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ORIGINAL ARTICLE

Phylogeny and biogeography of the African genus *Virectaria* Bremek. (Sabiceeae s.l., Ixoroideae, Rubiaceae)

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Abstract The phylogenetic relationships of the tropical African genus *Virectaria* with its associated genera within the tribe Sabiceeae s.l. (Ixoroideae and Rubiaceae) were inferred from the combined analysis of nuclear ITS and chloroplast *rpo*C1 and *trn*T-F nucleotide sequence data. Phylogenetic relationships within *Virectaria* were investigated using combined analyses of ETS (nrDNA), ITS, *rpo*C1 and *trn*T-F sequence data. The present analyses further show that *Hekistocarpa* is sister to the *Tamridaea–Virectaria–Sabicea* clade, *Tamridaea* and *Virectaria* are sister genera, and *Sabicea* s.l. is sister to the *Tamridaea–Virectaria* clade. Our results strongly support the monophyly of *Virectaria* and the sister-group relationships between *V. multiflora* and *V. herbacoursi*, *V. angustifolia* and *V. procumbens*, and *V. major* and *V. belingana*. Our analyses indicate a tropical African origin for Sabiceeae s.l., a long isolated evolution for *Tamridaea* and a wide range of dispersal of *Virectaria* species in the Lower-Guinean, Upper-Guinean and Congolian regions, without a clearly defined direction of migration.

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Introduction

When the pantropical tribe Sabiceeae was established by Bremekamp (1966), only *Sabicea* was included. It was characterized by simple stipules, axillary inflorescences and very narrow exotesta cells. Since then, conflicting circumscriptions of Sabiceeae have been proposed (Andersson 1996; Bremer and Thulin 1998; Dessein et al. 2001a; Robbrecht and Manen 2006). More recently, Khan et al. (2008) performed phylogenetic analyses based on sequence data from the chloroplast *trn*T-F region and the nuclear ribosomal internal transcribed spacers. The results of that study have led to the establishment of new tribal and generic circumscriptions of Sabiceeae, which comprises four genera: the monospecific *Hekistocarpa* Hook. f., restricted to Cameroon and Nigeria (Dessein et al. 2001a), the most species-rich *Sabicea* s.l. (170 species including *Ecpoma* K. Schum., *Pseudosabicea* N. Hallé, *Schizostigma* Arn. ex Meisn. and *Stipularia* P. Beauv.) distributed in mainland Africa, Madagascar, São Tomé and Príncipe, Central and South America and Sri Lanka, the monospecific *Tamridaea* Thulin and B. Bremer, confined to Socotra of Yemen, and the tropical African *Virectaria* Bremek. (eight species, Dessein et al. 2001b). The intergeneric relationships within this newly delimited Sabiceeae, entirely based on molecular data, were not addressed in Khan et al. (2008) mainly due to the lack of sufficient resolution and a limited sampling of *Virectaria*.

The Guineo–Congolian wide (Robbrecht 1996) genus *Virectaria* Bremek., the second largest genus of the tribe, is characterized by its herbaceous or subshrubby habit, lack of raphides, truncate stigmata, internal indumentum (Verdcourt 1958) with flattened hairs, elongated floral disc and fruits with one persistent and one deciduous valve during dehiscence. All proposed species circumscriptions of *Virectaria* are summarized in Table 1 [see Dessein et al. (2001b) for more information on the taxonomic history of the genus]. For this study, we adopted the circumscription of Dessein et al. (2001b), who presented a morphology-based analysis of *Virectaria*, in which the seven of the eight *Virectaria* species were resolved in two major clades.

This is the first phylogenetic study of the genus *Virectaria* based on combined morphological and molecular (ETS, ITS, *rpo*C1 and *trn*T-F) data. This study was undertaken with four goals: (1) to assess rigorously the phylogenetic relationships between *Virectaria* and the other genera of Sabiceeae sensu Khan et al. (2008), (2) to test the monophyly of the genus *Virectaria,* (3) to test the interspecific relationships within *Virectaria* postulated by Dessein et al. (2001b) and (4) to infer the biogeography of *Virectaria*.

Materials and methods

Plant sampling

Twenty-one species representing *Virectaria* and its allied genera were included in this study for the combined analyses of ITS, *rpo*C1 and *trn*T-F sequence data including morphological data to examine the relationships within Sabiceeae and to test the monophyly of *Virectaria*. Ten additional sequences of six *Virectaria* species were included for the separate analysis of each of the ITS, *rpo*C1 and *trn*T-F data sets, as well as their combined analysis to compare the results. Two species of subfamily Ixoroideae s.l., *Mussaenda pinatubensis* Elmer (tribe Mussaendeae) and *Warszewiczia coccinea* Klotzsch (tribe Condamineeae) were used as outgroup taxa because they were shown to belong to the sister clade of Sabiceeae s.l. by Khan et al. (2008). Eleven species including 16 individuals of *Virectaria* representing six species were included in the combined analyses of molecular and morphological data to assess the phylogenetic relationships within the genus. Five species, *Hekistocarpa minutiflora* Hook. f., *Tamridaea capsulifera* (Balf. f.) Thulin and B. Bremer, *Sabicea becquetii* (N. Hallé) Razafim., B. Bremer, Liede and Khan, *Sabicea elliptica* (Schweinf. ex Hiern) Hepper, and *Sabicea xanthotricha* Wernham were used as outgroup taxa. Materials for *Virectaria salicoides* (C. H. Wright) Bremek., known only from the type and *Virectaria tenella* J. B. Hall were not available.

DNA isolation, amplification and sequencing

DNA isolation, amplification and sequencing of the ITS region were accomplished following the protocols described in Alejandro et al. (2005) and Hassan et al. (2005) except the concentration of dH_2O (15.8 μL) and DNA samples (1.0 μL) following Khan et al. (2008). The amplification and sequencing of the *trn*T-F region were performed following the protocols outlined in Razafimandimbison and Bremer (2002). The amplification and sequencing of the ETS region were accomplished according to the protocols described in Razafimandimbison et al. (2005). The *rpo*C1 exon 1 including *rpo*C1 intron (partial) was amplified using the two DNA barcoding primers *rpo*C1.2f (5′ GGC AAA GAG GGA AGA TTT CG 3′) and *rpo*C1.4r (5′ CCA TAA GCA TAT CTT GAG TTG G 3′). For each 25 μL PCR reaction we added 16.3 μL dH_2O, 1 μL $MgCl_2$ (25 mM), 2 μL dNTP (2 mM), 1 μL each of forward (*rpo*C1.2f) and reverse (*rpo*C1.4r) primer, (10 pmol/μL), 2.5 μL PCR buffer (10X), 0.2 μL Taq (QIAGEN) DNA polymerase and 1 μL DNA sample.

Table 1 Species circumscriptions of *Virectaria*

Species	Bremekamp (1952)	Verdcourt (1953)	Hallé (1966)	Dessein et al. (2001b)
V. angustifolia	*V. angustifolia*	*V. angustifolia*	*V. angustifolia*	*V. angustifolia*
V. belingana			*V. belingana*	*V. belingana*
V. herbacoursi			*V. herbacoursi*	*V. herbacoursi*
V. heteromera	*V. heteromera*	–[a]		
V. kaessneri	*V. kaessneri*	–[b]		
V. major		*V. major*	*V. major*	*V. major*
V. multiflora	*V. multiflora*	*V. multiflora*	*V. multiflora*	*V. multiflora*
V. procumbens	*V. procumbens*	*V. procumbens*	*V. procumbens*	*V. procumbens*
V. salicoides		*V. salicoides*	*V. salicoides*	*V. salicoides*
V. tenella				*V. tenella*

[a] Lumped in *Virectaria angustifolia*

[b] Merged in *Virectaria* major

PCR reaction was done with initial denaturation for 3 min. at 94°C, followed by 30 cycles for 1 min. at 93°C, 1 min. at 55°C, and finishing with 72°C for 2 min. Using the same primers, the sequencing reactions were conducted with ABI PRISM big dye terminator cycle sequencing kit (Applied Biosystems, Bayreuth, Germany). ABI Prism Model 310, version 3.0, sequencer was used for sequencing.

