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Evolutionary history of the Afro-Madagascan *Ixora* species (Rubiaceae): species diversification and distribution of key morphological traits inferred from dated molecular phylogenetic trees

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• *Background and Aims* Previous work on the pantropical genus *Ixora* has revealed an Afro-Madagascan clade, but as yet no study has focused in detail on the evolutionary history and morphological trends in this group. Here the evolutionary history of Afro-Madagascan *Ixora* spp. (a clade of approx. 80 taxa) is investigated and the phylogenetic trees compared with several key morphological traits in taxa occurring in Madagascar.
• *Methods* Phylogenetic relationships of Afro-Madagascan *Ixora* are assessed using sequence data from four plastid regions (*petD*, *rps16*, *rpoB-trnC* and *trnL-trnF*) and nuclear ribosomal external transcribed spacer (ETS) and internal transcribed spacer (ITS) regions. The phylogenetic distribution of key morphological characters is assessed. Bayesian inference (implemented in BEAST) is used to estimate the temporal origin of *Ixora* based on fossil evidence.
• *Key Results* Two separate lineages of Madagascan taxa are recovered, one of which is nested in a group of East African taxa. Divergence in *Ixora* is estimated to have commenced during the mid Miocene, with extensive cladogenesis occurring in the Afro-Madagascan clade during the Pliocene onwards.
• *Conclusions* Both lineages of Madagascan *Ixora* exhibit morphological innovations that are rare throughout the rest of the genus, including a trend towards pauciflorous inflorescences and a trend towards extreme corolla tube length, suggesting that the same ecological and selective pressures are acting upon taxa from both Madagascan lineages. Novel ecological opportunities resulting from climate-induced habitat fragmentation and corolla tube length diversification are likely to have facilitated species radiation on Madagascar.

Key words: Rubiaceae, *Ixora*, Afro-Madagascan, molecular phylogenetics, molecular dating, biogeography, ETS, ITS, *petD*, *rps16*, *rpoB-trnC*, *trnL-trnF*.

INTRODUCTION

The pantropical genus *Ixora* is one of the largest genera in Rubiaceae, with approx. 530 species of shrubs and small trees that typically grow in humid rain forest (Davis *et al.*, 2009). The centre of species diversity for the genus is in South-East Asia, in particular Borneo (Lorence *et al.*, 2007). Although no modern monograph of *Ixora* exists, there have been a number of revisions focusing on specific geographical regions (e.g. De Block, 1998, revision of continental African *Ixora* spp.; De Block, 2013, revision of Madagascan *Ixora* spp.). Phylogenetic studies of *Ixora* have primarily focused on the tribal placement and circumscription of the genus (Andreasen and Bremer, 1996, 2000; Mouly, 2007; Mouly *et al.*, 2009*a*). Most recently, Mouly *et al.* (2009*b*) identified some well-supported, geographically defined lineages, including an 'Afro-Madagascan' clade.

There are approx. 80 Afro-Madagascan *Ixora* spp. distributed equally between continental Africa and Madagascar (De Block, 1998). In continental Africa, *Ixora* mainly occurs in the Guineo-Congolian Regional Centre of Endemism (RCE) (following White, 1983), but also in the Afromontane archipelago-like RCE, and extends into the Zambezian RCE, the Swahilian RCE and the Swahilian/Maputaland regional transition zone (RTZ) (De Block, 1998). In Madagascar, Rubiaceae are most numerous and species rich in the evergreen humid forests (Davis and Bridson, 2003). *Ixora* is no exception to this, occurring most frequently in the humid evergreen forest (littoral, lowland and montane) on the eastern coast of Madagascar, although *Ixora* spp. also occur in the semi-deciduous forest of Madagascar (De Block, 2003, 2013).

Ixora is one of the most easily recognizable genera in Rubiaceae, in part due to the often striking inflorescences and tetramerous flowers (Fig. 1). Diagnostic features for the genus (adapted from De Block, 2007) include articulated petioles, narrowly tubular tetramerous flowers, bilobed stigmas, bilocular ovaries and fruits (or, rarely, with more than two locules),

