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

**Arnaud Mouly**, **Sylvain G. Razafimandimbison**, **Frederic Achille**, **Thomas Haevermans**, and **Brigitte Bremer**

1UMS 2700 CNRS – USM 0602 MNHN: Taxonomie et Collections, Département de Systématique et Évolution, Muséum National d’Histoire Naturelle, CP39, 16 rue Buffon, 75231 Paris cedex 05, France;
2US 084 IRD BIODIVIAL: Biodiversité végétale tropicale: connaissance et valorisation, Institut de Recherche pour le Développement, Antenne MNHN, France;
3The Bergius Foundation at the Royal Swedish Academy of Sciences, P.O. Box 50017, SE-104 05 Stockholm, and Botany Department Stockholm University, Sweden
4Author for correspondence (mouly@mnhn.fr)

**Communicating Editor:** Sara B. Hoot

**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 Isoroideae sensu lato. The species has axillary inflorescences, with valvate flower aestivation, drupaceous fruits with a single pendulous ovule per locule, and 3-pororate 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 Isoroideae 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), Tirvengadum (1991) transferred *Rhopalobrachium congestum* to the genus *Pelagodendron* Seem. (*Gardenieae*). In a later re-vison 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 Tirvengadum (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.

Tirvengadum (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 Tirvengadum (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 Isoroideae 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 Tirvengadum (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 Weibel (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 (Robbecht 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 acetylated (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 electron microscopes (SEM; JEOL JSM 640 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.

**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, Condamineeae, Cremas-poreae, Gardeniaeae, Ixoreae, Mussaeae, Octotropideae, Pavetteae, Retiniphyleae, Sabileae (sensu Bremer & Thulin, 1998), Sipaneeae, and Vanguerieae, were included in the rps16 analysis to assess the tribal position of *Rhopalobrachium fragrans*. Chiococca alba and Gaettedra crisypilosa of subfamily Cinchonoideae and Lucalia guttissima, 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 (*Eroospermopsis* Robyns, *Eoerista* S.T. Reynolds & R.J.F. Hend., *Peraanastos* Robyns ex RiDL., *Tennocalyx* 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): *Albertia magna* (Alberteae), *Ixora coccinea* (Ixoreae), and *Mussaenda erythrophylla* (Mussaeae). We were unable to test the monophyly of *Rhopalobrachium* sensu Schlchter 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-Maroof 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 rpsF1/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 trnL-trnF region was amplified with the primer pair A1/1 (Razafimandimbison and Bremer 2000; Bremer et al. 2002) and the trnT-trnL-trnF region with the pair C/F (Taberlet et al. 1991). Sequencing reactions were prepared using the two external primer pairs A1/1 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 Sequencer 3.1.1 and edited manually. All sequences (Appendices 2–3) were aligned manually with Se-Al v1.0a1 (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 + I) 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 Rhopalobrachium fragrans and the available literature.