Morphological data

Morphological characters were recorded from 180 herbarium specimens of different herbaria that belong to the species listed in Table 2. The reproductive parts were studied after boiling in hot water for better pliability. Twenty-six characters (Table 3) were coded for the morphological matrix (Table 4) that was included in the combined ETS–ITS–*rpo*C1–*trn*T-F-morphologial analyses for examining the relationships within the genus. The autapomorphic characters or fully or partially overlapping characters were excluded from the analysis. A somewhat different morphological matrix (available from the corresponding author) comprising 28 coded characters, mostly of Table 3, was also used in the combined ITS–*rpo*C1–*trn*T-F-morphologial analyses to test the monophyly of the genus and its relationship with its allied genera. Before selecting the final characters for the study, a morphological matrix of 51 coded characters (not shown) including some seed and palynological characters used by Dessein et al. (2001b) was included in the preliminary analyses to assess the influence of these characters on the resolution of the phylogenetic analyses. The characters of seed exotesta of the species of *Sabicea* were studied by SEM (Philips XL-30) following the procedure outlined in Alejandro et al. (2005) and those of other genera (excluding *M. pinatubensis* and *W. coccinea*) were based on Dessein et al. (2001a, 2001b).

Data analyses

The forward and reverse sequences of the ETS, ITS, *rpo*C1 and *trn*T-F were assembled in Perkin Elmer sequence Navigator, version 1.0.1 and Sequencher 3.1.1. The consensus sequences were aligned and modified manually. Potentially informative indels were coded using the simple gap coding method (Simmons and Ochoterena 2000). Maximum parsimony analyses (MPA) of the combined ITS–*rpo*C1–*trn*T-F and ETS–ITS–*rpo*C1–*trn*T-F matrices, including and excluding the morphological matrix, were performed in PAUP, version 4.0b (Swofford 2000). All data matrices were analyzed using the following heuristic search settings: MULTREES option on, tree-bisection-reconnection (TBR) branch swapping, swap on best only in effect, and 5,000 random addition sequences. Consistency index (CI, Kluge and Farris 1969) and retention index (RI, Farris 1989) were calculated to estimate homoplasy. Bootstrap analyses were performed using 10,000 replicates, MULTREES option on, TBR branch swapping and five random addition sequences to assess the support of the resolved clades. In all analyses, we finally used the baseline matrices avoiding the coding of indels; however, to compare the results, we performed additional parsimony analyses including the coded indels, but excluding the coded positions and the results are mentioned only when these differed from those based on baseline matrices. In final analyses, all characters were given equal weight, gaps were treated as missing data, and only parsimony-informative characters were included. To explore the combinability of all data sets included in the ITS–*rpo*C1–*trn*T-F and ETS–ITS–*rpo*C1–*trn*T-F matrices, we conducted the ILD test as implemented in PAUP*, and compared the tree topologies generated from separate analyses of each data set.

To evaluate the statistically potential monophyletic groups, Bayesian analyses (BA) were performed in MrBayes, version 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) using the substitution model parameters: Prset statefreqpr = dirichlet (1,1,1,1), Lset nst = 6 rates = equal, selected for the best fit model (GTR + I+G) by both hierarchical likelihood ratio tests (hLRT) and Akaike information criterion (AIC) in MrModeltest, version 2.2 (Nylander 2004) for the uncoded and combined ITS–*rpo*C1–*trn*T-F and ETS–ITS–*rpo*C1–*trn*T-F data sets. In the combined analyses including morphological matrix, the morphological character partition was treated as standard and the model parameters lset applyto = (1/DNA) nst = 6 rates = invgamma; unlink shape = (all) pinvar = (all) statefreq = (all) revmat = (all); prset ratepr = variable; were applied. In all searches, the default settings (MrBayes, version 3.1.2) were used for all active parameters for the corresponding substitution models as well as for the heating scheme. Eight chains under two simultaneous runs with 100 sample frequencies were executed and monitored up to 3.5–4.5 $\times$ 10^6 Markov Chain Monte Carlo (mcmc) generations for arriving at the stationary phase. Exactly 25% of the samples were discarded as burn-in. The graphical presentations of summarized resulting trees were generated in PAUP* and Tree View (Page 1996). Internodes with posterior probabilities of more than 95% were considered as strongly supported.

To assess the evolution of morphological characters, selected characters were plotted on the strict consensus tree, generated from the parsimony analysis of ETS–ITS–*rpo*C1–*trn*T-F matrix, through importing it into MacClade, version 4.0 (Madison and Madison 2000).

Table 2 List of specimens used in this study, voucher information and GenBank accession numbers

Taxa	Country origins	Voucher information/ reference	ETS	ITS	*rpo*C1	*trn*T-F
Hekistocarpa minutiflora Hook. f. (1)	Cameroon	Sonké et al. 2708 (BR)	FM160674	AM981273	AM982749	AM982730
Hekistocarpa minutiflora Hook. f. (2)	Cameroon	Etuge and Thomas 143 (WAG)		AM981274	AM982750	AM982731
Mussaenda pinatubensis Elmer	Philippines	Alejandro 098 (UBT)		AJ846851	AM982780	AJ847365
Sabicea aspera Aubl.	French Guiana	Andersson et al. (2003) (NY)		AM409008	AM982751	AM409143
Sabicea becquetii (N. Hallé) Razafim., B. Bremer, Liede and Khan	Burundi	Reekmans 11116 (WAG)		AM409049	AM982752	AM409167
Sabicea caminata N. Hallé	Gabon	Wilde and Sosef 10311 (WAG)		AM409010	AM982753	AM409118
Sabicea ceylanica Puff	Sri Lanka	Jongkind et al. 1516 (UPS)		AM981275	AM982754	AM982732
Sabicea elliptica (Schweinf. ex Hiern) Hepper	Democratic Republic of Congo	Lisowski 56663 (BR)		AM409058	AM982755	AM409169
Sabicea hierniana Wernham	Gabon	Wilde 11714 (WAG)		AM981276	AM982756	AM982733
Sabicea medusula K. Schum. ex Wernh.	Cameroon	Andel et al. 3555 (WAG)		AM409047	AM982757	AM409163
Sabicea mildbraedii Wernham	Gabon	Wieringa 5032 (WAG)		AM409051	AM982758	AM409137
Sabicea mexicana Wernham	Mexico	Mendoza et al. 1329 (NY)		AM981277	AM982760	AM982734
Sabicea nobilis Good	Gabon	Valkenburg 2604 (WAG)		AM409052	AM982759	AM409165
Sabicea xanthotricha Wernham	Cameroon	Sonké 1082 (BR)		AM409045	AM982762	AM409151
Sabicea venosa Benth.	Central African Republic	Sonké and Benia 2797 (WAG)		AM409041	AM982761	AM409134
Tamridaea capsulifera (Balf. f.) Thulin and B. Bremer	Yemen	Miller et al. 10087 (UPS)		AM409059	AM982763	AM409170
Virectaria angustifolia (Hiern) Bremek.	Gabon	Wieringa 4730 (WAG)	FM160675	AM981278	AM982764	AM982735
Virectaria belingana N. Hallé (1)	Gabon	Parmentier 2336 (BRLU)	FM160676	AM981279	AM982765	AM982736
Virectaria belingana N. Hallé (2)	Equatorial Guinea	Parmentier 3675 (BRLU)	FM160666	AM981280	AM982766	AM982737
Virectaria belingana N. Hallé (3)	Equatorial Guinea	Obama and Lejoly 620 (BRLU)	FM160667	AM981281	AM982767	AM982738
Virectaria herbacoursi N. Hallé var. *petrophila* (1)	Equatorial Guinea	Parmentier and Esono 3375 (BRLU)	FM160668	AM981284	AM982768	AM982739
Virectaria herbacoursi N. Hallé var. *petrophila* (2)	Equatorial Guinea	Lejoly and Elad 98/73 (BRLU)	FM160669	AM981285	AM982769	AM982740
Virectaria major (K. Schum.) Verdc. subsp. *spathulata* (Verdc.) Dessein & Robbr. (1)	Democratic Republic of Congo	Lejoly 2934 (BR)	FM160670	AM981282	AM982770	AM982741
Virectaria major (K. Schum.) Verdc. subsp. *major* (2)	Tanzania	Kayombo 1842 (BR)	FM160671	AM981283	AM982771	AM982742
Virectaria multiflora (Sm.) Bremek. (1)	Ivory Coast	Leeuwenberg 2295 (UPS)	FM160672	AM409060	AM982772	AM409171
Virectaria multiflora (Sm.) Bremek. (2)	Liberia	Adams 606 (UPS)	FM160673	AM981286	AM982773	AM982743
Virectaria multiflora (Sm.) Bremek. (3)	Congo	Champluvier S109 (BR)	AM981184	AM981287	AM982774	AM982744
Virectaria multiflora (Sm.) Bremek. (4)	Gabon	Sosef et al. 551(WAG)	AM981185	AM981288	AM982775	AM982745

