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THE TRIBAL PLACEMENT OF THE MONOSPECIFIC TROPICAL AFRICAN GENUS PETITIOCODON (RUBIACEAE) BASED ON MOLECULAR DATA AND MORPHOLOGY

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SUMMARY

A first phylogenetic placement of *Petitiocodon* based on molecular sequence data from three plastid regions (*accD-psa1*, *rpl16* and *trnL-F*) is presented, in conjunction with a reassessment of morphology for the genus. Our results do not support an evolutionary affinity between *Petitiocodon* and *Tricalysia* (Coffeeae) as suggested by previous studies, but they confirm other research that *Petitiocodon* and *Didymosalpinx* are distinct genera. Placement of *Petitiocodon* in tribe Octotropideae is well-supported on the basis of molecular data and floral and carpological characters.

Key words: Octotropideae, African flora, *accD-psa1*, *rpl16*, *trnL-F*, molecular phylogenetics, placentation.

INTRODUCTION

Petitiocodon Robbr. consists of a single species, *Petitiocodon parviflora* (Keay) Robbr., which is endemic to the mid and high altitude rain forest of south-east Nigeria and south-west Cameroon (Robbrecht 1988a). The species was first described by Keay (1958) in his treatment of west African *Gardenia* Ellis and *Randia* L. He placed this taxon within the newly erected genus *Didymosalpinx* Keay, along with four other species previously recognized in *Gardenia*. These five species were united by the presence of axillary inflorescences paired at the nodes and large, funnel-shaped corollas (Fig. 1).

Hallé (1968) commented on how *Didymosalpinx parviflora* Keay differed from the other species of *Didymosalpinx* in a number of characters. The vegetative parts of *D. parviflora* are pubescent, the flowers are subtended by bracts and bracteoles, the anthers are exserted, and the connective is elongated forming a sterile apical appendage (Hallé 1968). The other four *Didymosalpinx* species have glabrous vegetative parts, no bracts or bracteoles and the anthers are included in the corolla throat (Hallé 1968, Robbrecht 1988a). Hallé (1968) suggested that '*Didymosalpinx parviflora*' might share greater affinity with the Afro-Madagascan genus *Tricalysia* A.Rich. than with *Didymosalpinx*.

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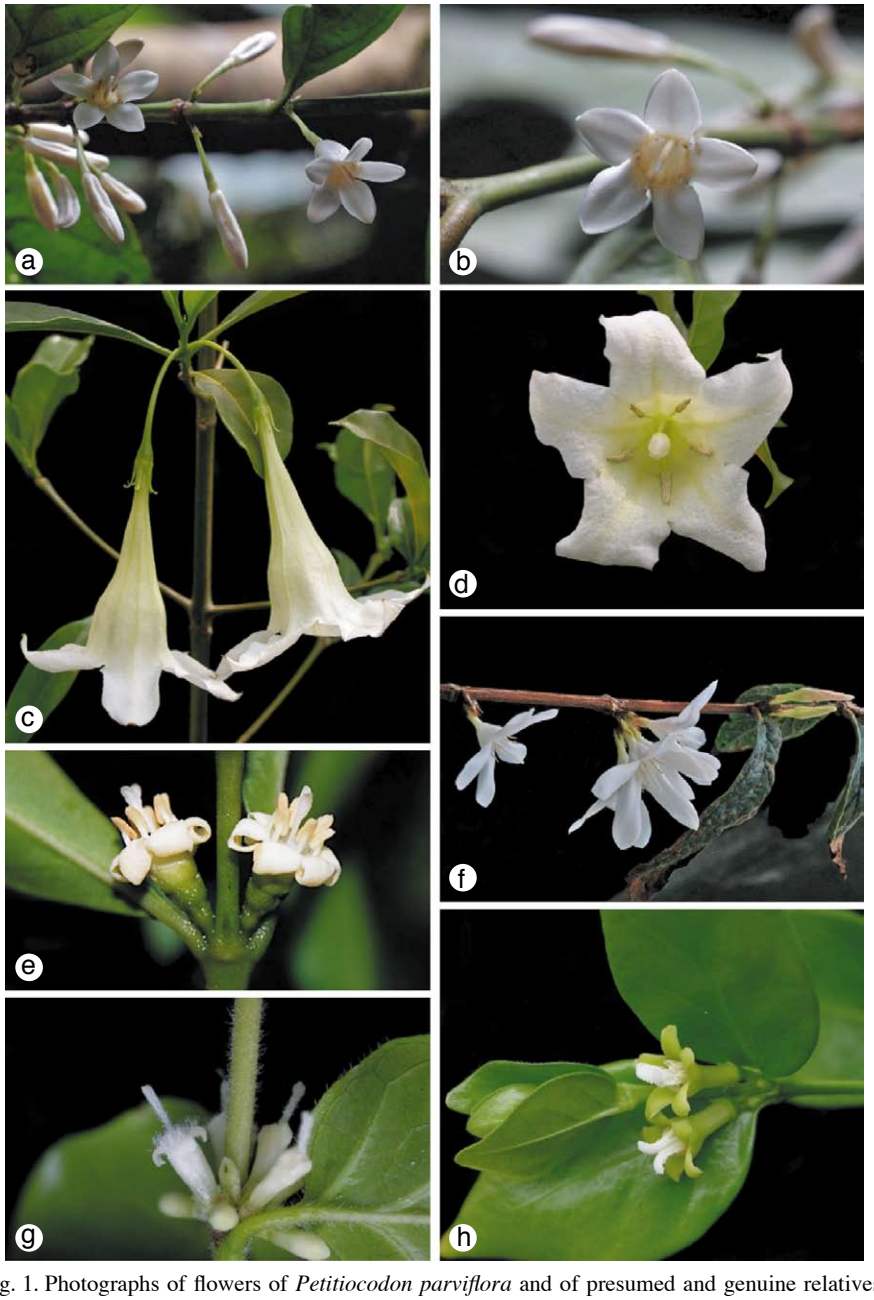

Fig. 1. Photographs of flowers of *Petitiocodon parviflora* and of presumed and genuine relatives. a. Flowering branch of *Petitiocodon parviflora*; b. front view of flower of *Petitiocodon parviflora*; c. flowering branch of *Didymosalpinx norae* (tentatively included in tribe Octotropideae); d. front view of flower of *Didymosalpinx norae*; e. flowering node of *Tricalysia cryptocalyx* Baker (tribe Coffeeae); f. flower of *Feretia aeruginescens* Stapf (tribe Octotropideae); g. flowering node of *Polysphaeria parvifolia* Hiern (tribe Octotropideae); h. flowering node of *Kraussia floribunda* Harv. (tribe Octotropideae). Photos: a, b by S. Dessein; c, d, f–h by F. Van Caekenberghe; e by P. De Block.

