--}25 \mu\text{m}$ in diameter. The type of pollen for each tribe is reported in Table 1.

The rps16 Analyses of Ixoroideae s.l. The analyses of *rps16* data included 804 characters (out of the 1,011 including ambiguous sites), of which 143 were parsimony informative and 17 were indels. The maximum pairwise distances of the *rps16* sequences between the ingroup and outgroup taxa and between the ingroup taxa were 0.01 (uncorrected p) and 0.06, respectively. The strict consensus trees from the maximum parsimony (MP) and Bayesian analyses (BA) had similar overall tree topologies. The only discrepancies occurred in three unsupported nodes, mainly along the basal nodes of the ingroup taxa. *Rhopalobranchium fragrans* was resolved with high

support (BS = 89; PP = 1.00) as sister to *Cyclophyllum* in a strongly supported (BS = 99; PP = 1.00) clade containing the representatives of Vanguerieae (Fig. 2). The Vanguerieae taxa, plus *R. fragrans*, shared six unambiguous substitution synapomorphies (CI=1) and a 5-bp insertion. The *rps16* sequence of *R. fragrans* had ca. 0.02 pairwise divergence with the other investigated Vanguerieae, 0.045 with Alberteae, and ca. 0.05 with Gardenieae.

The *trnT-F* Analyses of Vanguerieae. The analyses of *trnT-F* data included 1,779 characters (out of the 2,336 including ambiguous sites), of which 136 were parsimony informative, and 39 were indel positions. The computed MP and BA consensus trees (Fig. 3) shared the same overall tree topologies, with the exception of a single unresolved node between the *Vangueria* group and the *Robynsia-Pygmaeothamnus* within group J in the parsimony analysis (as noted by * in Fig. 3). Several internal nodes were moderately supported with the parsimony analysis, while the Bayesian analysis gave them strong support (PP > 0.95), notably clade A and the subclades F and G (Fig. 3).

Vanguerieae was resolved into two main clades: Clade A including the investigated *Rhopalobrachium* and the genera *Afrocanthium* (Bridson) Lantz & B. Bremer, *Cyclophyllum*, *Keetia* E. Phillips, *Psydrax* Gaertn., *Pyrostria* Comm. ex A.L. Jussieu and relatives (BS = 79; PP = 1.00); and Clade B containing the type genus of the tribe, *Vangueria* Juss., and its allied genera (BS = 100; PP = 1.00). Within Clade A, five distinct groups, labelled C-G were resolved with high support (PP ≥ 0.99) as monophyletic: Subclade C forming *Psydrax dicoccos* and *P. paradoxa*; Subclade D containing *Afrocanthium burttii* and *A. keniense*; Subclade E consisting of *Keetia gueinzii* and *K. lukei*; Subclade F forming *Rhopalobrachium fragrans*, *Cyclophyllum balansae*, and *C. deplanchei* (PP = 1.00); and Subclade G containing *Pyrostria anjouanensis* to *Canthium pseudo-sitiflorum*. The three investigated *Pyrostria* species formed a poorly supported (PP = 0.89) monophyletic group within the Subclade G. The relationships between *Pyrostria*, *Scyphochlamys* Balf.f., *Pseudopeponidium* Arènes, *Leroya* Cavaco, and *Canthium cystiporon* Cavaco remained unresolved. The unresolved Subclade F, consisting of *Cyclophyllum* species and *Rhopalobrachium fragrans* shared a few synapomorphic mutations within the *trnL* intron. The three taxa constituting Subclade F were very similar to each other with maximum pairwise distances of 0.01. We successively failed to obtain the *trnT-L* spacer of the *trnT-F* region for *Rhopalobrachium fragrans*, which may account for unresolved subclade F. Clade B

consisted of three strongly supported (PP = 1.00) subclades H-J: *Canthium coromandelicum*, the investigated *Fadogia*, *Rytigynia*, *Cuviera*, *Multidentia*, *Pygmaeothamnus*, *Robynsia*, and *Vangueria* representatives.