Table 2 continued

Taxa	Country origins	Voucher information/ reference	ETS	ITS	*rpo*C1	*trn*T-F
Virectaria procumbens (Sm.) Bremek. (1)	Gabon	Tabak et al. 182/189 (WAG)	AM981186	AM981289	AM982776	AM982746
Virectaria procumbens (Sm.) Bremek. (2)	Equatorial Guinea	Obama and Lejoly 538 (BRLU)	AM981187	AM981290	AM982777	AM982747
Virectaria sp. 1	Liberia	Adams 453 (UPS)	AM981188	AM409061	AM982778	AM409172
Virectaria sp. 2	Cameroon	Nemba and Thomas 321 (WAG)	AM981189	AM982729	AM982779	AM982748
Warszewiczia coccinea Klotzsch	South America	Delprete 6437 (UPS)		AJ846884	AM982781	AJ847397

Table 3 Morphological characters and character states used in the phylogenetic analyses

Char. No.	Characters and character states
1.	Plant habit: 0-herb, often woody at the base, 1-liana or vine, 2-(sub-) shrub and 3-tree
2.	Stem: 0-erect, 1-climbing and 2-straggling
3.	Stipule's shape: 0-oblong to lingulate, 1-ovate to deltate, 2-triangular and 3-lanceolate
4.	Stipule orientation: 0-antrorse and appressed, 1-antrorse and spread, 2-moderately decurved and 3-recurved to slightly reflexed
5.	Lobes of stipules: 0-at least 2 lobes present and 1-lobes absent
6.	Length-width ratios of leaf blade: 0—<3, 1—3–6, 2—>6
7.	Shape of leaf blades: 0-elliptic to oblong, 1-lanceolate, 2-ovate to widely lanceolate and 3-very narrowly elliptic to obovate or oblanceolate
8.	Indument of upper surface of leaf blades: 0-covered with indument at least along the veins, 1-glabrescent and 2-glabrous
9.	Number of flower per inflorescence: 0-one, sometimes three, 1-few and 2-many
10.	Calyx: 0-campanulate, 1-tubes nearly indistinct and 2-infundibuliform
11.	Length–width ratios of calyx lobes: 0—<2, 1—2–5, 2—>5
12.	Apex of calyx lobes: 0-acuminate to apiculate, 1-obtuse and 2-(sub-)acute
13.	Hairiness of calyx lobes margins: 0-eciliate and 1-ciliate or ciliolate
14.	Indument of outer surface of calyx lobes: 0-covered with indument, 1-glabrescent and 2-glabrous
15.	Trichomes of calyx lobes: 0-appressed and ±straight, 1-erecto-patent and ±straight and 2-(sub-) appressed to erecto-patent and ± straight or curled
16.	Long, stiff trichomes on outside of calyx lobes: 0-absent and 1-present
17.	Length-width ratios of corolla lobes: 0—<1 and 1—>1
18.	Hairiness of corolla lobe margins: 0-eciliate and 1-ciliate or ciliolate
19.	Indument cover of outer surface of corolla: 0-covered with indument, 1-glabrous and 2-glabrescent
20.	Trichomes of outer surface of corolla: 0-appressed and ± straight, 1-erecto-patent and ± straight, 2–appressed to erecto-patent and ± straight and 3-(sub-) appressed to erecto-patent and curled
21.	Protrusion of anthers: 0-included in corolla tubes and apically with or without protrusion beyond the tubes and 1-completely protrusion beyond corolla tubes
22.	Protrusion of style: 0-exserted part is longer than the corolla lobes, 1-exserted part is not longer than the corolla lobes and 2-included in corolla tube with or without projecting tip of stigmatic lobes
23.	Flower disc: 0-divided into two bilobed parts, 1-undivided and cylindrical and 2-undivided and shallowly campanulate
24.	Fruit dehiscence: 0-fruits dehiscent but margins do not fold inwards, 1-fruits dehiscent and margins fold inwards and 2-fruits indehiscent
25.	Elongation of exotesta cells: 0-elongated and 1-strongly elongated
26.	Trichomes of flowering branchlets and lower surface of leaves: 0-long and 1-short

Table 4 Morphological matrix for *Virectaria* and outgroup taxa

Taxa	Character states for characters 1–26																									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Hekistocarpa minutiflora	0	1&2	3	1&3	1	1	3	0	2	0	0	2	0	1	2	0	0	0	1	-	0	2	2	0&2	0	1
Sabicea becquetii	1	1	1	1&3	0&1	0	0&1	0&1	1	0	1	0	1	0	2	0	0	0&1	0	3	0	2	2	2	1	0
S. xanthotricha	1	1	1	0&1	1	0	0	0	1&2	0	2	0	1	0	2	0	0	0	1	-	0	2	2	2	1	1
S. elliptica	2	0	1	0	1	0	0	2	1	0	0&1	2	1	0	0	0	0	0	1	-	0	1	2	2	1	0
Tamridaea capsulifera	2&3	0	2	0	1	0	0	2	1	0	1	1	0	1	0	0	0	0	0	0	0	2	2	0	1	1
Virectaria angustifolia	0&2	0	3	0&1	1	1&2	3	2	1	1	1	1&2	1	2	-	0	0	0	1	-	1	1	1	1	1	1
V. belingana 1	0&2	0	3	0&1	1	0	2	0&1	1&2	1	1	0&2	1	1	2	0	1	0	0	0	1	0	1	0&1	1	1
V. belingana 2	0&2	0	3	0&1	1	0	2	0&1	1&2	1	1	0&2	1	1	2	0	1	0	0	0	1	0	1	0&1	1	1
V. belingana 3	0&2	0	3	0&1	1	0	2	0&1	1&2	1	1	0&2	1	1	2	0	1	0	0	0	1	0	1	0&1	1	1
V. herbacoursi 1	0	0	0	2	0	1	3	0&1	2	1	1	2	0	0	1	1	1	0&1	0	1	1	0	0	0	0	0
V. herbacoursi 2	0	0	0	2	0	1	3	0&1	2	1	1	2	0	0	1	1	1	0&1	0	1	1	0	0	0	0	0
V. major 1	1	0&2	3	1	0&1	0&1	0&2	0	2	1	1&2	0&2	1	0	2	0&1	1	1	0	2	1	0	1	0	1	0
V. major 2	2	0&2	3	1	0&1	0&1	0&2	0	2	1	1&2	0&2	1	0	1	0&1	1	1	0	1	1	0	1	0	1	0
V. multiflora 1	0	0	3	1	0	0	2&3	0	2	1	2	2	1	0	1	1	1	1	0	2	1	0	0	0	0	0
V. multiflora 2	0	0	3	1	0	0&1	2&3	0	2	1	2	2	1	0	1	1	1	1	0	2	1	0	0	0	0	0
V. multiflora 3	0	0	3	1	0	1	2&3	0	2	1	2	2	1	0	1	1	1	1	0	2	1	0	0	0	0	0
V. multiflora 4	0	0	3	1	0	1	2&3	0	2	1	2	2	1	0	1	1	1	1	0	2	1	0	0	0	0	0
V. procumbens 1	0	0&2	3	3	1	0	0&2	0	1	1	0&1	1	1	0	0	0	0&1	1	0	2	1	1	1	1	1	1
V. procumbens 2	0	0&2	3	3	1	0	0&2	0	1	1	0&1	1	1	0	0	0	0&1	1	0	2	1	1	1	1	1	1
Virectaria sp. 1	0	0&2	3	3	1	0	0&2	0	1	1	0&1	1	1	0	0	0	0&1	1	0	2	1	1	1	1	1	1
Virectaria sp. 2	0&2	0	3	0&1	1	2	3	0	1	1	1	1&2	1	2	-	0	0	1	1	0	1	1	1	1	1	1