Robbrecht revised the genus *Tricalysia* in a series of papers (1979, 1982, 1983, 1987), and conducted a comparative study of the morphology of '*Didymosalpinx parviflora*' with respect to *Didymosalpinx* and *Tricalysia* (Robbrecht 1988a). He concluded that '*D. parviflora*' shares habit, inflorescence arrangement and fruit and seed characters with *Tricalysia*, but differs from *Tricalysia* in corolla morphology, fruit size and the nature of the endocarp (for a full comparison see Robbrecht 1988a: table 1).

According to Robbrecht (1988a) the key characters of '*Didymosalpinx parviflora*' include: paired axillary inflorescences; flowers subtended by pairs of bracts and bracteoles, with the lower pairs sometimes fused; corolla funnel-shaped, with contorted aestivation; anthers ± included, attached below the corolla-throat, with connective elongated into a short sterile apical appendage; ovary 2-locular, each locule with an elongated placenta, longitudinally attached to the base and septum, with ± 2 immersed lateral ovules, style exserted, (very) shortly bifid; fruits ± spindle-shaped, with a ± fleshy wall containing longitudinal sclerified strands, and one-seeded by abortion; seeds ellipsoidal with an inferior radicle.

Robbrecht (1988a) decided that '*Didymosalpinx parviflora*' merited its own genus, namely *Petitiocodon*, within the tribe Gardenieae subtribe Diplosporinae Miq., an assemblage of taxa transferred from Ixoreae s.l. (Bremekamp 1934) by Robbrecht & Puff (1986). Members of subtribe Diplosporinae were characterized by axillary inflorescences, bilocular ovaries, axile placentation with one to many ovules and an embryo with inferior radicle. The large funnel-shaped corollas of *Petitiocodon parviflora* were considered unique within subtribe Diplosporinae.

Our understanding of the tribal delimitation within Rubiaceae has changed considerably since the advent of molecular phylogenetics. A number of studies have led to the re-assessment of tribal boundaries, and this is particularly the case for the 'Gardenieae–Ixoreae complex' investigated by Robbrecht & Puff (1986). The molecular studies of Andreasen & Bremer (1996, 2000) and Persson (2000) demonstrated that several members of the Gardenieae subtribe Diplosporinae are in fact closely related to *Coffea* L. and *Psilanthus* Hook.f. The study of Andreasen & Bremer (2000) resulted in an enlargement of the tribe Coffeeae, modified to include *Diplospora* DC., *Sericanthe* Robbr., *Tricalysia* and *Discospermum* Dalzell. *Diplospora* and *Tricalysia* were re-transferred to Coffeeae on the basis of molecular data, *Sericanthe* and *Discospermum* based on their morphological affinity to *Tricalysia* and *Diplospora*, respectively. Bridson & Verdcourt (2003) further modified the concept of Coffeeae, on the basis of morphological observations and unpublished data, to include *Argocoffeopsis* Lebrun, *Belonophora* Hook.f. and *Calycosiphonia* Pierre ex Robbr., and placed Gardenieae subtribe Diplosporinae in the synonymy of Coffeeae.

Davis et al. (2007) investigated the delimitation and characterization of the tribe Coffeeae, using a morphological and molecular dataset. Their study included all of the taxa previously placed in the tribe by Andreasen & Bremer (2000) and Bridson & Verdcourt (2003), as well as the three remaining members from the Gardenieae subtribe Diplosporinae (*Petitiocodon*, *Nostolachma* T.Durand and *Xantonneopsis* Pit.). In addition, their sampling included a wide selection of taxa from subfamily Ixoroideae Raf. s.s. For *Petitiocodon*, *Nostolachma* and *Xantonneopsis*, they were unable to obtain undegraded DNA from herbarium leaf material, and as a result the phylogenetic placement of these three genera was assessed on the basis of a combined molecular

and morphological investigation. *Nostolachma* was accepted within the tribe Coffeeae, *Xantonneopsis* was placed in Octotropideae (BS (= Bootstrap Support) 69) and *Petitiocodon* was provisionally placed within Gardenieae, although the support for this was very low (BS < 50).

In the present study, we aim to investigate the phylogenetic placement of *Petitiocodon* based on sequence data of three plastid regions (*accD-psa1*, *rpl16* and *trnL-F*). In addition, morphological observations are carried out that complement Robbrecht's previous work. In particular a study of placentation is made on recently collected herbarium vouchers and alcohol preserved material. This is the first study that includes molecular data for this enigmatic species.

MATERIALS AND METHODS

Taxon sampling

We included three accessions of *Petitiocodon parviflora*, two collected in the field (Cameroon) and one obtained from a herbarium sample (*Gereau 5497*, BR). To test whether *P. parviflora* is more closely related to *Tricalysia* or *Didymosalpinx*, we included sequence data for a large selection of Coffeeae taxa (i.e. former members of Gardenieae subtribe Diplosporinae) and produced novel sequence data for *Didymosalpinx norae* (Swynn.) Keay. These data were combined with sequences from representatives of Octotropideae, Pavetteae and Gardenieae, which collectively form the Gardenieae alliance (Robbrecht & Manen 2006). Vanguerieae (*Peponidium* (Baill.) Arènes and *Pyrostria* Comm. ex A.Juss.) and Ixoreae (*Ixora* L. and *Doricera* Verdc.) were selected as the outgroup in this investigation, based on broader molecular studies of the Rubiaceae (see Introduction).

In this study we incorporate novel sequence data (32 new sequences) with selected sequences taken from Davis et al. (2007) and Tosh et al. (In press). A complete list of accessions is given in Appendix 1.