DISCUSSION

The main goal of the present study is to determine the phylogenetic placement of *Rhopalobrachium* sensu Tirvengadam (1991) in Ixoroideae s.l. Our *rps16* (Fig. 2) and *trnT-F* (Fig. 3) trees are congruent with the previous analyses obtained from other chloroplast markers, *rbcl* and *trnL-F* and *rps16* (Andreasen and Bremer 2000; Rova 1999, respectively). Neither our molecular (Fig. 2) nor morphological data (Table 1) support the traditional tribal placement of *R. fragrans* in Alberteae (Schlechter and Krause 1908; Guillaumin 1930; Puff et al. 1984), which presently contains two genera, *Alberta* and *Nematostylis*. Alberteae have terminal thyrses, contorted, counter-clockwise aestivation, zygomorphic to infundibuliform corolla tubes, 2-locular ovaries with one ovule per carpel, and fruits elongated and dry (Table 1). In contrast, *R. fragrans* bears axillary and opposite cymose inflorescences, hypocrateriform corollas with valvate aestivation, and drupaceous fleshy fruits (Table 1). The initial confusion resulting in the placement of *Rhopalobrachium* sensu Schlechter and Krause (1908) in Alberteae appears to be due to its chimerical generic description. Effectively, a mix of characteristics from two distantly related taxa, *Aidia congesta* (Gardenieae) and *R. fragrans* (Vanguerieae), seems to have been used for the protologue of the genus. For example, uni-ovulate ovaries mentioned in the generic protologue are characteristic of *R. fragrans* specimens, while *A. congestum* has pluri-ovulate locules. On the other hand, contorted aestivation does not occur in *R. fragrans* and must have been observed from *A. congestum* only. This misinterpretation of the morphological features for the genus *Rhopalobrachium* and the rarity of the available material of *R. fragrans* are most likely the origin of its heretofore enigmatic placement and dubious identity of the species.

The combination of axillary inflorescences, valvate aestivation, a single ovule per locule, and 3-po(ro)rate pollen grains (Table 1; Fig. 1) all suggest a close affinity of *Rhopalobrachium fragrans* and Vanguerieae sensu Lantz & Bremer (2004). The placement of *R. fragrans* within Vanguerieae, and more precisely together with *Cyclophyllum*, is well supported by both the *trnT-F* MP and BA consensus trees (Fig. 3). Referring to Lantz and Bremer (2005), the cup-shaped stigma, also called the