<table>
<thead>
<tr>
<th>Lineage</th>
<th>Inflorescence</th>
<th>Aestivation</th>
<th>Stigma</th>
<th>Nb. ovule/locule</th>
<th>Seed</th>
<th>Fruit</th>
<th>Pollen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alberteae</td>
<td>Terminal</td>
<td>Contorted</td>
<td>Rounded bifid</td>
<td>1</td>
<td>Pendulous</td>
<td>Dry</td>
<td>3-colporate</td>
</tr>
<tr>
<td>Augusta group</td>
<td>Terminal</td>
<td>Contorted</td>
<td>Linear bifid</td>
<td>Many</td>
<td>Adaxial</td>
<td>Fleshy</td>
<td>3-colporate</td>
</tr>
<tr>
<td>Bertiereae</td>
<td>Terminal</td>
<td>Contorted</td>
<td>Linear bifid</td>
<td>Many</td>
<td>Adaxial</td>
<td>Fleshy</td>
<td>3-colporate</td>
</tr>
<tr>
<td>Coffeeae</td>
<td>Axillary</td>
<td>Contorted</td>
<td>Linear bifid</td>
<td>1</td>
<td>Adaxial</td>
<td>Fleshy</td>
<td>3-colporate</td>
</tr>
<tr>
<td>Condamineeae</td>
<td>Terminal</td>
<td>Valvate</td>
<td>Variable</td>
<td>Many</td>
<td>Adaxial</td>
<td>Dry</td>
<td>3-colporate</td>
</tr>
<tr>
<td>Cremasporaeae</td>
<td>Axillary</td>
<td>Contorted</td>
<td>Linear bifid</td>
<td>1</td>
<td>Adaxial</td>
<td>Fleshy</td>
<td>3-colporate</td>
</tr>
<tr>
<td>Gardenieae</td>
<td>Terminal/Axillary</td>
<td>Contorted</td>
<td>Linear bifid</td>
<td>1 to many</td>
<td>Variable</td>
<td>Fleshy</td>
<td>3-colporate/3-pororate</td>
</tr>
<tr>
<td>Greena group</td>
<td>Terminal</td>
<td>Contorted</td>
<td>Linear/Globose</td>
<td>Many</td>
<td>Adaxial</td>
<td>Dry</td>
<td>3-colporate</td>
</tr>
<tr>
<td>Ixoreae</td>
<td>Terminal</td>
<td>Contorted</td>
<td>Linear bifid</td>
<td>1</td>
<td>Adaxial</td>
<td>Fleshy</td>
<td>3-colporate</td>
</tr>
<tr>
<td>Mussaendaee</td>
<td>Terminal</td>
<td>Valvate</td>
<td>Linear bifid</td>
<td>Many</td>
<td>Adaxial</td>
<td>Fleshy/Dry</td>
<td>3-colporate</td>
</tr>
<tr>
<td>Octotropaeae</td>
<td>Axillary</td>
<td>Contorted</td>
<td>Linear bifid</td>
<td>1 to many</td>
<td>Variable</td>
<td>Fleshy</td>
<td>3-colporate</td>
</tr>
<tr>
<td>Pavetteae</td>
<td>Terminal</td>
<td>Contorted</td>
<td>Linear bifid</td>
<td>1 to many</td>
<td>Adaxial</td>
<td>Fleshy</td>
<td>3-colporate</td>
</tr>
<tr>
<td>Retiniphylleae</td>
<td>Terminal/Axillary</td>
<td>Contorted</td>
<td>Globose 5-merous</td>
<td>2</td>
<td>Adaxial</td>
<td>Fleshy</td>
<td>3-colporate</td>
</tr>
<tr>
<td>Salsicieae</td>
<td>Axillary</td>
<td>Valvate</td>
<td>Linear 5-merous</td>
<td>Many</td>
<td>Adaxial</td>
<td>Fleshy</td>
<td>3-colporate</td>
</tr>
<tr>
<td>Sipaneae</td>
<td>Terminal/Axillary</td>
<td>Contorted</td>
<td>Globose bifid</td>
<td>Many</td>
<td>Adaxial</td>
<td>Fleshy</td>
<td>3-colporate</td>
</tr>
<tr>
<td>Vanguerieae</td>
<td>Axillary</td>
<td>Valvate</td>
<td>Stylar head complex/</td>
<td>1</td>
<td>Pendulous</td>
<td>Fleshy 3-pororate</td>
<td></td>
</tr>
<tr>
<td>Rhopalobrachium</td>
<td>Axillary</td>
<td>Valvate</td>
<td>Rounded bifid</td>
<td>1</td>
<td>Pendulous</td>
<td>Fleshy</td>
<td>3-pororate</td>
</tr>
</tbody>
</table>

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 was GTR + I 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 assignation 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 Rhopalobrachium fragrans characteristics and relationships with other species.

Morphology. The plants of Rhopalobrachium 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 presenting, 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 Rhopalobrachium fragrans is 3-zono-pororate, bearing elliptic pores and a foveo-reticulate tectum, with larger luminae in polar axes. Grains are around 45–50 × 30–35 μm. The pollen of the Vanguerieae representatives is 3-zono-porurate, with circular to elliptic pores. The tectal ornamentation varies from slightly punctuate 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 35–50 × 25–35 μm. The pollen of tribes Alberteae, Coffeeae, and Ixoreae is, contrarily to former taxa, 3-zono-colporate, with smooth to rugulose and punctuate to foveolate tectum. Grains are of a small size, 15–25 μm 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. Rhopalobrachium 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 congestum 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 anjuanensis to Canthium pseudositiflorum. 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., Pseudopeonidium 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 coronandileicum, 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 Tirvengadum (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 *Nematostylus*. Albertae have terminal thyrses, contorted, counter-clockwise aestivation, zygomorphic to infundibuliform corolla tubes, 2-loccular 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 Albertae appears to be due to its chimerical generic description. Effectively, a mix of characteristics from two distantly related taxa, Aida 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 pluriovulate locules. On the other hand, contorted aestivation does not occur in R. fragrans and must have been observed from other chloroplast markers, rbcL and trnL-F and rps16. Referring to Lantz and Bremer (2005), the cup-shaped stigma, also called the...
“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 Ixoroidae, is not plesiomorphic in Vanguerieae, accord-
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.Halle & 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* F.