Morphological features

The taxa under investigation were subject to thorough morphological investigations (Robbrecht & Puff 1986, Robbrecht 1986, 1988a). Additional LM and SEM observations were made to investigate the placentation of *Petitiocodon* (spirit material, *Dessein & Sonké 1592* from Cameroon). Methods for microtome sectioning and SEM-investigations follow those described by Igersheim (1993). A fruit dissection was carried out on material from *Cheek et al. 3454* (K) and *Nemba et al. 570* (K).

DNA isolations, PCR amplification and sequencing

DNA samples were obtained from recent silica gel collections and leaf material taken from herbarium specimens (BR, K), or alternatively from living collections at the National Botanic Garden of Belgium (Appendix 1). Fresh leaf material and silica gel samples were isolated using a modified DNA Mini-extraction protocol (RBG Kew protocol, unpubl. data). In the case of herbarium material, DNA was obtained by a modification of the 2X CTAB protocol of Doyle & Doyle (1987). All DNA isolations

Table 1. Amplification primers for *accd-psa1*, *rpl16* and *trnL-F* plastid regions.

Region	Primer	Primer sequence (5'-3')	Reference
accD-psa1	Forward (769 F)	GGA AGT TTG AGC TTT ATG CAA ATG	Mendenhall 1994
	Reverse (75 R)	AGA AGC CAT TGC AAT TGC CGG AAA	
rpl16	Forward (71f)	GCT ATG CTT AGT GTG TGA CTC GTT G	Jordan et al. 1996
	Reverse (1661r)	CGT ACC CAT ATT TTT CCA CCA CGA C	
	Reverse (1516r)	CCC TTC ATT CTT CCT CTA TGT TG	Shaw et al. 2005
	Internal forward	GTA AGA AGT GAT GGG AAC GA	Davis et al. 2007
	Internal reverse	TCG TTC CCA TCA CTT CTT AC	
trnL-F	Forward (c)	CGA AAT CGG TAG ACG CTA CG	Taberlet et al. 1991
	Reverse (f)	AAT TGA ACT GGT GAC ACG AG	

were purified using Nucleospin columns (Macherey-Nagel) according to the manufacturers instructions, to remove potential PCR inhibitors.

Amplification of the *trnL-F*, *rpl16* and *accD-psa1* plastid regions were carried out using the primers listed in Table 1. Internal primers were required for complete amplification of some *rpl16* sequences. PCR and cycle sequencing reactions were performed using a Perkin Elmar GenAMP® 9700 thermocycler machine. Amplification of all three regions was carried out using the following profile: 94 °C for 3 min; 32 cycles of 94 °C for 1 min, 51 °C for 1 min, 72 °C for 2 min; final extension of 72 °C for 7 min. PCR master mixes were prepared using GoTaq® DNA polymerase, 5X reaction buffer, 1.5 μl 25 mM $MgCl_2$ and 2.5 μl dNTP's (Promega, Maddison, WI, USA). All amplification products were purified using Nucleospin purification columns. Cycle sequencing reactions were carried out using BigDye Terminator Mix (Applied Biosystems, Inc., Warrington, Cheshire, UK) and 26 cycles of: 10 s at 96 °C, 5 s at 50 °C and 4 min at 60 °C. Cycle sequence products were purified using an automated robot (Beckman Coulter Biomek NX S8) with the Promega Magnesil clean up system. Sequencing was performed using an AB 3730 DNA analyzer (Applied Biosystems). Electropherograms were assembled and edited using the Staden software package (Staden et al. 1998) and aligned manually in MacClade (version 4.04 © Maddison & Maddison 2002).

Phylogenetic analyses

Phylogenetic analyses were performed on the three separate plastid datasets and a combined three-region plastid matrix, using maximum parsimony and Bayesian inference. In all analyses, potential phylogenetically informative indels were coded according to the 'simple indel coding' method of Simmons & Ochoterena (2000).

Maximum parsimony

Heuristic tree searches were carried out in PAUP* version 4.0b10 (Swofford 2003). Each analysis consisted of tree-bisection-reconnection (TBR) branch swapping on 10,000 random taxon sequence addition replicates, holding 10 trees at each step, with delayed transformation (DELTRAN) optimization, MulTrees in effect and saving no

more than 10 trees per replicate. Support values for clades recovered in the analyses were estimated using bootstrap analysis (Felsenstein 1985). One thousand replicates of 100 random sequence additions, with TBR swapping, saving 10 trees per replicate were performed in PAUP*. We interpreted bootstrap values greater than 85% as being well supported, 75–84% as being moderately supported and 50–74% as having low support.

Bayesian inference

The most appropriate substitution model for each plastid region was selected using Modeltest v.3.06 (Posada & Crandall 1998) under the Akaike Information Criterion. The HKY + I nucleotide substitution model was selected for *trnL-F* and the HKY + I + G model was selected for *accD-psa1* and *rpl16*. The restriction site (binary) model was implemented for indel data, following the recommendation of Ronquist et al. (2005). Two independent Bayesian searches, each consisting of two simultaneous parallel analyses, were carried out using MrBayes 3.1 (Huelsenbeck & Ronquist 2001). Four Markov Chains (one cold, three heated) were run simultaneously in each analysis for a total of 2,000,000 generations, with tree sampling occurring every 100 generations. The initial 25% of trees were discarded as a conservative 'burn in'. The post 'burn in' trees from the four independent analyses were pooled together and summarized by majority rule consensus using PAUP* version 4.0b10 (Swofford 2003). Nodes with posterior probabilities of 0.95 or higher were considered well supported.

RESULTS

Genetic variation of plastid regions

Low levels of genetic variation across all three regions enabled sequences to be aligned without difficulty. The *rpl16* region proved to be the most variable of the three plastid regions, with 10.04% of the total number of characters being potentially parsimony informative (Table 2). However, *rpl16* also proved to be the most difficult marker to amplify due to two poly-A stretches, one near the 5' end and the other near the 3' end of the sequence. Considerable length variation was observed in both the *accD-psa1* and *rpl16* regions (Table 2). *Peponidium* and *Pyrostria* species (outgroup taxa from tribe Vanguerieae) shared a 274 base pair insertion/deletion in the *rpl16* region, which accounted for a large amount of the total length variation observed in this region. In the case of *accD-psa1*, three genera (*Didymosalpinx*, *Empogona* and *Petitiocodon*) contained large, though non-homologous, deletions in the aligned sequence matrix. The *accD-psa1* region was rich in potentially informative insertion and deletion events, and 16 indels were coded in the final analysis (Table 2).