Table 5 Descriptions of combined maximum parsimony analyses and resulting trees

Data partitions and analyses	Outgroup/ingroup taxa	No. informative characters	Length	CI	RI	No. MP trees
ITS + *rpo*C1 + *trn*T-F	2/21	293	546	0.685	0.853	2
ITS + *rpo*C1 + *trn*T-F + morphology	2/21	321	673	0.633	0.814	1
ETS + ITS + *rpo*C1	5/16	200	324	0.750	0.888	1
ETS + ITS + *rpo*C1 + morphology	5/16	226	421	0.708	0.863	4
ITS + *rpo*C1 + *trn*T-F	5/16	239	376	0.758	0.883	6
ITS + *rpo*C1 + *trn*T-F + morphology	5/16	265	475	0.726	0.864	6
ETS + ITS + *rpo*C1 + *trn*T-F	5/16	293	462	0.751	0.886	2
ETS + ITS + *rpo*C1 + *trn*T-F + morphology	5/16	319	542	0.720	0.867	1

Results

Phylogenetic analyses

Description of all MPA of the combined data sets and resulting trees are summarized in Table 5.

Separate analysis of ITS, rpoC1 and trnT-F data. The results of the ILD test supported the combinability of the ITS, *rpo*C1 and *trn*T-F data sets of the 23 taxa used in evaluating the relationships between the genera of Sabiceeae and testing the monophyly of *Virectaria* (Table 6). However, the tree topologies of the strict consensus trees resulted from the separate analyses of ITS, *rpo*C1 and *trn*T-F data (results not shown) appeared conflicting regarding the positions of *Hekistocarpa* and *Tamridaea*. But neither of the positions was supported by more than 50% BS. In both ITS and *trn*T-F analyses (results not shown), *Hekistocarpa* was resolved as sister (BS 100) to the clade of *Tamridaea*: *Sabicea* and *Virectaria* (BS 57, ITS, BS 54, *trn*T-F). In *rpo*C1 analysis, *Tamridaea*, instead of *Hekistocarpa* (ITS or *trn*T-F tree), was resolved as sister (BS 98) to an unsupported *Hekistocarpa–Virectaria Sabicea* clade (BS <50). In an analysis of our *trn*T-F data (results not shown) *Tamridaea* and *Sabicea* s.l. were resolved as a weakly supported monophyletic group (BS 60) in the unsupported *Tamridaea–Sabicea–Virectaria* clade (BS <50).

Table 6 Scores of incongruency length difference (ILD) test for the combinability of ITS and *trn*T-F data partitions (*$P < 0.05$) without excluding any taxa

Data partitions	*P* values	Significance
ITS, *rpo*C1and *trn*T-F of 23 taxa	0.916000	Congruent
ETS, ITS and *rpo*C1 of 21 taxa	0.132000	Congruent
ITS, *rpo*C1 and *trn*T-F of 21 taxa	0.088000	Congruent
ETS, ITS, *rpo*C1 and *trn*T-F of 21 taxa	0.004000	Incongruent
ETS, ITS, *rpo*C1 and *trn*T-F of 17 taxa (excluding *T. capsulifera*, *S. becquetii*, *S. elliptica* and *S. xanthotricha*)	0.002000	Incongruent

Based on the results of the ILD test and lack of support for the topological conflicts, we combined the ITS, *rpo*C1 and *trn*T-F data sets.

Combined ITS–rpoC1–trnT-F analyses. The strict consensus of two most parsimonious trees generated from the combined analyses of the ITS, *rpo*C1 and *trn*T-F data of 23 taxa (Fig. 1) exhibited strong support to the clade of Sabiceeae sensu Khan et al. in which two *Hekistocarpa* accessions were shown to be sister to a weakly to moderately supported clade comprising *Tamridaea*, six species of *Virectaria* and twelve species of *Sabicea* s.l. This *Tamridaea–Virectaria–Sabicea* clade was further resolved into two major clades, the moderately to strongly supported *Tamridaea–Virectaria* clade, which was resolved with weak to moderate support as sister to the *Sabicea* clade. Within the *Tamridaea–Virectaria* clade, *Tamridaea* was consistently shown to be sister to the strongly supported monophyletic group comprising all *Virectaria* species (hereafter *Virectaria* clade). Within the *Virectaria* clade, *V. herbacoursi* N. Hallé and *V. multiflora* (Sm.) Bremek. formed a strongly supported clade (hereafter *V. herbacoursi–V. multiflora* clade), which was further resolved as sister to the strongly supported clade formed by *V. angustifolia* (Hiern) Bremek. *V. procumbens* (Sm.) Bremek. *V. belingana* N. Hallé and *V. major* (K. Schum.) Verdc. (hereafter *V. angustifolia–V. procumbens–V. belingana–V. major* clade). This clade of four *Virectaria* accessions was resolved into two subclades, the strongly supported *V. angustifolia–V. procumbens* subclade and the moderately supported *V. belingana–V. major* subclade. The *Tamridaea–Virectaria* clade or a *Tamridaea* resolved as sister to *Virectaria* was weakly supported when 28 morphological characters (not shown) were included in the analyses. The topology of the most parsimonious tree generated from the combined ITS–*rpo*C1–*trn*T-F tree was similar to that resulting from the separate analysis of the ITS data set, except for *S. hierniana* Wernham, *S. caminata* N. Hallé, *S. ceylanica* Puff and *S. nobilis* Good or the *trn*T-F data set, except for *T. capsulifera*, and few accessions of