![Figure 2](image-url)  
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 compared to Bayesian analysis; if ‘-‘, support below 50%), numbers to the right are the Bayesian posterior probabilities. The gray box indicates tribe Vangueriæ. Bold branches identify the clades currently recognized at the tribal level within the Ixoroideae s.l.
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.

Alberta magna

Pachystigma pygmeum
Vangueria madagascariensis
Tapiphyllum cinerascens
Vangueria parviflora
Lagnias dryadum
Lagnias lasiantha
Rytignya fuscocetulosa
Vangueriopsis cf. longiflora
Tapiphyllum velutinum
Pygmaeoanthamus zeyheri
Robynsia glabrata
Multidentia concrescens
Multidentia fanshawei
Ancyplants rubiginosus
Cuviera angolensis

Fadogia tetraquerta
Fadogaeila stigmatoloba
Fadogia verdecourtii
Hutchinsonia barbata
Rytignya bagshawei

Canthium coromandelicum
Pygmaeoanthamus cf. chamaedendrum

Pyrostria anjuanensis
Pyrostria bibracteata
Pyrostria phyllantoidea
Canthium cystiporin
Scyphochlamys revoluta
Pseudopedonimium ampiojoroense
Leroya cf. richardiae
Peponidium comorensa
Peponidium horridum
Canthium pseudositiflorum
Cyclophyllum balansae
Rhopalobrachium fragrans
Cyclophyllum deplanchei

Keetia gueinzi
Keetia tukei
Afrocanthium hurttii
Afrocanthium kemense
Psydrax diccocos
Psydrax paradoxa
Ixora cocinea

Mussaenda erythrophylla

DIOECIOUS

CLADE

0.01
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 hermaphrodism in Cyclophyllum deplanchei, as for Pyrostria biflora in Pyrostris, 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 Rhopalobracium 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 Rhopalobracium sensu Tirvengadum (1991). Rhopalobracium 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 Pygmaeothamnus 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 Pyrostris 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 Rhopalobracium sensu Tirvengadum (1991) as member of the tribe Vanguerieae dioecious clade with close relationships with Cyclophyllum.

**Acknowledgements.** The authors wish to thank Anbar Khodabandeh for technical help for bio-molecular works; Pr. Joël Jérémie (MNHN, Paris) and Dr. Jacques Florence (IRD, Paris) for their useful comments on the manuscript; Pr. Philippe Morat (MNHN, Paris) for the funding of fieldwork; Pr. Philippe Bouchet, (MNHN, Paris) who has given his agreement for this study; Mrs. Monique Calapin (MNHN, Paris) for the technical assistance with pollen preparation; Dr. Gordon McPherson for his communication of the knowledge of the indigenous flora and the organization of the fieldwork; the authorities of the Northern Province of New Caledonia, for granting access to the field and authorization to collect specimens, as well as the tribal authorities who allowed us to work in their territories; the Missouri Botanical Garden providing biological material in preserved in silica-gel for bio-molecular studies; the team of the IRD botany lab in Nouméa for granting access to the herbarium and arranging accommodation; the Director and the team of the Geneva Herbarium for their help and for granting access to their collections; the Director and the team of the Botanical Garden of Meise (Belgium) for access to their collections. Financial support was provided by the UMS-CNRS 2707 – MNHN 060. “Taxonomie et Collections”; Department “Systématique et Evolution”, MNHN, Paris to A. Mouly and the Swedish Research Council to B. Bremer.

**Literature Cited**


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.


APPENDIX 2. List of species included in the *pslF* 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 an *.