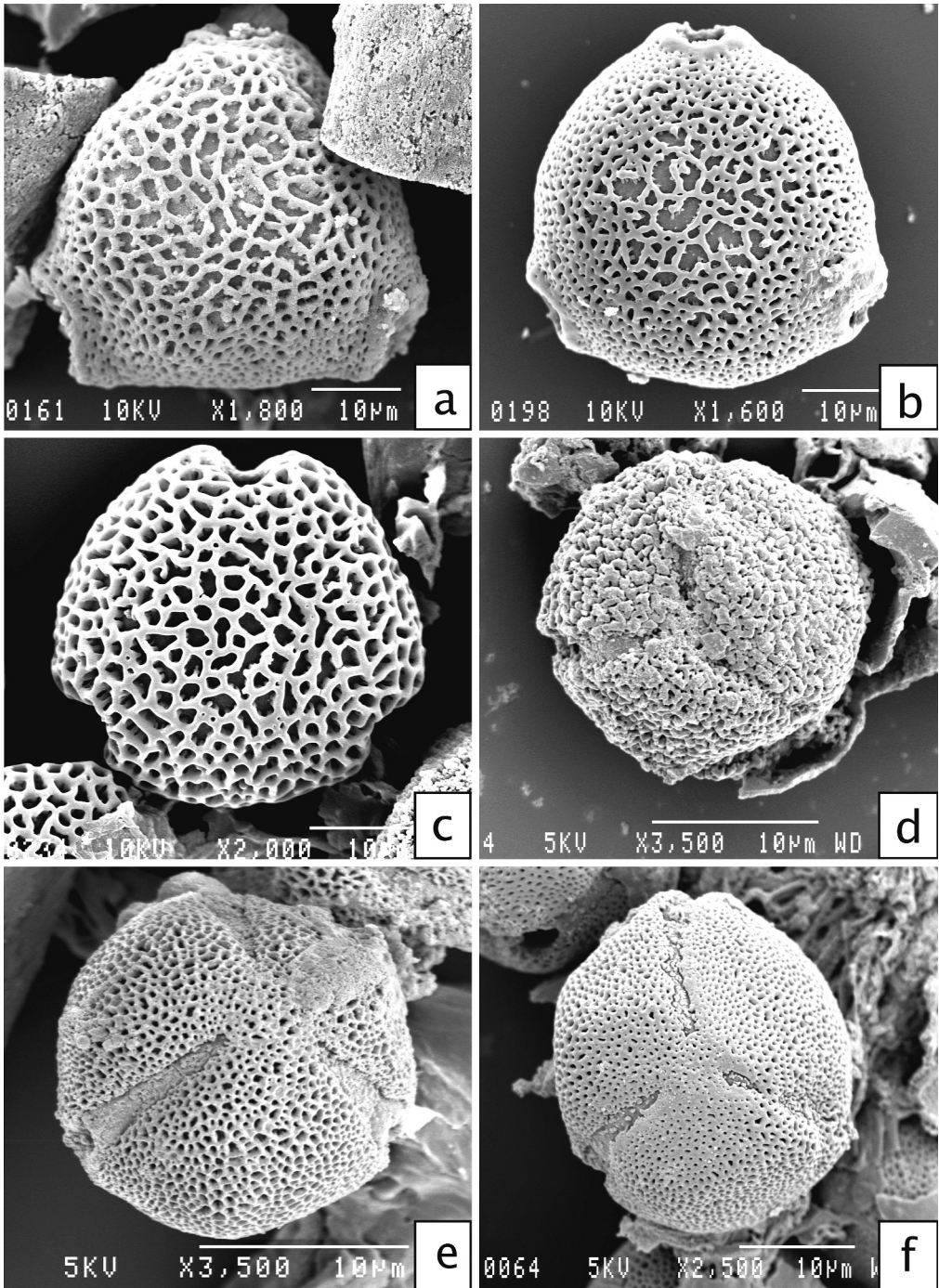


FIG. 1. Pollen grains in polar view in SEM. a. *Rhopalobrachium fragrans*, 3-porate. b. *Cyclophyllum deplanchei*, 3-porate. c. *Psydrax odorata*, 3-porate. d. *Coffea liberica*, 3-colporate. e. *Ixora yaouhensis*, 3-colporate. f. *Alberta minor*, 3-colporate.

“stylar head” complex (Igersheim 1993), only occurs in Vanguerieae and was considered a morphological synapomorphy of the tribe. Our data do not support this. In *Cyclophyllum*, *Everistia*, *R. fragrans* and some *Pyrostria* species, the stigmatic

heads are rounded to campanulate and are attached to the style at its convex base (Reynolds and Henderson 2001). This adapted shape to secondary pollen presentation, common in Ixoroideae, is not plesiomorphic in Vanguerieae, accord-