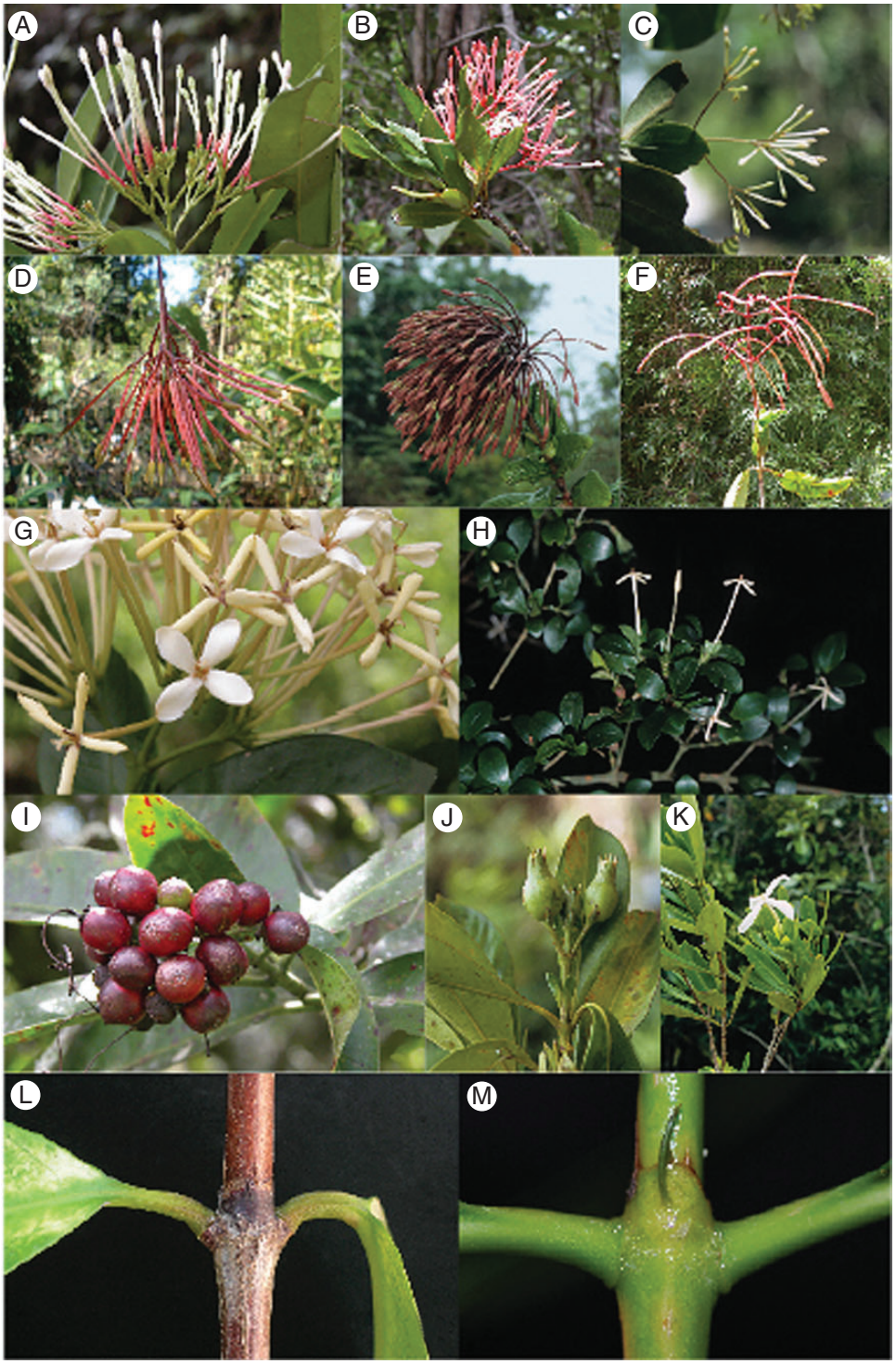

FIG. 1. Example of morphological variation in *Ixora*. (A) Inflorescence of *Ixora regalis*. (B) Inflorescence of *Ixora elliotii*. (C) Inflorescence of *Ixora emirnensis*. (D) Pendulous inflorescence of *Ixora mangabensis*. (E) Inflorescence of *Ixora densithyrsa*. (F) Inflorescence of *Ixora siphonantha*. (G) Front view of a flower of *Ixora guillotii*. (H) Flowering branch of *Ixora rakotonasoloi*. (I) Mature fruits of *Ixora guillotii*. (J) Fruits of *Ixora quadrilocularis*. (K) Flowering node of *Ixora homolleae*. (L) Articulate petioles of *Ixora finlaysoniana*. (M) Articulate petioles of *Ixora homolleae*.

uniovulate locules and seeds with a large adaxial hilar cavity. In contrast, identification at the species level is difficult, with species distinguished on the basis of minor and often continuous characters, typically involving features of the inflorescence and flowers (De Block, 1998, 2003). This is particularly the case for the African representatives of the genus, which De Block (1998) described as 'extremely homogeneous' in their characters. On Madagascar, there are several morphological traits

occurring in *Ixora* that are absent in the continental African taxa and rare in the genus as a whole. These include: (1) reduction of the number of flowers per inflorescence towards solitary flowers; (2) increase from two- to four-locular ovaries; and (3) increase towards large flowers (corolla tubes >15 cm long) and fruits (De Block, 2007, 2008, 2013).

In the present study, we further investigate the phylogenetic relationships of Madagascan and continental African *Ixora* spp. using molecular sequence data from four plastid regions (*petD*, *rps16*, *rpoB-trnC* and *trnL-trnF*) and nuclear ribosomal external transcribed spacer (ETS) and internal transcribed spacer (ITS) regions. The purpose of this study is to improve taxon sampling of both African and Madagascan species in order to: (1) test existing hypotheses concerning the evolutionary affinities within and between African and Madagascan species; (2) assess the distribution of key morphological innovations of the Madagascan species on our molecular phylogenetic trees; and (3) investigate the age of species diversification and dispersal using molecular dating techniques.

MATERIALS AND METHODS

Taxon sampling and DNA preparation

Extensive fieldwork was undertaken in eastern and northern Madagascar in order to collect herbarium, alcohol and DNA material of Madagascan *Ixora* spp. This material was used in the molecular and the morphological study. We included 67 *Ixora* accessions, representing approx. 50 species that occur throughout the global distribution of the genus (Table 1). In particular, our taxon sampling is focused on Madagascan and African species. Where possible, we included multiple accessions for each Madagascan species to test species monophyly. Thirty-eight Madagascan accessions were included, representing 24 species. We included 16 accessions (14 species) from continental Africa, and from west and east tropical Africa. The remaining 12 *Ixora* accessions are Asian (three species), Mascarene (two species), Neotropical (four accessions from three species) and Pacific Island (three species) taxa. *Vangueria madagascariensis*, representing the closely related tribe Vanguerieae (e.g. Kainulainen *et al.*, 2013) was selected as an outgroup.