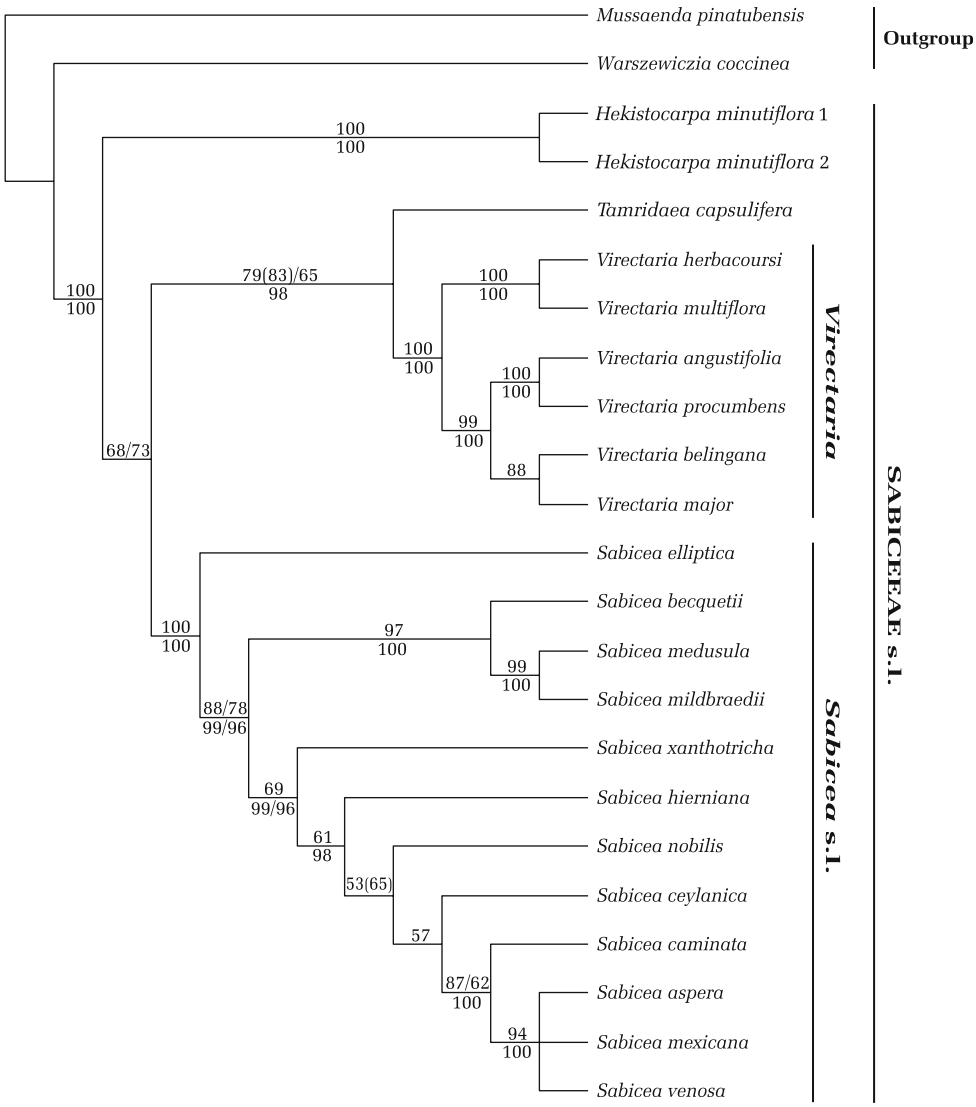

Fig. 1 Strict consensus tree based on the combined phylogenetic analysis of the ITS–*rpo*C1–*trn*T-F data. The numbers *above* the branches are bootstrap support values (>50%), those *below* the branches are Bayesian posterior probabilities (>95%), those *after slash* are the support from morphological data, and those *in brackets* are the supports due to the indels. The taxa shown in *boldface* are the sequenced individuals of monospecific *Hekistocarpa* and *Tamridaea*

Sabicea (e.g. *S. hierniana*, *S. nobilis* and *S. mexicana* Wernham) and *Virectaria* (e.g. *V. angustifolia*, *V. major*, and *V. procumbens*). The strict consensus tree generated from a separate analysis of *rpo*C1 data (not shown) was unresolved except for the clades of Sabiceeae s.l. and *Sabicea* s.l.

Separate analysis of the ETS, ITS, rpoC1 and trnT-F data. The results of the ILD test did not support the combinability of ETS, ITS, *rpo*C1 and *trn*T-F data sets to assess the infraspecific relationships and biogeography within the genus. But the combinability of either the ETS, ITS and *rpo*C1 or the ITS, *rpo*C1 and *trn*T-F data sets were supported (Table 6). On the other hand, the separate analyses of each of the ETS, ITS and *trn*T-F data sets and the ETS–ITS–*rpo*C1, ITS–*rpo*C1–*trn*T-F and ETS–ITS–*rpo*C1–*trn*T-F matrices (results not shown) imparted the unsupported topological conflicts in resolving the two *V. herbacoursi* accessions as sister group to the subclade of *V. multiflora* accessions (e.g. ITS, *trn*T-F, ITS–*rpo*C1–*trn*T-F, ETS–ITS–*rpo*C1–*trn*T-F trees) versus the clade of all *Virectaria* accessions (e.g. ETS tree). Based on the lack of clear-cut evidence to the reason of incongruence of ETS, ITS, *rpo*C1 and *trn*T-F data sets or the topological conflicts, we combined these four data sets. Finally, to describe the infraspecific relationships within the genus, we present our results based on the combined ETS–ITS–*rpo*C1–*trn*T-F analyses.

Combined ETS–ITS–rpoC1–trnT-F analyses. In the most parsimonious ETS–ITS–*rpo*C1–*trn*T-F tree (Fig. 2), the sampled *Virectaria* formed a strongly supported monophyletic group, in which all individuals were resolved in two major clades: the moderately to strongly supported *Virectaria herbacoursi–Virectaria multiflora* clade (hereafter Clade A; Fig. 2), and the strongly supported

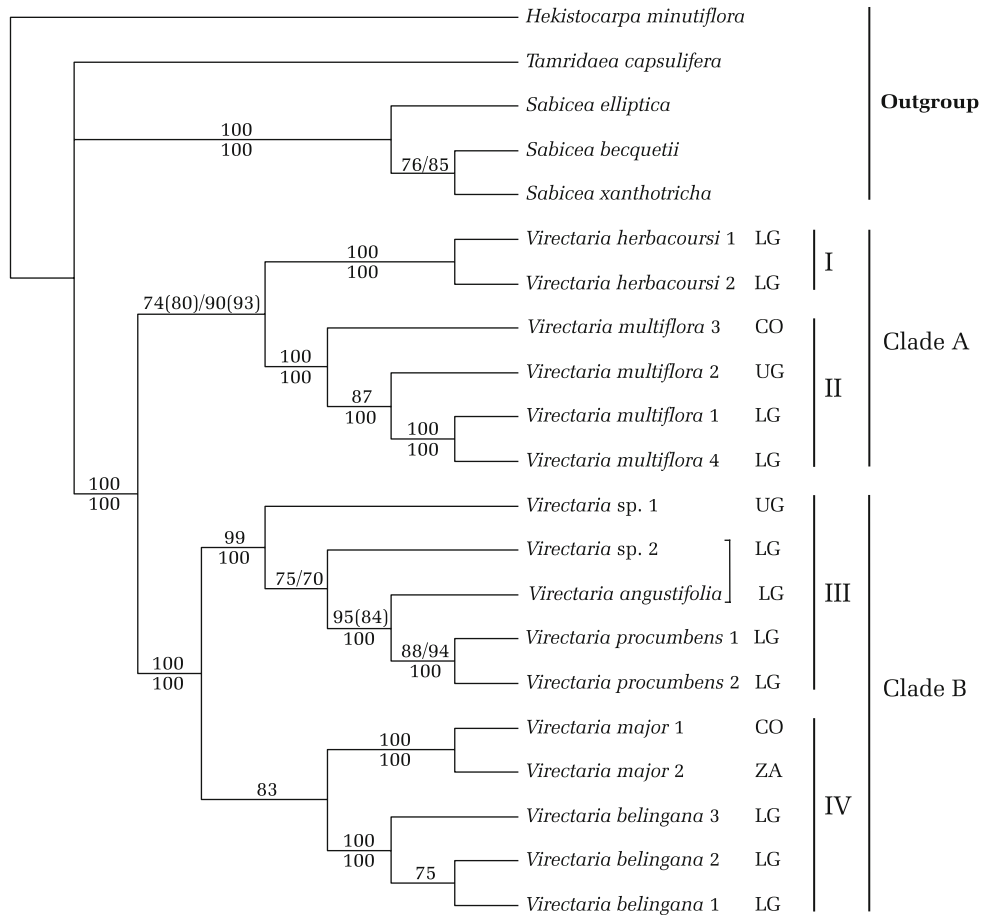

Fig. 2 Strict consensus tree based on the combined phylogenetic analysis of the ETS–ITS–*rpo*C1–*trn*T-F data. The numbers *above* the branches are bootstrap support values (>50%), those *below* the branches are Bayesian posterior probabilities (>95%), those *after slash* are the support from morphological data, and those *in brackets* are the supports due to the indels. *Virectaria* sp. 2 and *V. angustifolia*, delimited with bracket, form a subclade when morphological data are included in the analyses. *V. herbacoursi* (=*V. herbacoursi* var. *petrophila*); *V. major* 1 (=*V. major* subsp. *spathulata*); *V. major* 2 (=*V. major* subsp. *major*). *Clade A* = *V. herbacoursi–V. multiflora* clade; *Clade B* = *Virectaria* sp.–*V. angustifolia–V. procumbens–V. major–V. belingana* clade; *I* = *V. multiflora* subclade; *II* = *Virectaria* sp.–*V. angustifolia–V. procumbens* subclade; *III* = the *V. major–V. belingana* subclade. *CO* = Congolian element, *LG* = Lower-Guinean element, *UG* = Upper-Guinean element and *ZA* = Zambezian element

Virectaria sp.–*Virectaria angustifolia–Virectaria procumbens–Virectaria major–Virectaria belingana* clade (hereafter Clade B; Fig. 2). Clade A is further resolved as sister to Clade B. Within Clade A, two accessions of *V. herbacoursi* were resolved as sister (hereafter subclade AI; Fig. 2) to the strongly supported subclade comprising all sampled *V. multiflora* accessions (hereafter subclade AII; Fig. 2).