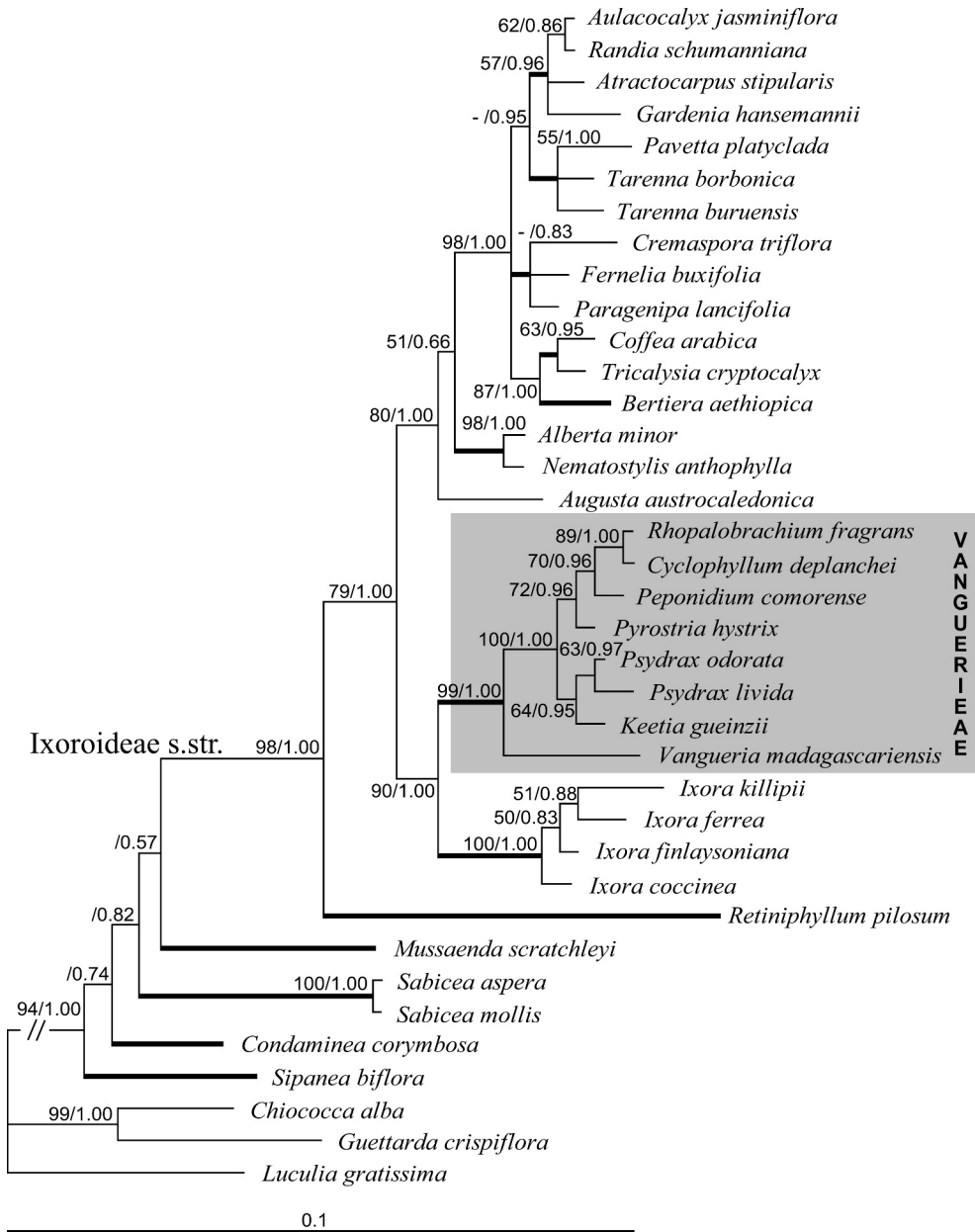


FIG. 2. Majority rules consensus tree resulting from the Bayesian analyses (2M generations) of Ixoroideae s.l. generated from the *rps16* dataset. Numbers above nodes on the left are bootstrap support from the parsimony analysis of identifiable nodes (if missing, different resolution compare to Bayesian analysis; if '-', support below 50%), numbers to the right are the Bayesian posterior probabilities. The gray box indicates tribe Vanguerieae. Bold branches identify the clades currently recognized at the tribal level within the Ixoroideae s.l.

ing to our results, but appears to be secondarily and independently derived compared to other Ixoroideae (Fig. 3). The evolution of this character is probably related to the sexual behaviour. *Rhopalobrachium* appears functionally dioecious. The male and female flowers have the same morphology, except for anther reduction and sterility in the latter and undeveloped stigmatic

lobes and reduced ovaries in the former. Herbarium studies and field observations suggest that the following species of *Cyclophyllum* are functionally dioecious: *C. balansae*, *C. barbatum* (G.Forst.) N.Hallé & J.Florence, *C. coprosmoides* (F.Muell.) S.T.Reynolds & R.J.F.Hend., *C. henriettae* (Baill.) Guillaumin, *C. maritimum* S.T.Reynolds & R.J.F. Hend, *C. pancheri* (Baill.) Guillaumin, *C. sagittatum*