Phylogenetic analyses

The tree topologies of the three individual plastid analyses were examined by eye and found to be consistent. The three datasets were then combined for all subsequent analyses. The aligned combined matrix had a total length of 3464 base pairs, of which 316 characters (9.03%) were potentially phylogenetically informative. The combined analysis also included 37 indel characters.

Table 2. Tree statistics and characterization of individual and combined datasets.

	accd-psa1	*rpl16*	*trnL-F*	Combined
Number of taxa	45	44	45	46
Total length (base pairs)	1319	1265	880	3464
Sequence length variation	694–1084	898–1121	786–827	–
Number of constant characters	1121	1038	752	2911
Number of potentially PI characters	116	127	73	316
(% of total characters)	(8.69)	(10.04)	(8.30)	(9.03)
Number of PI indels	16	11	10	37
Tree length	291	348	174	821
CI	0.852	0.802	0.845	0.821
RI	0.875	0.849	0.897	0.861
Number of trees saved	72642	207	22803	457

The maximum parsimony (MP) analysis of the combined plastid dataset generated 457 most parsimonious trees with a length of 821 steps (Table 2). The topologies of the MP strict consensus tree and the Bayesian majority rule consensus tree were more or less congruent with each other, only differing in the placement of the genus *Rutidea* DC. (Pavetteae).

In all three individual plastid analyses (trees not shown) and the combined analyses (Fig. 2), *Petitiocodon* is sister to *Polysphaeria* Hook.f., within a clade containing *Canephora* Juss., *Chapelieria* A.Rich., *Cremaspora* Benth. and *Didymosalpinx* (hereafter referred to as Clade I, Fig. 2). In the combined analyses, the sister relationship between *Petitiocodon* and *Polysphaeria* is supported by a bootstrap value (BS) of 96% and posterior probability (PP) of 1.00. The clade of *Canephora* and *Chapelieria* is strongly supported (BS 100, PP 1.00), although there is only weak support for its sister relationship to the clade of *Petitiocodon* and *Polysphaeria* (BS 73). There is strong support for the clade containing *Canephora*, *Chapelieria*, *Cremaspora*, *Petitiocodon* and *Polysphaeria* (BS 93, PP 1.00). The sister relationship of *Didymosalpinx* to all the other taxa within Clade I is also strongly supported (BS 90, PP 0.99).

In the Bayesian analysis, *Rutidea* (Pavetteae) is sister to Clade I, although this relationship receives no support (PP 0.60). In the MP analysis (not shown), *Rutidea* is sister to the clade of Gardenieae taxa, but again this relationship is not supported (BS < 50). The sister relationships of *Schumanniophyton* to *Gardenia* and *Hyperacanthus* is not supported (BS < 50, PP 0.77), as is the case for the sister relationship between *Gardenia* and *Hyperacanthus* (BS < 50, PP 0.90).

The monophyly of Coffeeae (Clade II) is strongly supported (BS 99, PP 1.00), as is the clade of *Coffea* and *Psilanthus* (BS 100, PP 1.00), which is sister to the remaining Coffeeae taxa (BS 86, PP 1.00). *Argocoffeopsis* and *Calycosiphonia* form a well-supported clade (BS 100, PP 1.00), as do *Empogona* and *Diplospora* (BS 100, PP 1.00) and *Discospermum* and *Xantonnea* (BS 97, PP 1.00). The monophyly of *Sericanthe* (BS 100, PP 1.00) and *Tricalysia* (BS 100, PP 1.00) are confirmed. There is strong BS (87) and high PP (1.00) for the sister relationship between *Bertiera* Aubl. and Coffeeae.