Total genomic DNA was isolated from either silica gel collections, fresh leaf material from the living collections of the National Botanic Garden of Belgium (NBGB) or herbarium material (BR, MO, P) using a standard cetyltrimethyl ammonium bromide (CTAB) protocol (Doyle and Doyle, 1987). As reported elsewhere (e.g. Rajaseger *et al.*, 1997), *Ixora* leaves may be highly coriaceous and can contain high levels of phenolic compounds that may affect the quality of isolated DNA. Therefore, we purified isolated DNA using Nucleospin purification columns (Macherey-Nagel), following the manufacturer's instructions.

Amplification and sequencing

Primers for amplification of plastid and nuclear ribosomal DNA (nrDNA) regions are listed in Table 2. PCR and cycle sequencing was performed using a Perkin Elmer GeneAMP® 9700 thermocycler. Plastid PCR mixes were made up to 25 μL, and contained 1 μL of each primer (100 ng μL^{-1}), 0·35 μL of Biotaq DNA polymerase, 2·5 μL of 10× NH_4 reaction buffer, 1·5 μL of 50 mM $MgCl_2$, 2·5 μL of 10 mM dNTPs, 1 μL of bovine serum albumin (BSA; 0·4 %) and 2 μL of total genomic DNA. Amplification of *petD*, *rps16* and *trnL-trnF* used the following temperature profile: 94 °C for 3 min; 32 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1·5 min; final extension of 72 °C for 7 min. The amplification profile for *rpoB-trnC* was: 94 °C for 3 min; 32 cycles of 94 °C for 1 min, 53 °C for 1 min, 72 °C for 2 min; final extension of 72 °C for 7 min.

PCR mixes for nuclear regions were the same as for plastid regions, except that 1 μL of dimethylsulfoxide (DMSO) was added per 25 μL. The ITS amplification profile was: 94 °C for 3 min; 32 cycles of 94 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min; final extension of 72 °C for 7 min. The ETS amplification profile was: 97 °C for 1 min; 40 cycles of 97 °C for 10 s, 55 °C for 30 s, 72 °C for 30 s; final extension of 72 °C for 7 min. All amplification products were purified using Nucleospin purification columns and sent to Macrogen Inc. (Seoul, South Korea) for sequencing.

Sequence alignment and phylogenetic analyses

Contiguous sequences were assembled and edited using the Staden software package (Staden *et al.*, 1998). Sequences were manually aligned in MacClade v. 4·04 (Maddison and Maddison, 2002) without difficulty due to low levels of sequence variation. All variable nucleotide positions were verified against the original electropherograms. Gaps were treated as missing data; potentially informative indels were coded using the 'simple indel coding' method of Simmons and Ochoterena (2000). To minimize the time and computational effort required for phylogenetic analyses, we excluded duplicate accessions of a species if the sequences from each accession were identical across all six data sets.

Congruence of the data sets was assessed using the partition homogeneity test implemented in PAUP* v. 4·0b10 (Swofford, 2003). All constant and uninformative characters were excluded. One thousand permutation cycles were run, each consisting of a heuristic maximum parsimony (MP) search of ten random sequence addition replicates with TBR (tree bisection and reconstruction) branch swapping, holding ten trees at each step and saving no more than five trees per replicate.

Maximum parsimony analyses were performed with PAUP* v. 4·0b10. We conducted equal weighted parsimony heuristic tree searches on: (*a*) individual data sets; (*b*) a combined plastid data set; and (*c*) a combined plastid–nuclear DNA data set. Each analysis consisted of 1000 random sequence addition replicates, holding ten trees at each step, with TBR branch swapping and MulTrees in effect, DELTRAN optimization and saving no more than ten trees per replicate. Support for clades was evaluated with 1000 full-heuristic bootstrap pseudo-replicates (Felsenstein, 1985), using the same settings as outlined above.

Bayesian analyses were implemented in MrBayes 3·1 (Huelsenbeck and Ronquist, 2001). The model of DNA substitution for each region was determined using Modeltest v. 3·06 (Posada and Crandall, 1998) under the Akaike information criterion (AIC; Supplementary Data Table S1). Four independent Bayesian analyses with four chains were run for each data set,