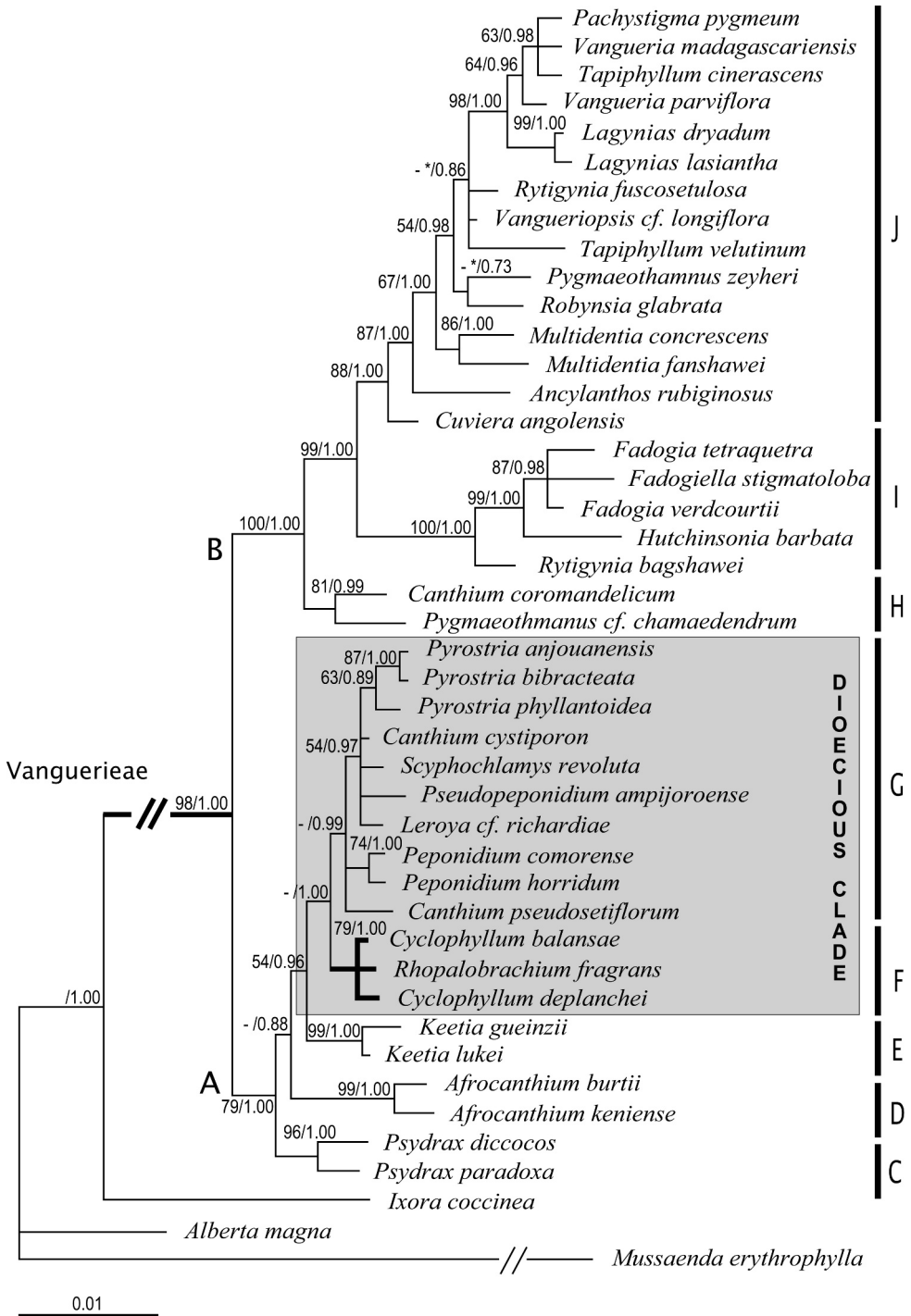


FIG. 3. Majority rules consensus tree resulting from the Bayesian analyses (2M generations) of Vanguerieae generated from the *trnT-F* dataset. Numbers above nodes to the left are bootstrap support from the parsimony analysis of identifiable nodes (if missing, different resolution compare to Bayesian inference; if '-', support below 50%), number to the right are the Bayesian posterior probabilities. Capital letters below the nodes indicate the considered monophyletic lineages; capital letters on the right are the main clades of Vanguerieae. The gray box indicates the 'dioecious clade' of Vanguerieae.

Guillaumin, *C. subalatum* (Baill.) Guillaumin, and *C. tenuipes* Guillaumin. Accordingly, Lantz and Bremer's (2004) dioecious clade should be enlarged to include both *Cyclophyllum* and *R. fragrans* (Fig. 3). The finding of secondary hermaphroditism in *Cyclophyllum deplanchei*, as for *Pyrostria bibracteata* in *Pyrostria*, is inconsistent with Bridson (1987) and Davis and Ruhsam (2005), who regarded all *Cyclophyllum* as hermaphroditic.