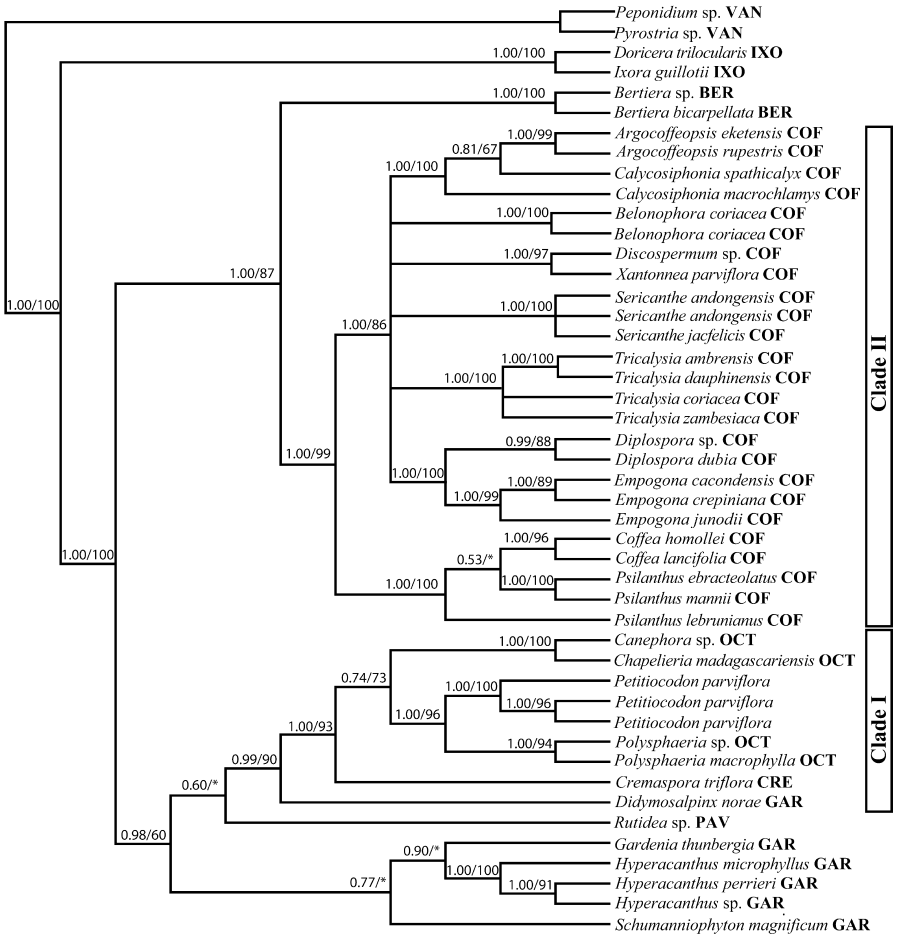

Fig. 2. Bayesian majority rule consensus tree. Support values (Bayesian Posterior Probabilities/ MP Bootstrap) are indicated above the branches. Asterisks (*) denote nodes that have bootstrap support < 50 in the MP analysis. Tribal ranking is annotated after species names. **VAN** – Vanguerieae, **IXO** – Ixoreae, **BER** – Bertiereae, **CRE** – Cremasporeae **COF** – Coffeeae, **OCT** – Octotropideae, **PAV** – Pavetteae, **GAR** – Gardenieae. **Clade I** corresponds to the Octotropideae sensu Robbrecht & Manen (2006), *Petitiocodon* and *Didymosalpinx*. **Clade II** corresponds to the tribe Coffeeae.

Ixora and *Doricera* (tribe Ixoreae), which Mouly (2007) considers to be congeneric, are sister (BS 100, PP 1.00) to all members of the 'Gardenieae alliance' (sensu Robbrecht & Manen 2006).

Morphological observations

The paired axillary inflorescences of *P. parviflora* are placed 3–6 mm above the node and are thus supra-axillary (Fig. 1a). The flowers are hermaphrodite and 5-merous, and the corolla lobes in bud (i.e. aestivation) are contorted to the left (Fig. 1a, b). The corolla has a rather thick, waxy texture, and the inside of the corolla tube is finely

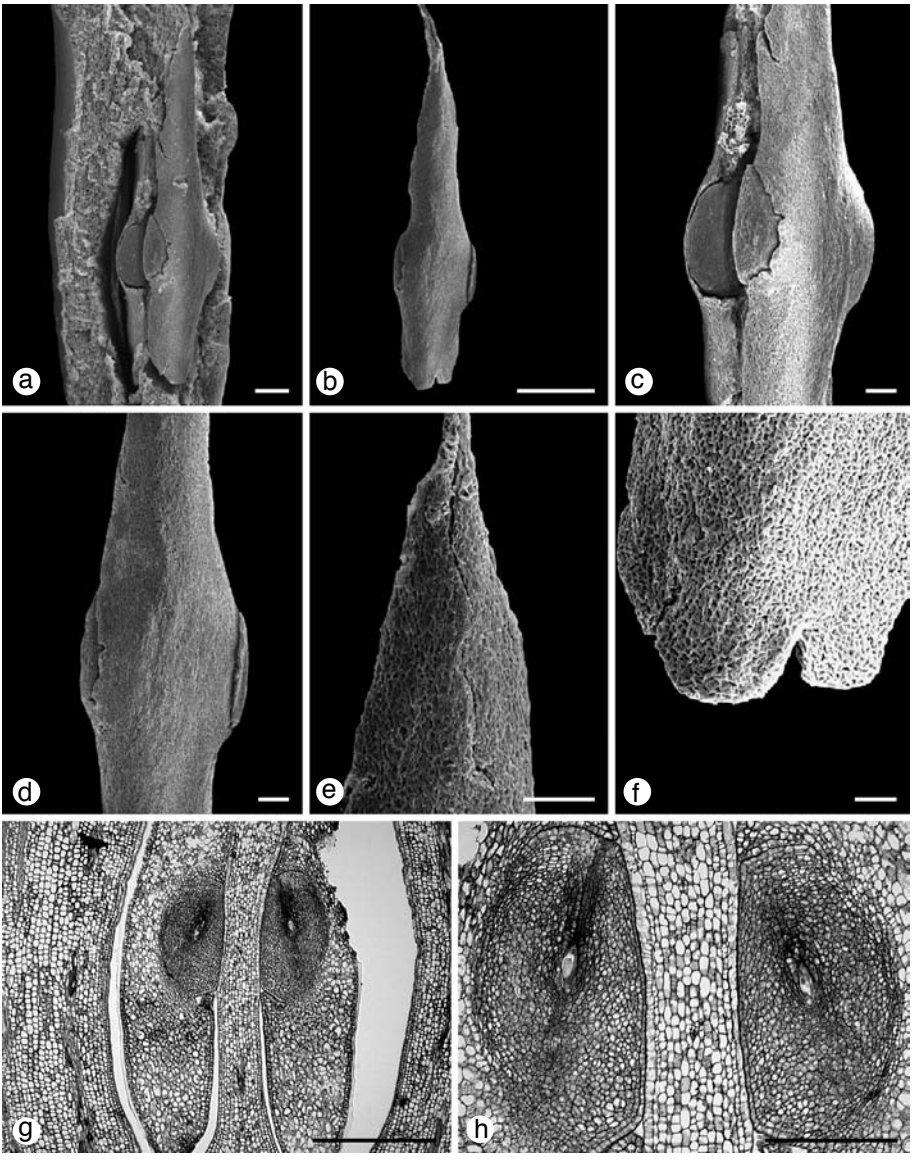

Fig. 3. Placentation in *Petitiocodon parviflora*. a. Opened bilocular ovary showing two placentas; b. front view of placenta with two lateral ovules; c. detail of central part of the two placentas showing the insertion of the ovules; d. detail of front view of the placenta showing the position of the two ovules; e. elongated tip of the placenta; f. base of the placenta; g. longitudinal section of bilocular ovary perpendicular to septum showing the two placentas; h. detail of g, showing the ovules with superior micropyles directed towards the septum. — Scale bars: a, h = 200 μm; b, g = 500 μm; c–e = 100 μm; f = 50 μm.

Table 3. Salient morphological characters of *Petitiocodon* and its putative relatives.