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

Taxon	Voucher/Herbarium/Country of origin	ETS	ITS	*petD*	*rps16*	*rpoB-trnC*	*trnL-trnF*
Vangueria madagascariensis J.F.Gmel.	Van Caekenberghe 82 (BR), Mozambique	—	—	HG315109	HG315176	HG315244	HG315312
Ixora aluminicola Steyerm.	Prévost 4160 (P), French Guiana	HG315378	HG315441	—	HG315177	HG315245	—
I. ambrensis De Block	Gautier 5006 (BR), Madagascar	HG315379	HG315442	HG315110	HG315178	HG315246	HG315313
I. amplidentata De Block*	De Block 976 (BR), Madagascar	HG315380	HG315443	HG315111	HG315179	HG315247	—
I. ankazobensis De Block*	Tosh 30 (BR), Madagascar	HG315381	HG315444	HG315112	HG315180	HG315248	HG315314
I. ankazobensis De Block*	Tosh 245 (BR), Madagascar	HG315382	HG315445	HG315113	HG315181	HG315249	HG315315
I. ankazobensis De Block*	De Block 943 (BR), Madagascar	HG315383	HG315446	HG315114	HG315182	HG315250	HG315316
I. ankazobensis De Block*	De Block 857 (BR), Madagascar	HG315384	HG315447	HG315115	HG315183	HG315251	HG315317
I. ankazobensis De Block*	Tosh 256 (BR), Madagascar	HG315385	HG315448	HG315116	HG315184	HG315252	HG315318
I. batesii Wernham	Dessein 1455 (BR), Cameroon	—	HG315449	HG315117	HG315185	HG315253	HG315319
I. borboniae Mouly & B.Bremer	Van Caekenberghe 42 (BR), Mauritius	HG315386	HG315450	HG315118	HG315186	HG315254	HG315320
I. brachypoda DC.	Bradley 1022 (MO), Gabon	HG315387	HG315451	HG315119	HG315187	HG315255	HG315321
I. brachypoda DC.	Walters 1437 (MO), Gabon	—	HG315452	HG315120	HG315188	HG315256	HG315322
I. brevifolia Benth.	Delprete s.n. (BR), Brazil	HG315388	HG315453	HG315121	HG315189	HG315257	HG315323
I. capuroniana De Block*	Tosh 400 (BR), Madagascar	HG315389	HG315454	HG315122	HG315190	HG315258	HG315324
I. cauliflora Montrouz.	Mouly 267 (P), New Caledonia	HG315390	HG315455	HG315123	HG315191	HG315259	HG315325
I. chinensis Lam.	Van Caekenberghe 316 (BR), China	—	—	HG315124	HG315192	HG315260	HG315326
I. collina (Montrouz.) Beauvis.	Mouly 236 (P), New Caledonia	HG315391	HG315456	HG315125	HG315193	HG315261	HG315327
I. crassipes Boivin ex De Block	Groeninckx 80 (BR), Madagascar	HG315392	HG315457	HG315126	HG315194	HG315262	HG315328
I. cremixora Drake	Mouly 659 (P), Comoro Islands	HG315393	HG315458	HG315127	HG315195	HG315263	HG315329
I. cremixora Drake	De Block 987 (BR), Madagascar	HG315394	HG315459	HG315128	HG315196	HG315264	HG315330
I. densithyrsa De Block	De Block 1773 (BR), Madagascar	HG315395	HG315460	HG315129	HG315197	HG315265	HG315331
I. elliotii Drake ex De Block	De Block 1977 (BR), Madagascar	HG315396	HG315461	HG315130	HG315198	HG315266	HG315332
I. emirnensis Baker	De Block 1786 (BR), Madagascar	HG315397	HG315462	HG315131	HG315199	HG315267	HG315333
I. emirnensis Baker	De Block 1788 (BR), Madagascar	HG315398	HG315463	HG315132	HG315200	HG315268	HG315334
I. ferrea (Jacq.) Benth.	Merello 1716 (MO), Commonwealth of Dominica (Lesser Antilles)	HG315399	HG315464	HG315133	HG315201	HG315269	HG315335
I. ferrea (Jacq.) Benth.	Taylor 11693 (MO), Puerto Rico	HG315400	HG315465	HG315134	HG315202	HG315270	HG315336
I. foliosa Hiern	Onana 566 (P), Cameroon	HG315401	HG315466	HG315135	HG315203	HG315271	HG315337
I. foliicalyx Guédès	Tosh 352 (BR), Madagascar	HG315402	HG315467	HG315136	HG315204	HG315272	HG315338
I. foliicalyx Guédès	De Block 696 (BR), Madagascar	HG315403	HG315468	HG315137	HG315205	HG315273	HG315339
I. francii Schltr.	Mouly 241 (P), New Caledonia	HG315404	HG315469	HG315138	HG315206	HG315274	HG315340
I. guillotii Hoch.	De Block 2091 (BR), Madagascar	HG315405	HG315470	HG315139	HG315207	HG315275	HG315341
I. guillotii Hoch.	Tosh 408B (BR), Madagascar	HG315406	HG315471	HG315140	HG315208	HG315276	HG315342
I. guineensis Benth.	Gereau 5601 (MO), Ghana	HG315407	HG315472	HG315141	HG315209	HG315277	HG315343
I. hartiana De Block	Bamps 4320 (BR), Angola	HG315408	HG315473	HG315142	HG315210	HG315278	HG315344
I. hiernii Scott-Elliot	Adam 23101 (P), Sierra Leone	HG315409	HG315474	HG315143	HG315211	HG315279	HG315345
I. hippoperifera Bremek.	Dessein 1669 (BR), Cameroon	HG315410	HG315475	HG315144	HG315212	HG315280	HG315346
I. hippoperifera Bremek.	Van Valkenburg 3083 (WAG), Gabon	HG315411	HG315476	HG315145	HG315213	HG315281	HG315347
I. homolleae De Block & Govaerts[†]	Tosh 107 (BR), Madagascar	HG315412	HG315477	HG315146	HG315214	HG315282	HG315348
I. homolleae De Block & Govaerts[†]	Tosh 207 (BR), Madagascar	HG315413	HG315478	HG315147	HG315215	HG315283	HG315349
I. lagenifructa De Block*	De Block 2036 (BR), Madagascar	HG315414	HG315479	HG315148	HG315216	HG315284	HG315350
I. macilenta De Block	Dessein 1404 (BR), Cameroon	HG315415	HG315480	HG315149	HG315217	HG315285	HG315351
I. mangabensis DC.	Tosh 128 (BR), Madagascar	HG315416	HG315481	HG315150	HG315218	HG315286	HG315352
I. mangabensis DC.	Tosh 130 (BR), Madagascar	HG315417	HG315482	HG315151	HG315219	HG315287	HG315353
I. mangabensis DC.	De Block 2040 (BR), Madagascar	HG315418	HG315483	HG315152	HG315220	HG315288	HG315354
I. mangabensis DC.	De Block 2053 (BR), Madagascar	HG315419	HG315484	HG315153	HG315221	HG315289	HG315355
I. masoalensis De Block*	Razafimandimbison 654 (BR), Madagascar	HG315420	HG315485	HG315154	HG315222	HG315290	HG315356
I. microphylla Drake	De Block 985 (BR), Madagascar	HG315421	—	HG315155	HG315223	HG315291	HG315357
I. minutiflora Hiern	Dessein 1440 (BR), Cameroon	HG315422	HG315486	HG315156	HG315224	HG315292	HG315358
I. mocquerysii DC.	Malcomber 2805 (MO), Madagascar	HG315423	HG315487	HG315157	HG315225	HG315293	HG315359
I. moramangensis De Block*	Tosh 255 (BR), Madagascar	HG315424	—	HG315158	HG315226	HG315294	HG315360
I. moramangensis De Block*	De Block 837 (BR), Madagascar	HG315425	—	HG315159	HG315227	HG315295	HG315361
I. narcissodora K.Schum.	De Block 418 (BR), Kenya	HG315426	HG315488	HG315160	HG315228	HG315296	HG315362
I. nematopoda K.Schum.	Dessein 1449 (BR), Cameroon	HG315427	HG315489	HG315161	HG315229	HG315297	HG315363
I. nitens (Poir.) Mouly & B.Bremer	Friedmann 2631 (P), Mauritius	HG315428	HG315490	HG315162	HG315230	HG315298	HG315364
I. perrieri De Block*	De Block 841 (BR), Madagascar	HG315429	HG315491	HG315163	HG315231	HG315299	HG315365
I. perrieri De Block*	Tosh 232 (BR), Madagascar	HG315430	HG315492	HG315164	HG315232	HG315300	HG315366
I. platythyrsa Baker	De Block 773 (BR), Madagascar	HG315431	HG315493	HG315165	HG315233	HG315301	HG315367