Within Clade B, the two *Virectaria* sp., one *V. angustifolia*, and two *V. procumbens* accessions formed a strongly supported subclade (hereafter subclade BIII; Fig. 2) which was further resolved as sister to another moderately supported subclade consisting of two subspecies of *V. major* (*V. major* 1 = *V. major* (K. Schum.) Verdc. subsp. *spathulata* (Verdc.) Dessein & Robbr. *V. major* 2 = *V. major* (K. Schum.) Verdc. subsp. *major*) and three accessions of *V. belingana* (hereafter subclade BIV; Fig. 2). In subclade BIII, *Virectaria* sp. 1 was resolved as sister to the moderately supported group of *Virectaria* sp. 2, *V. angustifolia* and two *V. procumbens* accessions. Within this group, *Virectaria* sp. 2 was resolved with weak to moderate support as sister to the well-supported group of one *V. angustifolia* and two *V. procumbens* accessions, while *V. angustifolia* was further resolved with moderate to strong support as sister to the moderately to strongly supported monophyletic group of two *V. procumbens* accessions. Within subclade BIV, the two sampled subspecies of *V. major*, forming a strongly supported monophyletic group, were resolved as sister to the strongly supported monophyletic group of three *V. belingana* accessions (Fig. 2). The topology of the combined ETS–ITS–*rpo*C1 tree was similar to that of the strict consensus tree generated from the separate ETS analysis, except for the position of two *V. herbacoursi* accessions. The

resolution of each species exhibited in the most parsimonious tree generated from the combined analysis of molecular data sets was mostly compatible with species delimitation by morphological characters but resolution within the species was uncongenial with morphological distinctiveness.

The plotting of selected morphological characters on the strict consensus ETS–ITS–*rpo*C1–*trn*T-F tree indicated their evolution within the sampled taxa of Sabiceeae s.l., especially in *Virectaria*. Characters such as indistinct calyx tubes and completely exserted anthers (Fig. 3a), absence of campanulate flower disc (Fig. 3g) and dehiscent fruits (Fig. 3h) characterizes the clade of *Virectaria*. The 2–3-lobed stipules and calyx lobes covered with long and stiff trichomes and lanceolate to lingulate bilobed parts of flower disc support the clade of *V. herbacoursi* and *V. multiflora* (Fig. 3b), while the cylindrical flower disc (Fig. 3g) supports the clade of *V. angustifolia*, *V. belingana*, *V. major* and *V. procumbens*. The inward folded margins of valves characterizes the *V. angustifolia*–*V. procumbens* subclade (Fig. 3h).

The resolutions in the combined ITS–*rpo*C1–*trn*T-F and the ETS–ITS–*rpo*C1–*trn*T-F tree indicated some phytogeographical aspects within *Sabiceeae* s.l. and *Virectaria* that are discussed here following White's (1979, 1983) centres and subcentres of endemism.

Discussion

The main focus of this paper is the relationship of *Virectaria* with its associated genera of Sabiceeae sensu Khan et al. (2008), the monophyly of *Virectaria* and interspecific relationships between its species, and biogeography of the genus as inferred by our results.

Sequence characteristics

ETS and *rpo*C1 data are here used for the first time for the Sabiceeae, while the ITS and *trn*T-F data were used in our earlier study (Khan et al. 2008). Consequently the sequence characteristics of the ITS and *trn*T-F (Table 7) correspond closely to those in Khan et al. (2008). The range of variation in the ETS lengths and the percentage of GC contents (Table 7) appear close to the record for other Rubiaceae (469 bp, Nepokroeff et al. 2003, 51%, Negrón-Ortiz and Watson 2002). The non-coding sequences from the *rpo*C1 region have been frequently used in angiosperms, in intrafamilial (e.g. Apiaceae Downie et al. 1996a, Fabaceae Liston and Wheeler 1994) or infrageneric (e.g. *Lathyrus* L. Asmussen and Liston 1998, *Trifolium* Watson et al. 2000) phylogeny studies. However, in Rubiaceae only Samson et al. (2007) explored the implication of the *rpo*C1 region of *Coffea arabica* L. for phylogenetic relationships. Using the DNA barcoding primers (*rpo*C1.2f and *rpo*C1.4r), we could amplify only exon 1 and a tiny part of intron from *rpo*C1 region. The lack of *rpo*C1 intron in some angiosperms is reported (Downie et al. 1996b, Wallace and Cota 1996, Hansen et al. 2006). However, we are unable to conclude here, whether or not the *rpo*C1 intron or *rpo*C1 exon 2 is missing in the genera included in this study. The low variation of lengths and potentially informative characters of the aligned *rpo*C1 matrix (484 bp and 13 informative characters) appears close to the report for the *rpo*C spacer of the flowering plant genus *Styrax* (Styracaceae, Ebenales, Fritsch 2001). The mostly unresolved to moderately resolved trees resulting from the separate analysis of the *rpo*C1 data corresponds to its very low variation; however, it generates moderate to strong supports to the resolved clades (not shown), which indicates its potential phylogenetic implication at generic level in Rubiaceae.

Relationships between *Virectaria* and its associated genera of Sabiceeae s.l.

While the position of *Hekistocarpa* as sister to the *Tamridaea–Virectaria–Sabicea* clade is only weakly to moderately supported in the combined ITS–*rpo*C–*trn*T-F tree (Fig. 1), this sister-group relationship is consistently retained in the parsimonious trees generated from the combined analyses conducted in this study. Plus, it is highly supported (BS 82, PP 100) by the combined ITS–*trn*T-F analysis of Khan et al. (2008). However, this result is inconsistent with that of the *rbc*L analysis of Dessein et al. (2001a; Fig. 38), in which *Sabicea* is resolved with weak support (JK 66) as sister to a clade containing *Hekistocarpa*, *Tamridaea* and *Virectaria*. The sister-group relationships between *Tamridaea* and *Virectaria* shown by Khan et al. (2008; Fig. 3), but poorly supported is further corroborated by our results (Fig. 1). Both *Tamridaea* and *Virectaria* appear to share a sister-group relationship with *Sabicea* s.l., as the moderately to strongly supported *Tamridaea–Virectaria* clade was resolved as sister to the *Sabicea* clade with weak to moderate support. In sum, the present analyses have confirmed that *Hekistocarpa* is sister to the *Tamridaea–Virectaria–Sabicea* clade and *Tamridaea* and *Virectaria* are sister genera. It is important to stress that these relationships are clearly supported by molecular data only. There is no clear-cut support from morphological characters to these close relationships. *Virectaria* and its associated genera (*Hekistocarpa*, *Sabicea* s.l., *Tamridaea*) contain some autapomorphic characters and share mostly homoplasious characters (Table 3, 4; Fig. 3a–h; Khan et al. 2008; Appendix 2–3, Dessein et al. 2001b).