	Octotropideae I	Octotropideae II	Octotropideae III	*Cremaspora*	*Didymosalpinx*	*Petitiocodon*	*Tricalysia*
Habit	Shrub, treelet	Shrub, (treelet)	Shrub, (treelet)	Shrub	Shrub	Shrub, treelet	Shrub, tree
Thorns	Absent	Absent	Absent	Absent	Present	Absent	Absent
Leaf arrangement	Decussate	Decussate, (whorls of 3)	Decussate	Decussate	Decussate	Decussate	Decussate, ((whorls of 3))
Flower sex	Unisexual or Hermaphrodite	(Unisexual) or Hermaphrodite	Hermaphrodite	Hermaphrodite	Hermaphrodite	Hermaphrodite	(Unisexual) or Hermaphrodite
Secondary pollen presentation	(Absent), present	Present	Present	Present	Present	?	Present
Merosity	4–5	(3–)4–5	4–5(–7)	5–6	5	5	4–6(–12)
Flower size (cm)	< 1–2 (> 2)	< 1 (1–2)	(< 1–)1–2	1–2	5–8	3–4	< 1–2(–6)
Placentation	Axile or parietal	Axile	Axile	Axile	Parietal	Axile	Axile
Number of ovules	4–many	2–many	1–2(–3)	1	4–8	2	1–many
Micropyle orientation	Superior	Superior	Superior	Inferior*	Inferior	Superior	Inferior
Fruit size (cm)	(< 1) 1–> 2	< 1–2	< 1–2(> 2)	< 1	2–3	1–2	< 1–1
Mesocarp	Leathery, fleshy	Fleshy	(Woody), fleshy	Fleshy	Leathery	Fleshy	(Woody), fleshy
Endocarp	Membranous or chartaceous	Membranous, papery or chartaceous	Membranous, papery or chartaceous	Chartaceous	Chartaceous	Chartaceous	Membranous or chartaceous
Placenta at fruiting stage	–, ±, +	(–), ±	–, ±	+, ±	++	?	–, ±, (+)**
Rumination	Absent	Absent	Absent, present	Absent	Absent	Absent	Absent, (present)
Exotesta wrinkled	Absent, present	(Absent), present	Absent	Absent	Absent	Absent	Absent
Exotestal type	Type C and D	Type C and E	Type a, A, b and D	Type D	Type C***	Type a	Type a, A, (b)

Figures in single parentheses = rarely; figures in double parentheses = very rarely.

Placental fruiting stage: ++ placenta (and septa) forming a fleshy matrix (pulp) in which seeds are embedded; + placenta partially covering the seeds; ± placenta is ± absent; – placenta is no longer detected.

Exotestal type: Lower case denotes isodiametric cells, uppercase denotes strongly elongated cells; a/A no cellular thickening; b/B cellular thickening on the upper tangential well; C cellular thickening along the upper tangential and radial wall (at the angle of both upper and tangential wall); D Cellular thickening along the radial wall (at the angle of the lower tangential wall); E cellular thickening along the entire radial wall. For a more detailed review of the differing exotestal types in these taxa, consult Robbrecht & Puff (1986).

* Abnormally inferior position of micropyle.

** Massive placental development in *Tricalysia bifida*.

*** The multilayered, fibrous seed coat of *Didymosalpinx* is unique in Rubiaceae.

pubescent over its entire surface apart from a glabrous area about 2 mm from the base. The style is spindle-shaped, 10-ridged (where the anthers sit in 5 by 2 anther thecae, when the flower is in bud), and glabrous (Fig. 1b). The stigmatic lobes are adnate for most of their length and only the tips separate in mature styles (before maturity the tips are adnate). The stigmatic lobes are fused for 1/3 to 3/5 of their entire length, with two longitudinal grooves covered with papillae where they adjoin. The style and anther morphology strongly suggests secondary pollen presentation (Puff et al. 1996).

The ovary of *P. parviflora* is bilocular, with each locule containing a massive elongated axile placenta with two lateral ovules (Fig. 3a–f). The placenta is more or less spindle-shaped, widest in its lower half and becoming gradually narrower in the upper half. The tip is long acuminate and divided longitudinally for roughly half of the length above the insertion of the ovules (Fig. 3b, e). The base is rounded and divided longitudinally for a very short distance (Fig. 3f). The placenta is attached to the septum almost over its entire length. In the lower half of each placenta there are two lateral ovules, more or less immersed in the placenta. Each ovule possesses a superior micropyle directed towards the septum (Fig. 3g, h).

Mature and spirit preserved fruits remain unavailable, and so detailed embryographical and carpological observations could not be made. However, dissection of dried fruits revealed that each fruit contains two narrowly hemi-ellipsoidal seeds, with a smooth seed coat and entire endosperm.

DISCUSSION

The phylogenetic position of Petitiocodon parviflora

The results of our phylogenetic analyses do not support a close association between *Petitiocodon parviflora* and *Tricalysia* (Hallé 1968) or genera previously placed within Gardenieae subtribe Diplosporinae (Robbrecht 1986), i.e. Coffeeae (Davis et al. 2007). Our results support the claim by Hallé (1968) that *P. parviflora* does not belong in *Didymosalpinx*. Although we did not sequence the type species of *Didymosalpinx* (*D. abbeokutae* (Hiern) Keay), with the exception of *P. parviflora* the delimitation of the genus has never been doubted (see Introduction). Indeed, the genus is morphologically homogeneous and contains only four Afrotropical species. *Didymosalpinx norae* and *P. parviflora* are both placed in our Clade I, but they do not share a sister relationship (Fig. 2).

Instead, our data imply that *P. parviflora* is sister to *Polysphaeria* of tribe Octotropideae, a relationship that receives considerable support in the analyses (BS 96, PP 1.00). Even though our taxon sampling of Octotropideae, Pavetteae and Gardenieae is not exhaustive, Clade I receives high levels of support in both maximum parsimony and Bayesian Analyses (BS 90, PP 0.99) inferring a close relationship between *P. parviflora* and Octotropideae. In addition, the present study further confirms the sister group relationship between *Cremaspora* and Octropideae, as previously shown by Andreasen & Bremer (2000), Persson (2000) and Robbrecht & Manen (2006).

Table 3 is adapted from the comparative morphological dataset of Robbrecht & Puff (1986) and compares selected morphological characters between *P. parviflora*, other

members of Clade I and *Tricalysia*. In their survey of the Gardenieae and related tribes, Robbrecht & Puff (1986) recognized three informal groups within the Octotropideae (then known as Hypobathreae): Octotropideae I, a group of presumably primitive taxa characterized by large fruits with numerous seeds and thickening of the radial and outer (± inner) tangential walls of the exotestal cells; Octotropideae II, a group of core taxa with smaller fruits, relatively few ovules and thickening of the radial walls of the exotestal cells; and Octotropideae III, a group of presumably advanced taxa with small fruits, one or few ovules, ruminate endosperm and little or no thickening of the exotestal cells. Robbrecht (1980) concluded that the Octotropideae as a whole form 'a highly natural group, perhaps one of the most natural ones of the Rubiaceae'.