Continued

TABLE 1. *Continued*

Taxon	Voucher/Herbarium/Country of origin	ETS	ITS	*petD*	*rps16*	*rpoB-trnC*	*trnL-trnF*
I. praetermissa De Block	Dessein 1519 (BR), Cameroon	HG315432	HG315494	HG315166	HG315234	HG315302	HG315368
I. quadrilocularis De Block*	Tosh 85 (BR), Madagascar	HG315433	HG315495	HG315167	HG315235	HG315303	HG315369
I. rakotonasoloi De Block	Tosh 316 (BR), Madagascar	HG315434	HG315496	HG315168	HG315236	HG315304	HG315370
I. regalis De Block*	De Block 835 (BR), Madagascar	HG315435	HG315497	HG315169	HG315237	HG315305	HG315371
I. regalis De Block*	De Block 2083 (BR), Madagascar	HG315436	—	HG315170	HG315238	HG315306	HG315372
I. scheffleri K.Schum.	Luke 9162 (P), Tanzania	HG315437	HG315498	HG315171	HG315239	HG315307	HG315373
I. siphonantha Oliv.	Tosh 389 (BR), Madagascar	HG315438	HG315499	HG315172	HG315240	HG315308	HG315374
I. sp. 'Brunei'	Malcomber 2980 (MO), Brunei	—	HG315500	HG315173	HG315241	HG315309	HG315375
I. sp. 'Malaysia'	Billiet 7327 (BR), Malaysia	HG315439	HG315501	HG315174	HG315242	HG315310	HG315376
I. tanzaniensis Bridson	Luke 9304 (P), Tanzania	HG315440	HG315502	HG315175	HG315243	HG315311	HG315377

*sp. nov. ined.
†nom. nov. ined.