Both the morphological and molecular data support a close relationship between *Rhopalobrachium fragrans* and *Cyclophyllum* (Clade F, Fig. 3) within Vanguerieae, an affinity never proposed before. *Cyclophyllum* is an indigenous genus of the Pacific Islands and Australia, with an extensive radiation in New Caledonia where *R. fragrans* occurs. The multiple substitutions and indels supporting the *C. deplanchei* and *R. fragrans* clade (e.g., four substitutions and two indels within the *rps16* intron) are strong indications of a close relationship between *Cyclophyllum* and *Rhopalobrachium* sensu Tirvengadam (1991). *Rhopalobrachium* shares many morphological characters with *Cyclophyllum*, notably hypocotyliform and long corolla (from 1–4 cm), bearded corolla throat, obpyriform fruits and 3-porate pollen grains with foveolate tecta (Fig. 1; Lens et al. 2000).

The Vanguerieae genera that were not included in our analysis (*Eriosemopsis*, *Everistia*, *Neoleroya* Cavaco, and *Temnocalyx*) do not appear morphologically similar to *R. fragrans*. *Eriosemopsis* was recognized to be related to *Pygmaeoathamnus* s.s. (Lantz 2003); *Temnocalyx* is thought to be related to *Fadogia* and *Vangueriopsis* (Lantz 2003) and should therefore fall within the *Vangueria* group (Lantz and Bremer 2005); *Neoleroya* was shown embedded in the *Pyrostria* group (Lantz et al. 2002) and *Everistia* is presumably related to *Psydrax* (Reynolds and Henderson 2001; Lantz 2003). Thus, we do not expect these missing taxa to be closely related to *R. fragrans* and propose *Rhopalobrachium* sensu Tirvengadam (1991) as member of the tribe Vanguerieae dioecious clade with close relationships with *Cyclophyllum*.

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APPENDIX 1. List of species and specimens used for palynological investigation of Ixoroideae s.l. to complete the data from literature. The source of the material and the herbarium where the specimen used is deposited is mentioned.

Alberteae. *Alberta minor* Baill.: Madagascar, Baron 2019 (P); *Nematostylis anthophylla* (A.Rich.) Baill.: Madagascar, Keraudren 1 (P). Coffeaeae. *Coffea arabica* L.: Africa, Chealier 45086 (P); *Coffea liberica* Hiern: Africa, Berthe 422 (P). Ixoreae. *Ixora yaouhensis* Schltr.: New Caledonia, McPherson 6404 (P); *Myonima violacea* (Lam.) Verdc.: Mascarene Islands, Bosser 21865 (P). Vanguerieae. *Cyclophyllum balansae* (Baill.) Guillaumin: New Caledonia, McKee 4143 (P); *Cyclophyllum deplanchei* Hook.f.: New Caledonia, McKee 22091 (P); *Cyclophyllum maritimum* ST.Reynolds & R.J.F.Hend: Australia, Specht L1312 (BRI); *Cyclophyllum merrillianum* Guillaumin: New Caledonia, Bonati 997 (P); *Cyclophyllum saviense* Guillaumin: Vanuatu, Aubert de la Rue 450 (P); *Psydrax odorata* (G.Forst.) A.C.Sm. & S.P.Darwin: New Caledonia, Viroi 825 (P); *Psydrax paradoxa* (Viroi) Mouly: New Caledonia, McKee 16660 (P); *Pyrostria ankobeensis* Arènes ex Cavaco: Madagascar, Capuron 24339 SF (P); *Pyrostria major* (A.Rich. ex DC.) Cavaco: Madagascar, Decary 13716 (P); *Pyrostria media* (A.Rich. ex DC.) Cavaco: Madagascar, Saboureau 1509 RN (P). Incertae sedis. *Rhopalobranchium fragrans* Schltr. & K.Krause: New Caledonia, McKee 24582 (P).

APPENDIX 2. List of species included in the *rps16* molecular analysis of Ixoroideae s.l., origin of the sequences (publication or voucher) and accession numbers (EMBL). The newly published sequence accession numbers are indicated with a *.