The salient characters of Octotropideae are (adapted from Robbrecht 1988b, Bridson & Verdcourt 2003 and Ruhsam & Davis 2007): petioles articulated (rarely not); inflorescences paired at the nodes, axillary or supraxillary; flowers hermaphrodite (rarely functionally unisexual) with secondary pollen presentation; corolla bell- or salver-shaped, lobes contorted to the left; ovary 2-locular (rarely 1-locular); placentation axile or rarely parietal (*Villaria* Rolfe); placentas small or large and fleshy; ovules 1–many, often (but not exclusively) pendulous, if more than two ovules per locule then in two distinct rows; micropyle/radicle usually superior; style simple or club- to spindle-shaped, entire or 10 ridged/winged, adnate at the apex and sometimes very shortly bifid, or bifid; fruits mostly small, fleshy or rarely leathery; seeds 1–several (rarely more than 10); endosperm ruminate or entire; testa often appearing fibrous with a minute fingerprint-like pattern at low magnifications; exotesta mostly folded with regard to endotesta, composed of elongated cells with thickenings along the radial walls or less often isodiametric, parenchyma-like.

Based on our anatomical and morphological survey, it is now clear that *P. parviflora* has many of the salient characters of Octotropideae, including: inflorescences paired and supra-axillary; flowers hermaphrodite with secondary pollen presentation; corolla bell-shaped, lobes contorted to the left; placentation axile, ovary 2-locular; ovules 2 per locule, micropyle superior and directed towards the septum (e.g. Fig. 3g, h); radicle superior; style spindle-shaped, 10-ridged, adnate along its entire length but becoming very shortly bifid at maturity; fruits fleshy to leathery; seeds 2 per fruit; endosperm entire. The details of the seed-coat are as yet unknown.

Our observations on ovary, style and fruit morphology are particularly pertinent to our reassessment of *Petitiocodon*. In his study of *P. parviflora* Robbrecht (1988a) gave the orientation of the radicle in the seed as inferior (micropyle downward) but we now know that it is superior (micropyle upward). The orientation of the micropyle is a valuable systematic character in the Rubiaceae and is superior in Octotropideae, except in rare cases (Robbrecht & Puff 1986, Ruhsam & Davis 2007). Robbrecht (1988a: fig. 2d) noted the style of *P. parviflora* simply as 'exserted, (very) shortly bifid'. Our examinations based on living and alcohol preserved specimens show that the style is spindle-shaped, 10-ridged, very shortly bifid at maturity. Within the tribes of Ixoroideae s.s. this type of style is consistent in Bertiereae (K.Schum) Bridson, frequent in Octotropideae (e.g. *Chapelieria*, *Kraussia* Harv.), rare in Pavetteae and Gardenieae s.l., and absent in Coffeeae, Cremasporeae (Verdc.) S.P.Darwin, Ixoreae A.Gray and Vanguerieae Dumort. Our observation of two-seeded fruits in *Petitiocodon* indicates

that the one-seeded condition (by abortion) of fruits as mentioned by Robbrecht (1988a: table 1) may not be consistent for the genus.

Whereas the Octotropideae were once thought to possess a more or less uniform ovary morphology (pendulous ovules attached to an apical placenta; Robbrecht & Puff 1986), we now know this is not the case for all representatives of the tribe. *Petitiocodon* is one example, as is the Madagascan genus *Flagenium* Baill. In this genus Ruhsam & Davis (2007) described a placenta bearing two distinct rows of ovules, with the uppermost ovules erect and the lower ovules pendulous, and in some species there are also a set of lateral ovules. However, complete data on ovary morphology for the Octotropideae are lacking, and variation is obviously greater than previously recorded (Robbrecht & Puff 1986). In some respects the ovary morphology of *Petitiocodon* is similar to other Octotropideae, and in particular *Kraussia* (Octotropideae III). *Kraussia speciosa* Bullock has a large (hemi-ellipsoid not spindle-shaped) placenta and two ovules per locule (Bridson & Verdcourt 1988) but in contrast to *Petitiocodon*, each placenta is only attached for a small part to the apex of the septum. *Kraussia* and *Petitiocodon* also both possess non-articulated petioles, a 10-ridged/winged style, and seeds with entire endosperm. In our molecular analyses *Petitiocodon* is sister to *Polysphaeria* (Octotropideae III), although a close relationship is not envisaged following a more complete sampling of Octotropideae, as *Polysphaeria* has an ovary with a small placenta attached to the apex of the septum, a single ovule per locule, an undivided style, and seeds with ruminate endosperm (Robbrecht & Puff 1986, Bridson & Verdcourt 1988, 2003).

Based on our morphological reassessment it is clear that *Petitiocodon* falls within Octotropideae and has no association with other tribes of the Ixoroideae sampled in this investigation. This assumption is strongly supported by our molecular data, which convincingly place *Petitiocodon* with representatives of Octotropideae (e.g. BS 96, PP 1.00).

Conclusions

This study provides the first phylogenetic placement of *Petitiocodon* based on molecular sequence data. Our results do not support an evolutionary affinity between *Petitiocodon* and *Tricalysia* but they confirm that *Petitiocodon* and *Didymosalpinx* are distinct genera. Furthermore, molecular and morphological data strongly indicate that *Petitiocodon* belongs to the Octotropideae. Therefore, tribal delimitation of the Octotropideae is amended to incorporate this rare monospecific African genus.

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Appendix 1. Taxon accession data (only first collector listed for voucher).