TABLE 2. *Amplification primers for plastid and nuclear regions*

Region	Primer	Primer sequence (5'–3')	Reference
petD	*PetB*1365	TTGACYCGTTTTTATAGTTTAC	Löhne and Borsch (2005)
	*PetD*738	AATTTAGCYCTTAATACAGG	
rpoB-trnC	*rpoB*-F	CACCCRGATTYGAACTGGGG	Shaw *et al.* (2005)
	trnC-R	CKACAAAAYCCYTCRAATTG	
rps16	*rps16*-F	AAACGATGTGGTARAAAGCAAC	Shaw *et al.* (2005)
	rps16-R	AACATCWATTGCAASGATTCGATA	
trnL–trnF	*trnL*-c	CGAAATCGGTAGACGCTACG	Taberlet *et al.* (1991)
	trnF-f	ATTTGAACTGGTGACACGAG	
ETS	18S-ETS	GCAGGATCAACCAGGTGACA	Negrón-Ortiz and Watson (2002)
	ETS-ERIT	CTTGTATGGGTTGGTTGGA	Baldwin and Markos (1998)
ITS	ITS 1	TCCGTAGGTGAACCTGCGG	White *et al.* (1990)
	ITS 4	TCCTCCGCTTATTGATATGC	

starting from random trees, for 5 million generations, sampling trees every 1000 generations. TRACER v. 1·4 (Rambaut and Drummond, 2007) was used to assess if the search had reached stationarity and to check each parameter had an effective sample size (ESS) >100. The initial 1250 (25 %) trees were discarded as a conservative burn-in. Post-burn-in trees from the four independent analyses were pooled and summarized by a 50 % majority rule consensus tree using PAUP* v4·0b10 to obtain posterior probabilities (PPs).

Morphology and optimization of morphological characters

The material for the taxonomical and morphological study of *Ixora* in Madagascar consists of preserved samples and herbarium material of the following institutions: BM, BR, G, K, MO, P, S, TAN, TEF, UPS, W, WAG and Z (abbreviations of institutions follow Holmgren *et al.*, 1990). In total, >1000 herbarium collections, each with several duplicates, were studied. Morphological terminology generally follows Robbrecht (1988). For the Madagascan species, morphological characters were scored on herbarium material available for this species. For the continental African species, morphological characters were taken from the revision of African *Ixora* (De Block, 1998). We chose morphological traits of interest (i.e. uniflorous inflorescences, four-locular ovaries, extreme corolla tube length) and characters of potential taxonomic significance (Table 3). We assessed the distribution of these morphological characters by mapping unambiguous character state changes onto our combined Bayesian MJ consensus tree using MacClade v. 4·04 (Maddison and Maddison, 2002).

Divergence time estimation

A Bayesian approach was applied to infer the temporal framework of the evolution of *Ixora*. Due to the limited fossil record that could be unequivocally assigned to the most recent common ancestor of *Ixora*, an expanded family-level data set was constructed and divergence time estimation was inferred based on fossils (see the following section for more detail). This large-scale analysis allowed estimation of the temporal origin of *Ixora* together with a 95 % confidence interval, which was subsequently used as a prior to perform a second analysis focusing only on this genus. The family-level data set included representatives of all the major lineages in Rubiaceae (see Appendix 1) and was based on the group II plastid introns *petD*, *rps16* and the *trnL-trnF* spacer (Groeninckx, 2009). In the large-scale analysis, a sub-set of taxa representative of the main clades in *Ixora* was included. These taxa were selected based on preliminary phylogenetic analyses. We opted for this approach rather than pooling all the data sets and taxa together mainly to avoid encountering problems related to missing data. In addition to the group II plastid introns, the *Ixora* data set