Ingroup taxa: *Alberta minor* Baill. ex K.Schum.: Madagascar, Razafimandimbison 558 (UPS); EF205637*. *Atractocarpus stipularis* (F.Muell.) Puttock, Persson (2000); AF200979. *Augusta austrocaledonica* (Brongn.) J.H.Kirkbr.: New Caledonia, Mouly and Innocente 237 (P); EF205638*. *Aulacocalyx jasmiflorus* Hook.f.: Africa, Schmidt et al. 1672 (MO); EF205639*. *Bertiera aethiopica* Hiern: Dessen et al. (2001); AF332365. *Coffea arabica* L.: Andersson and Rova (1999); AF004038. *Condaminea corymbosa* (Ruiz & Pav.) DC.: Andersson and Rova (1999); AF004039. *Cremaspora triflora* K.Schum.: Persson (2000); AF200990. *Cyclophyllum deplanchei* Hook.f.: New Caledonia, Mouly and Innocente 228 (P); EF205640*. *Fernelia buxifolia* Lam.: Rova et al. (2002); AF244892. *Gardenia hansemanii* K.Schum. Bremer et al. (2002); AJ320077. *Ixora coccinea* L.: Cultivated Stockholm, Bremer 3104 (S); EF205641*. *Ixora ferrea* Benth.: Caribbean Islands, Taylor 11693 (UPS); EF205642*. *Ixora finlaysoniana* Wall ex G.Don: Kenya, Luke

9042 (UPS); EF205643*. *Ixora killipii* Standl.: Persson (2000); AF201001. *Keetia gueinzii* (Sond.) Bridson: Africa, Schmidt et al. 1701 (MO); EF205644*. *Mussaenda scratchleyi* Wernham: Bremer et al. (2002); AJ320079. *Nematostylis anthophylla* Baill.: Madagascar, Razafimandimbison et al. 360 (UPS); EF205645*. *Paragenipa lancifolia* (Boj. ex Bak.) Tirveng. & Robbr.: Andersson and Rova (1999); AF004066. *Pavetta platyclada* K.Schum. & Lauterb.: Bremer et al. (2002); AJ320082. *Peponidium comorense* Arènes: Mayotte (France), Labat, et al. 3299 (P); EF205646*. *Psydrax livida* (Hiern) Bridson: Madagascar, Lantz 109 (UPS); EF205647*. *Psydrax odorata* (G.Forst.) A.C.Smith & S.P.Darwin: Andersson and Rova (1999); AF004031. *Pyrostria hystrix* (Bremek.) Bridson: Cultivated Uppala (UPS); EF205648*. *Randia shumanniana* Merr. & L.M.Perry: Bremer et al. (2002); AJ320087. *Retiniphyllum pilosum* Müll.Arg.: Andersson and Rova (1999); AF004076. *Rhopalobranchium fragrans* Schltr. & K.Krause: New Caledonia, Mouly et al. 90 (P); EF205649*. *Sabicea aspera* Aubl.: Andersson and Rova (1999); AF004079. *Sabicea mollis* K.Schum. ex Wernham: Dessen et al. (2001); AF332371. *Sipanea biflora* Cham. & Schlecht.: Andersson and Rova (1999); AF004085. *Tarenna borbonica* (EG. & A.Henderson) Verdc.: Persson (2000); AF201024. *Tarenna buruensis* Merr.: Bremer et al. (2002); AJ320088. *Tricalysia cryptocalyx* Baker: Andersson and Rova (1999); AF004088. *Vangueria madagascariensis* J.F.Gmel.: Rova et al. (2002); AF243033.

Outgroup taxa: *Chiococca alba* (L.) Hitchc.: Andersson and Rova (1999); AF004034. *Guettarda crispiflora* Vahl: Andersson and Rova (1999); AF004052. *Luculia gratissima* (Wall.) Sweet: Bremer et al. (2002); AJ431036.

APPENDIX 3. List of species included in the *trnT-F* molecular analysis of Vanguerieae, origin of the sequences (publication or voucher) and the accession number (EMBL). The newly published sequences have an accession number with a *.