Taxon	Voucher	*accD-psa1*	*rpl16*	*trnL-F*
Argocoffeopsis eketensis (Wernham) Robbr.	*Davis 3031* (K), Cameroon	DQ180497	DQ180531	DQ180566
Argocoffeopsis rupestris (Hiern) subsp. *thonneri* (Lebrun) Robbr.	*Harris 8168* (K), Central African Republic	DQ180496	DQ180532	DQ180567
Belonophora coriacea Hoyle	*Maurin 5* (K), Cameroon	DQ180499	DQ180534	DQ180569
Belonophora coriacea Hoyle	*Maurin 19* (K), Cameroon	DQ180500	DQ180535	DQ180570
Bertiera bicarpellata (K.Schum.) N.Hallé	*Davis 3051* (K), Cameroon	DQ180502	DQ180537	DQ180572
Bertiera sp.	*Davis 3017* (K), Cameroon	DQ180504	DQ180539	DQ180574
Calycosiphonia macrochlamys (K.Schum.) Robbr.	*Davis 3036* (K), Cameroon	DQ180506	DQ180541	DQ180575
Calycosiphonia spathicalyx (K.Schum.) Robbr.	*Davis 2925* (K), Tanzania	DQ180509	DQ180544	DQ180578
Canephora sp.	*Davis 2727* (K), Madagascar	DQ180510	AM999523	DQ180579
Chapeliera madagascariensis A.Rich ex DC.	*Rakotonasolo 62* (K), Madagascar	FM177151	FM177162	NA
Coffea homollei J.-F.Leroy	*Davis 2305* (K), Madagascar	DQ153402	DQ153651	DQ153769
Coffea lancifolia A.Chev.	*Davis 2307* (K), Madagascar	DQ153403	DQ153652	DQ153770
Cremaspora triflora (Thonn.) K.Schum.	Cult. NBGB 19770366, Nigeria	FM177152	FM177163	FM177173
Didymosalpinx norae (Swynn.) Keay	Cult. NBGB 19761047, voucher: *Van Caekenberghe 62* (BR), Zimbabwe	FM160621	AM999522	AM999465
Diplospora dubia (Lindl.) Masam.	Cult. NBGB 2000195065, voucher: *Van Caekenberghe 49* (BR), origin unknown	AM999388	AM999526	AM999468
Diplospora sp.	*Bremer 15238* (K), Borneo (Brunei)	DQ180511	DQ180546	DQ180580
Discospermum abnorme (Korth.) S.J.Ali & Robbr.	*Sidiyasa 2148* (K), Borneo (Kalimantan)	AM999380	AM999528	AM999469
Doricera trilocularis (Balf.f.) Verdc.	*Friedmann 2939* (K), Mascarenes (Rodrigues)	DQ180513	DQ180548	DQ180582
Empogona cacondensis (Hiern) J.Tosh & Robbr., comb. nov. ined.	*Dessein 1031* (BR), Zambia	AM999355	FM160588	AM999482
Empogona crepiniana (De Wild. & T.Durand) J.Tosh & Robbr., comb. nov. ined.	*De Wilde 888* (K), Cameroon	FM177158	FM177169	FM177179
Empogona kirkii subsp. *junodii* (Schinz) J.Tosh & Robbr., comb. nov. ined.	*Van Caekenberghe 79* (BR), Zimbabwe	AM999369	FM160602	AM999496

Gardenia thunbergia L.f.	1961-29703 (K), SE Africa	DQ180514	DQ180549	DQ180583
Hyperacanthus microphyllus (K.Schum.) Bridson	*Goyder 5024* (K), Mozambique	AM999387	AM999520	AM999464
Hyperacanthus perrieri (Drake) Rakotonas. & A.P.Davis	*Davis 2584* (K), Madagascar	FM160619	AM999519	AM999462
Hyperacanthus sp.	*Davis 2586* (K), Madagascar	FM160620	AM999521	AM999463
Ixora guillotii Hochr.	*Tosh 408B* (BR), Madagascar	FM160624	AM999518	AM999461
Peponidium sp.	*Davis 2705* (K), Madagascar	FM177149	FM177160	FM177171
Petitiocodon parviflora (Keay) Robbr.	*Gereau 5497* (BR), Cameroon	NA	FM177164	FM177174
Petitiocodon parviflora (Keay) Robbr.	*Dessein 1597* (BR), Cameroon	FM177153	FM177165	FM177175
Petitiocodon parviflora (Keay) Robbr.	*Dessein 1612* (BR), Cameroon	FM177154	FM177166	FM177176
Polysphaeria macrophylla K.Schum.	*Maurin 50* (K), Cameroon	FM177150	FM177161	FM177172
Polysphaeria sp.	*Mvungi 15* (K), Tanzania	DQ180517	DQ180552	DQ180586
Psilanthus ebracteolatus Hiern	*Billiet 53054* (BR), Ivory Coast	AM999392	AM999530	AM999471
Psilanthus lebrunianus (Germ. & Kesler) J.-F.Leroy ex Bridson	*Louis 272* (K), D.R. Congo	FM177155	FM177167	FM177177
Psilanthus mannii Hook.f.	Cult. NBGB 19770367, voucher: *Van Caekenberghe 78* (BR), Ghana	FM160623	AM999531	AM999472
Pyrostria sp.	*Davis 2709* (K), Madagascar	DQ180519	DQ180554	DQ180588
Rutidea sp.	*Davis 3056* (K), Cameroon	DQ180521	DQ180556	DQ180590
Schumanniophyton magnificum (K.Schum.) Harms.	Cult. NBGB 19850094, D.R. Congo	FM177156	FM177168	FM177178
Sericanthe andongensis (Hiern) Robbr.	*Bidgood 3490* (K), Tanzania	DQ180522	DQ180557	DQ180591
Sericanthe andongensis (Hiern) Robbr.	*Dessein 1097* (BR), Zambia	FM177157	AM999532	AM999473
Sericanthe jacfelicis (N.Hallé) Robbr.	*Carvalho 4169* (K), Gulf of Guinea Islands	DQ180523	NA	DQ180592
Tricalysia ambrensis Randriamb. & De Block	*De Block 1313* (BR), Madagascar	AM999349	FM160582	AM999477
Tricalysia coriacea (Benth.) Hiern	*Dessein 1283* (BR), Zambia	AM999358	FM160591	AM999485
Tricalysia dauphinensis Randriamb. & De Block	*De Block 694* (BR), Madagascar	AM999361	FM160594	AM999488
Tricalysia zambesiaca Robbr.	*Fanshawe 3600* (K), Kenya	FM177159	FM177170	FM177180
Xantonnea parviflora (Kuntze) Craib	*Craib 895* (K), Thailand	DQ180530	NA	DQ180599