TABLE 3. *Selected morphological characters*

	Taxon	Inflorescence		Calyx		Flower		
		Sessile/pedunculate	Lax/compact	Tube length (mm)	Lobe length (mm)	Number	Corolla tube length (cm)	Ovary
African taxa	*I. batesii*	Sessile	Lax	0·4–0·5	0·4–0·6	30–70	0·5–1·3	Bilocular
	I. brachypoda	Pedunculate	Lax	0·4–0·8	0·3–1·0	50–200	3·3–11	Bilocular
	I. foliosa	Pedunculate	Compact	0·3–0·5	0·4–0·7	45–90	0·9–2·3	Bilocular
	I. guineensis	Sessile	Lax	0·3–0·8	0·2–0·6	30–90	0·5–2·3	Bilocular
	I. hartiana	Pedunculate	Lax	0·2–0·5	0·3–0·6	9–30	1·2–2·4	Bilocular
	I. hiernii	Sub-sessile	Lax	0·2–0·7	0·2–0·5	30–90	1·4–3·3	Bilocular
	I. hippoperifera	Pedunculate or sessile	Compact	0·2–0·4	0·2–0·4	30–120	1·6–2·7	Bilocular
	I. macilenta	Sessile	Lax	0·2–0·5	0·2–0·6	9–20	0·9–2·2	Bilocular
	I. minutiflora	Sessile	Compact	0·2–0·4	0·1–0·4	9–30	1·0–2·0	Bilocular
	I. narcissodora	Sessile	Lax	0·8–1·5	0·2–0·6	50–100	3·0–7·5	Bilocular
	I. nematopoda	Pedunculate	Lax	0·3–0·6	0·8–1·2	20–50	0,4–0,9	Bilocular
	I. praetermissa	Sessile	Lax	0·2–0·5	0·3–0·8	30–70	1·1–2·5	Bilocular
	I. scheffleri	Pedunculate	Compact	0·4–0·6	0·6–1·0	30–90	0·8–2·2	Bilocular
	I. tanzaniensis	Sessile	Compact	0·3–0·7	0·2–0·4	20–50	2·2–3·2	Bilocular
Madagascan taxa (Clade 2)	*I. amplidentata*	Pedunculate	Lax	0·2–0·4	0·5–1·25	9–60	1·5–3·3	Bilocular
	I. densithyrsa	Pedunculate	Compact	0·5–1·0	2·5–4·0	50–120	18–23	Bilocular
	I. foliicalyx	Sub-sessile	Compact	2·0–4·0	1·5–6·0	45–90	3·5–8·5	Bilocular
	I. guillotii	Pedunculate	Lax	0·5–1·0	0·75–2·5	50–150	5·0–8·0	Bilocular
	I. homolleae	(Sub-)sessile	Uniflorous	5·0–10	5·0–12	1	2·6–4·7	Four-locular
	I. lagenifructa	Pedunculate	Lax	5·0–8·0	1·5–2·0	3(–15)	3·5–6·5	Four-locular
	I. microphylla	(Sub-)sessile	Lax	0·5–2·0	0·5–1·5	(1–)3–9	2·2–4·0	Bilocular
	I. mocquerysii	Pedunculate	Compact	0·5–1·0	2·0–6·0	3–45	5·5–11(–16)	Bilocular
	I. platythyrsa	Pedunculate	Lax	0·2–0·4	0·9–1·4	50–150	1·4–2·5	Bilocular
	I. quadrilocularis	Pedunculate	Lax	4·0–7·0	(3–)5–15	3(–9)	4·0–8·0	Four-locular
	I. regalis	Pedunculate	Lax	0·5–1(–1·5)	1–5(–9)	50–120	1·3–5·5	Bilocular
	I. siphonantha	Pedunculate	Lax	0·5–1	3·0–11	12–80	(10–)15–22	Bilocular
Madagascan taxa (Clade 3)	*I. ambrensis*	Pedunculate	Lax	0·4–0·5	0·2–0·35	25–50	3·2–3·9	Bilocular
	I. ankazobensis	Sessile (± pedunculate)	Lax	0·5–0·75	0·3–0·75	7–18	4·0–6·3	Bilocular
	I. capuroniana	Sessile	Lax or compact	0·3–0·6	0·1–0·8	40–90	1·5–3·5	Bilocular
	I. crassipes	Sub-sessile	Lax	0·75–1·25	0	15–50	17–22·5	Bilocular
	I. cremixora	Sessile	Lax	0·2–0·75	<0·3	50–120	4·5–7·8	Bilocular
	I. elliotii	Sessile	Lax	0·2–0·4	0·3–0·5	30–90	1·8–3·1	Bilocular
	I. emirnensis	Pedunculate	Lax	0·2–0·3	0·3–0·4	9–50	1·0–1·5	Bilocular
	I. mangabensis	Pedunculate	Lax	0·2–0·5	0·5–1·0	9–30	2·0–3·2	Bilocular
	I. masoalensis	Sessile	Lax	0·5–2·0	0·5–1·5	8–25	2·2–4·0	Bilocular
	I. moramangensis	Pedunculate	Lax	0·25–0·5	0·25–0·8	9–30	2·1–3·5	Bilocular
	I. perrieri	Pedunculate	Lax	0·3–0·5	0·2–0·4	30–90	4·5–7·5	Bilocular
	I. rakotonasoloi	Sessile	Uniflorous	0·3–0·5	0·3–0·5	1	2·2–2·8	Bilocular

Adapted from De Block (1998, 2013).

included one additional plastid (*rpoB-trnC*) and two nuclear (ETS and ITS) regions. These regions proved useful to resolve phylogenetic relationships in *Ixora* further.

With the exception of the number of runs and length of the Monte Carlo Markov chain (MCMC), the settings of the Bayesian analyses were identical for the two data sets. A partitioned Bayesian MCMC analysis was conducted in BEAST v. 1·7 with a relaxed log-normal molecular clock and a Yule speciation model. The partitions were unlinked for the model of evolution, but linked for the estimation of the molecular clock and the tree topology. The other parameters were set as default (see Drummond and Rambaut, 2007). The best-fit models for each DNA region were kept identical to those in the MrBayes analyses (see above). In the case of the family-level data set, four runs of 10 million generations were performed, sampling a tree every 1000 generations. To improve the tree topology research, the MrBayes consensus tree was provided as the starting tree in the BEAST family-level analysis. In the case of the *Ixora* data set (which was rooted using *Vangueria infausta*), two runs of 5 million generations were performed, sampling a tree every 1000 generations, and the analyses were seeded with a random tree. For each parameter, convergence of runs was confirmed by the examination of their respective posterior distributions in TRACER v. 1·4 (Rambaut and Drummond, 2004). In addition, we considered the MCMC sampling sufficient when the ESS was >200 using TRACER v. 1·4 (Rambaut and Drummond, 2004). A maximum clade credibility tree with median branch lengths and 95 % confidence interval on nodes was built using TreeAnnotator v. 1·5·4 (Drummond and Rambaut, 2007) based on the set of trees after burn-in (for each run, a burn-in period of 1 million was applied).

Fossil calibration

To estimate absolute ages for lineage divergences in the case of the family-level data set, we have used seven fossil calibration points (see below) in Rubiaceae to set age constraints for several nodes. As in Buerki *et al.* (2011), for each calibration point, the oldest fossil record was selected and the upper (younger) bound of the geological interval (Gradstein *et al.*, 2004) in which the fossil was found was used to represent the age constraint. In the family-level Bayesian analysis, all the calibration points were modelled as follows: log-normal distribution, mean = 0·5, s.d. = 1, offset = age fossil (see below).