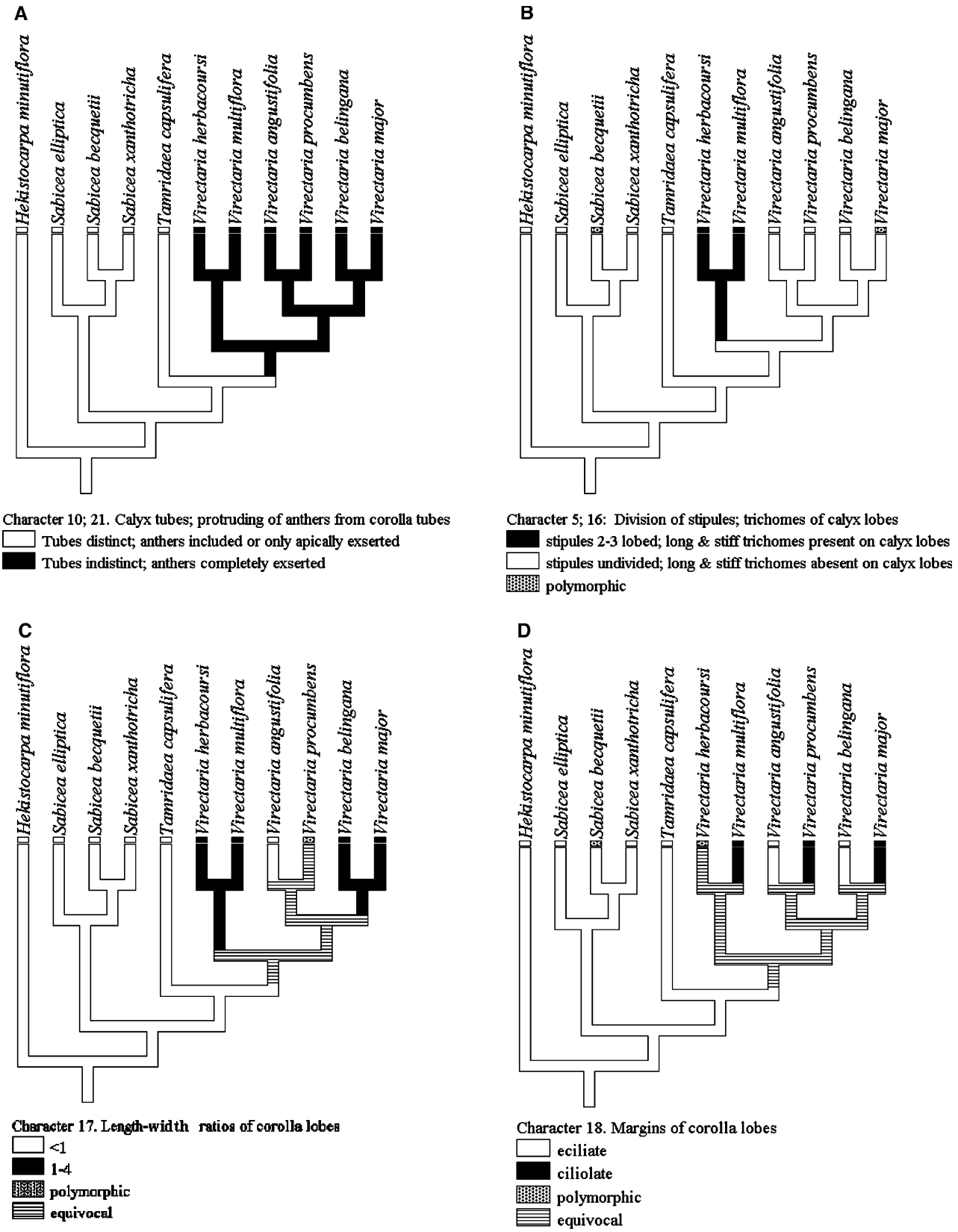

Fig. 3 Distribution patterns of some important characters on the strict consensus tree generated from the combined analysis of ETS–ITS–*rpo*C1–*trn*T-F data sets; **a** length of calyx tubes and position of anthers, **b** division of stipules and long stiff trichomes on calyx lobes, **c** length–breadth ratios of corolla lobes, **d** margins of corolla lobes, **e** external surface of corolla lobes, **f** protruding of styles, **g** division and shape of floral disc, **h** fruit dehiscence and folding of valves

Fig. 3 continued

Monophyly of *Virectaria*

The morphological characteristics (Verdcourt 1953; Table 4; Dessein et al. 2001b) of the herbaceous to semi-woody genus *Virectaria* support its position in Rubiaceae and Sabiceeae, which is confirmed by molecular data (Bremer and Thulin 1998; Khan et al. 2008). Although the monophyly of the genus has never been doubted, it has also never been assessed using molecular phylogenetic analysis. In Khan et al. (2008), two *Virectaria* species (*V. multiflora* and *V. procumbens*) form a monophyletic group. In all analyses of the present study including morphological data

Table 7 Characteristics of the non-aligned sequences and their description in alignments

Markers	Length ranges of sequences (bp)	Ranges of GC contents in sequences (%)	Number of positions in alignments	Parsimony-informative characters in alignments	Parsimony-uninformative variable characters in alignments
ETS region	358–449	46.7–50.01	–/455	–/54	–/15
ITS region	584–713	53.7–65.5	638/616	164/140	82/60
ITS1	186–294	52.7–68.7	245/228	83/75	42/28
S5.8	147–165	53.3–66.1	165/165	05/05	00/00
ITS2	156–279	53.6–71.6	228/223	76/60	36/32
*rpo*C1 region	493–519	41–43	484/484	12/06	04/03
*rpo*C1 exon 1	399–416	41.3–43.3	399/400	12/06	04/03
*rpo*C1 intron (partial)	94–103	44.7–46.8	85/85	00/00	04/00
*trn*T-F region	1292–1669	28.2–36.7	1,810/1760	117/93	186/70
*trn*T-L spacer	388–774	24.1–32.5	838/826	71/53	110/50
*trn*L spacer	537–615	36.7–44.1	641/606	22/13	36/10
*trn*L intron	455–530	35.3–42.8	556/521	22/13	32/08
*trn*L-F spacer	313–327	32.1–36.1	331/328	24/27	40/10

The values before the slash mark correspond to the combined ITS–*rpo*C1–*trn*T-F matrix and those after the slash mark to the ETS–ITS–*rpo*C1–*trn*T-F matrix

(Figs. 1, 2), all sampled *Virectaria* are constantly resolved as one strongly supported monophyletic group.

Morphological characters like eciliate or ciliolate margins of corolla lobes (Fig. 3d), glabrous or hairy external surfaces of corolla (Fig. 3e) and length of exserted part of style (Fig. 3f) seem to have evolved independently in the sampled taxa of Sabiceeae s.l. and are thus not useful for phylogenetic conclusions. However, there are several morphological characters supporting the genus *Virectaria*, such as completely exserted anthers (Fig. 3a), truncated stigmata, internal indumenta with flattened hairs, presence of elongated but non-campanulate flower disc (Fig. 3g) and dehiscent fruits (Fig. 3h) with capsules splitting into one persistent and one deciduous valve. Therefore, the monophyly of the genus *Virectaria* is strongly supported by both molecular and morphological analyses and easily identified by the several distinct morphological characters. On the other hand, the constant resolving of all sampled species of *Sabicea* as a monophyletic group conforms to *Sabicea* s.l. (Khan et al. 2008).

Relationships within *Virectaria*

The previous studies discussing the relationships within the genus *Virectaria* (Verdcourt 1953; Dessein et al. 2001b) were exclusively based on morphological data. The overall tree topology of our most parsimonious ETS–ITS–*rpo*C1–*trn*T-F tree or ETS–ITS–*rpo*C1–*trn*T-F-morphology tree (Fig. 2) is mostly consistent to that of Dessein et al. (2001b). The groups of *Virectaria* species resolved in two major clades (Clade A and Clade B) are strongly supported by both molecular and morphological data.

Virectaria herbacoursi–Virectaria multiflora clade (Clade A). Within Clade A (Fig. 2), *V. herbacoursi* is well resolved as sister (Fig. 2: AI) to *V. multiflora* (Fig. 2: AII), consistent with Dessein et al. (2001b). This clade is distinct from its sister Clade B by the following four morphological characters: 2–3 distinct lobes of stipules, long and stiff trichomes on outer calyx lobe surfaces, and two lanceolate and bilobed parts of floral disc (Figs. 3b, g), and broad exotesta cells and smaller pollen (Dessein et al. 2001b). In other words, the close relationship of *V. herbacoursi* with *V. multiflora* is strongly supported by both molecular and morphological data. *V. herbacoursi* can easily be distinguished from *V. multiflora* by its 1–2 trichomes consistently present on the outer calyx lobe surface in contrast to almost more than two, usually few to many trichomes of *V. multiflora* calyx lobes. All four accessions of *V. multiflora* from Gabon, Congo and Liberia form a strongly supported subclade (Fig. 2: AII). The resolution of four *V. multiflora* accessions within this subclade, i.e. the resolving of *V. multiflora* 3 as sister to other three accessions (*V. multiflora* 2, *V. multiflora* 1, and *V. multiflora* 4) or *V. multiflora* 2 as sister to *V. multiflora* 1 and *V. multiflora* 4, is unsupported by their morphological characters, consistent with Dessein et al. (2001b).

A close relationship between *V. herbacoursi* and *V. tenella* based on the characters of floral disc and trichomes of calyces was postulated by Dessein et al. (2001b), but this relationship is yet to be tested with molecular data. The two

species contain notable autapomorphies such as creeping habit, erect branches, narrowly elliptic or lingulate, long (>20 mm) leaves and linear calyx lobes of *V. herbacoursi* in contrast to prostrate habit without erect branches, widely ovate and shorter (<15 mm) leaves, deltoid, foliaceous or spathulate calyx lobes for *V. tenella*. *V. tenella* also resembles *V. belingana* in the relatively small leaves and the short trichomes.