Ingroup taxa: *Afrocanthium burtii* (Bullock) Lantz: Lantz and Bremer (2004); AJ620120. *Afrocanthium kenienne* (Bullock) Lantz: Lantz and Bremer (2004); AJ620126. *Canthium coromandelicum* (Burm.f.) Alston: Lantz and Bremer (2004); AJ620122. *Canthium cypriporon* Byn. Ex Cavaco: Mayotte (France), Labat et al. 3280 (P); EF205629*. *Canthium pseudosetiflorum* Bridson: Lantz and Bremer (2004); AJ620131. *Cuviera angolensis* Welw. ex K.Schum.: Lantz and Bremer (2004); AJ620134. *Cyclophyllum balansae* (Baill.) Guillaumin: New Caledonia, Mouly 181 (P); EF205630*. *Cyclophyllum deplanchei* Hook.f.: New Caledonia, Mouly and Innocente 228 (P); EF205631*. *Fadogia tetraquetra* K.Schum. & Krause: Lantz and Bremer (2004); AJ620139. *Fadogia verdcourtii* Tennant: Lantz and Bremer (2004); AJ620140. *Fadogiella stigmatoloba* (K.Schum.) Robyns: Lantz and Bremer (2004); AJ620141. *Hutchinsonia barbata* Robyns: Lantz and Bremer (2004); AJ620142. *Keetia gueinzii* (Sond.) Bridson: Lantz and Bremer (2004); AJ620143. *Keetia lukei* Bridson: Lantz and Bremer (2004); AJ620144. *Leroya cf. richardiae* Cavaco: Lantz and Bremer (2004); AJ620148. *Multidentia concrescens* (Bullock) Bridson & Verdc.: Lantz and Bremer (2004); AJ620150. *Multidentia fanshawei* (Tennant) Bridson: Lantz and Bremer (2004); AJ620151. *Peponidium comorense* Arènes: Mayotte (France), Labat et al. 3299 (P); EF205632*. *Peponidium horridum* (Baill.) Arènes: Lantz and Bremer (2004); AJ620153. *Pseudo-peponidium ampiporoense* Arènes: Lantz and Bremer (2004); AJ719193. *Psydrax dicoccos* Gaertn.: Indonesia, Jacques 18 (P); EF205633*. *Psydrax paradoxa* (Viroi) Mouly: A. Mouly and E. Innocente 231 (P); EF205634*. *Pygmaeoathamnus cf. chamaedendrum* Robyns: Lantz and Bremer (2004); AJ620165. *Pygmaeoathamnus zeyheri* Robyns ex Good: Lantz and Bremer (2004); AJ620166. *Pyrostria anjouanensis* Arènes ex Cavaco:

- Mayotte (France), *Pignal 1808* (P); EF205635*. *Pyrostria bibracteata* (Baker) Cavaco: Lantz and Bremer (2004); AJ620167. *Pyrostria phyllantoidea* (Baill.) Bridson: Lantz and Bremer (2004); AJ620169. *Rhopalobrachium fragrans* Schltr. & K.Krause; New Caledonia, Mouly et al. 90 (P); EF205636*. *Robynsia glabrata* Hutchinson: Lantz and Bremer (2004); AJ620170. *Rytigynia bagshawei* (S.Moore) Robyns: Lantz and Bremer (2004); AJ620171. *Vangueria cinerascens* (Welw. Ex Hiern) Lantz: Lantz and Bremer (2004); AJ620177. *Vangueria dryadum* S.Moore: Lantz and Bremer (2004); AJ620146. *Vangueria fuscocetulosa* (Verdc.) Lantz: Lantz and Bremer (2004); AJ620173. *Vangueria lasiantha* (Sond.) Sond.: Lantz and Bremer (2004); AJ620147. *Vangueria madagascariensis* J.F.Gmel.: Lantz and Bremer (2004); AJ620184. *Vangueria paroifolia* Sond.: Lantz and Bremer (2004); AJ620181. *Vangueria pygmaea* Schltr.: Lantz and Bremer (2004); AJ620152. *Vangueria rubiginosa* (Desf.) Lantz: Lantz and Bremer (2004); AJ620119. *Vangueria zambesiaca* Lantz: Lantz and Bremer (2004); AJ620178. *Vangueriopsis cf. longiflora* Verdc.: Lantz and Bremer (2004); AJ620183.
- Outgroup taxa:** *Alberta magna* E.Mey.: Lantz and Bremer (2004); AJ620118. *Ixora coccinea* L.: Lantz and Bremer (2004); AJ620117. *Mussaenda erythrophylla* Schumach. & Thonn.: Lantz and Bremer (2004); AJ620116.