Virectaria sp.–Virectaria angustifolia–Virectaria procumbens–Virectaria major–Virectaria belingana clade (Clade B). Within Clade B (Fig. 2), subclade BIII (forming *Virectaria* sp. 1, *Virectaria* sp. 2, *V. angustifolia*, *V. procumbens*) is supported by two important characters: smaller corolla tubes and inward folding of valves, and presumably the presence of a tectum with elongated and curved or more rounded sexine elements and pollen P/E < 1.2 (Dessein et al. 2001b). *Virectaria* sp. 1, resolved here with low support as sister to a clade consisting of *Virectaria* sp. 2, *V. angustifolia*, and two accessions of *V. procumbens*, seem morphologically distinct from all other members of this subclade by its dwarf (15–18 cm long) semi-erect habit, upto 1 mm long trichomes, densely leafy branches, small leaves (0.8–2 (−2.5) × 0.4–1.1 cm), and 4–5 mm long corolla tubes. Morphologically, *Virectaria* sp. 1 seems an intermediate between *V. procumbens* and the Ghanian *V. tenella*. Its growth habit, shape and size of leaves, longer trichomes and structure of inflorescence appear similar to those of *V. tenella*, whereas its floral characters seem similar to those of *V. procumbens*; however, we find no molecular support for this, as *V. angustifolia* is resolved to be the closest relative of *V. procumbens*.

Morphologically *Virectaria* sp. 2, resolved as sister to the group of *V. angustifolia* and two accessions of *V. procumbens*, appears closely related to *V. angustifolia* and *V. salicoides*. The duplicate of the sequenced specimen of *Virectaria* sp. 2 (Nemba and Thomas 321 at BR) is included under *V. angustifolia* by Dessein et al. (2001b). This taxonomic decision is not supported by the analyses of our molecular data. The analysis adding morphological data to the molecular data seems to support the close relationship between *Virectaria* sp. 2 and *V. angustifolia* (Fig. 2: BIII). However, *Virectaria* sp. 2 has truely spathulate calyx lobes as opposed to linear to lanceolate or triangular ones of *V. angustifolia* var. *angustifolia* and somewhat widely linear ones of *Virectaria angustifolia* var. *schlechteri*.

V. angustifolia and *V. salicoides* appear closely related morphologically by their similar length–width ratios of leaves and the length ratios of corolla lobes and tubes, narrowly elliptic to lingulate or oblanceolate leaves, and short trichomes, etc. On the other hand, *V. angustifolia* is distinct from *V. salicoides* by its short (usually 4–4.5 mm long; "less than 0.5 mm long" in Dessein et al. is a clerical error) corolla tubes and the entire stipules, externally glabrous corolla [Hiern 1877: PL 12 (2&3), Hallé 1966], narrowly lingulate to triangular calyx lobes and exserted part of stamens and styles not longer than corolla lobes.

The recognition of two varieties [*V. angustifolia* (Hiern) Bremek. var. *angustifolia* Bremek., *V. angustifolia* (Hiern) Bremek. var. *schlechteri* Verdc.] within *V. angustifolia* based on leaf shape and size as described by Verdcourt (1953) appears unjustified, as the variation in leaf length (0.8–5 cm), leaf width (0.2–1 cm) or length–width ratio of leaves is continuous within this species. The variation in leaf shape or apices is also overlapping. Within subclade BIII (consisting of *Virectaria* sp. 1, *Virectaria* sp. 2, *V. angustifolia* and *V. procumbens;* Fig. 2) the *V. angustifolia–V. procumbens* subclade is diagnosed by two potential morphological synapomorphies: exserted part of style longer than corolla lobes and margins of valves folded inwards (Fig. 3f, h). Their notable distinct characters include narrowly elliptic to lingulate or oblanceolate leaves, lanceolate to triangular calyx lobes, and glabrous to glabrescent upper surface of leaves in *V. angustifolia* rather than ovate to widely lanceolate leaves, spathulate calyx lobes and sparsely strigulose upper surface of leaves in *V. procumbens*.

In subclade BIV (*V. major*, *V. belingana*; Fig. 2), the sister-group relationship between *V. major* and *V. belingana* accessions supported by our analyses is not consistent to Verdcourt's (1953) placement of *V. major* in the central line of his scheme and between *V. angustifolia* and *V. procumbens*. This result is also not consistent with Dessein et al. (2001b) who postulated *V. major* as the basal species within Clade B. On the other hand, this relationship is poorly supported in the Bayesian analyses and appears unsupported by any morphological synapomorphy. The recognition of two subspecies *V. major* subsp. *major* (=*V. major* 1) and *V. major* subsp. *spathulata* (=*V. major* 2) seems warranted due to their restricted distributions and dissimilarity in shape of calyx lobes, as described by Dessein et al. (2001b).

Preliminary biogeographic hypotheses of *Virectaria*

Khan et al. (2008; Fig. 3) and all combined analyses performed for this study, including the combined ITS–*rpo*C1–*trn*T-F analysis (Fig. 1), consistently indicates that *Hekistocarpa* is sister to the *Tamridaea–Virectaria–Sabicea* clade. This seems to indicate a tropical African and possibly a Guineo–Congolian origin for the whole tribe, as *Hekistocarpa* is known to be restricted to the Lower-Guinean subcentre of endemism (Dessein et al. 2001a). The fruits of *Hekistocarpa* are dry, small and crowned with persistent calyx lobes and hairs, which might be dispersed by wind or by adhering to the bodies of animals or by

sticking to the feathers of birds. *Tamridaea* is restricted to Socotra (Bremer and Thulin 1998). Socotra is of Gondwanian origin; however, dating of its separation from Africa and Arabia is still debated with estimates ranging from 10 mya (Miller and Morris 2004) to 65–70 mya (Kopp 1999; Mies 2001). Recent geological studies suggest an age between 35 and 15 mya (Fleitmann et al. 2004; Thiv and Meve 2007). The origin of Rubiaceae is placed in the Danian at 61–64 mya (Wikström et al. 2001) and 78 mya (Bremer et al. 2004). However, estimates for differentiation of subfamilies and tribes are not yet available. Thus, it cannot be said with certainty whether *Tamridaea* is the result of vicariance and subsequent evolution in isolation or whether it arrived in Socotra by a long-distance dispersal event. Its high number of autapomorphies, both molecular and morphological, testifies either for a long isolated evolution or a rapid radiation.

The resolution of the sampled *Virectaria* species in our most parsimonious tree resulting from a combined analysis (Fig. 2) indicates some biogeographic facts for the genus. In this tree, neither the Upper-Guinean (e.g. *V. multiflora* 2, and *Virectaria* sp. 1), nor the Lower-Guinean (e.g. *V. herbacoursi*, *V. angustifolia* and *V. belingana*), nor the Congolian elements (e.g. *V. multiflora* 3, and *V. major* 1) form a monophyletic group, indicating that the species of any of these three subcentres of endemism (White 1979) or domains are not closely related. In contrast, two of the four subclades (Fig. 2: AII and BIV) contain elements of all three domains, and one subclade (Fig. 2: BIII) of two domains. In all three subclades, the Congolian and Upper-Guinean elements are sister to the Lower-Guinean elements. Regarding Clade A (Fig. 2), a Lower-Guinean element (*V. herbacoursi*) is sister to a group with members in all three domains. This pattern suggests an ongoing dispersal of taxa between the three domains, without a clearly defined direction of migration. On the other hand, although *V. multiflora* is a Guineo–Congolian species, its Upper-Guinean element (*V. multiflora* 2) is nested within its Congolian and Lower-Guinean elements (*V. multiflora* 3, and *V. multiflora* 1 and *V. multiflora* 4, respectively). This indicates that the Upper-Guinean population of *V. multiflora* might have had radiated from its Congolian or Lower-Guinean population. Similar results are not reflected by the Guineo–Congolian species *V. procumbens* or the Guineo–Congolian–Zambezian species *V. major*, because their Upper-Guinean elements are not included in the analyses.

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