Before detailing the fossils used here, we would like to report the current knowledge on fossil records that have been tentatively assigned to *Ixora*. Palaeobotanical remains of *Ixora* are poorly known, and Graham (2009) reported the presence of *Ixora* pollen dating from the Miocene on the Marshall Islands (Micronesia). In his study, Graham (2009) stressed that this evidence could be challenged and that further studies have to be conducted to assign these pollen unequivocally to existing taxa. Therefore, we have not considered this record in our divergence time estimations. The following calibration points were selected: (*a*) the stem group of Rubiaceae was set with an offset of 54 million years (Ma), following the first occurrence of Rubiaceae fossils during the Eocene (Malcomber, 2002); (*b*) the stem of the *Faramea* clade was set with an offset of 40 Ma, based on distinctive diporate pollen from the late Eocene (Graham, 2009); (*c*) the *Coprosma* clade was constrained with an offset of 23·8 Ma based on fossil records of pollen from the Oligocene (Graham, 2009); (*d*) the stem of the *Galium* and *Rubia* clade was assigned an offset of 5·3 Ma, following the first occurrence of fossil pollen during the late Miocene for this group (Graham, 2009); (*e*) the stem of the *Chiococca* clade was calibrated with an offset of 5·3 Ma based on leaf material from the late Miocene (Graham, 2009); (*f*) the stem of the *Emmenopterys* clade was set with an offset of 48 Ma, following the first record of Rubiaceae fruits in the mid Eocene (Graham, 2009); and (*g*) an offset of 14·5 Ma was assigned to the *Gardenia* clade, based on pollen data from the mid Miocene (Graham, 2009). Based on the posterior distribution of the dating uncertainty on the stem of *Ixora*, the most recent common ancestor of the ingroup in the second BEAST analysis was set with a normal distribution, a mean of 18 Ma and an s.d. of 2·5.

RESULTS

The 368 novel sequences generated in this study were combined with 31 sequences previously generated by Mouly (2007), resulting in a total combined data set of 399 sequences, representing approx. 50 *Ixora* spp. Levels of genetic variation between species were generally low across all six regions investigated (Table 4). The total number of potentially parsimony informative characters ranged from 21 in *rps16* and *trnL-trnF* to 58 in the ITS. In terms of percentage variability, the ETS proved to have the highest proportion of potentially parsimony informative characters (13·4 %). The lowest percentage variability was observed in *petD* (2·3 %). Our taxon sampling included multiple accessions for 13 *Ixora* spp. We observed no intraspecific sequence variation in nine of these species. In contrast, varying amounts of intraspecific sequence divergence were observed in the other five species; multiple accessions from these five species were included in all subsequent phylogenetic analyses.

Phylogenetic analyses

Plastid data sets. Phylogenetic analyses of individual plastid data sets (*petD*, *rpoB-trnC*, *rps16*, *trnL-trnF*) generated largely unresolved and poorly supported phylogenetic trees (data not shown). The partition homogeneity test did not reveal any significant incongruence between plastid data sets ($P = 0{\cdot}69$) and there was no supported incongruence (Bayesian PP >0·95), so these four regions were combined for all subsequent analyses.

The characteristics of the individual and combined plastid data sets are listed in Table 4. In both the MP and Bayesian analyses (Fig. 2), the Asian and Pacific Ocean taxa are sister to the rest of the genus [bootstrap (BS) 70, PP 1·00]. The Mascarene *Ixora* clade is in turn sister to the Neotropical and Afro-Madagascan *Ixora* spp. (BS 77, PP 1·00). The sister relationship between the three Neotropical Central–South American taxa and the Afro-Madagascan taxa is weakly to moderately supported in the MP (BS 56) and Bayesian (PP 0·96) analyses (Fig. 2). In the Afro-Madagascan group, we identified a clade containing African and Madagascan taxa (BS 72, PP 1·00; Clade 1) and an exclusively Madagascan clade (BS 59, PP 0·99; Clade 2). There is a lack of resolution and weak node support in Clades 1 and 2 due to a paucity of potentially parsimony informative characters (Table 4). In Clade 1 there is

TABLE 4. *Characteristics of individual and combined data sets and tree statistics*

	petD	*trnL-trnF*	*rps16*	*rpoB-trnC*	Plastid	ETS	ITS	nrDNA	Total
Number of taxa	56	57	57	55	57	52	52	49	57
Total length (bp)	1023	828	722	1016	3589	409	631	1040	4629
Non-parsimony informative characters	49	41	46	75	211	51	71	122	333
Potentially parsimony informative characters (% of total)	24 (2·3 %)	21 (2·5 %)	21 (2·9 %)	33 (3·2 %)	99 (2·7 %)	55 (13·4 %)	58 (9·2 %)	108 (10·4 %)	212 (4·6 %)
Indels	3	2	1	3	9	0	0	0	9
Tree length	81	68	76	140	376	159	196	357	758
Consistency index	0·914	0·956	0·921	0·850	0·872	0·780	0·791	0·759	0·801
Retention index	0·955	0·969	0·917	0·811	0·889	0·898	0·819	0·839	0·849
Number of trees saved	100	68	511	4887	8722	1151	9580	3464	5362

strong support for a clade of Madagascan and tropical East African taxa (BS 85, PP 1·00; Clade 3).

nrDNA data sets. We were unable to obtain ETS and ITS sequences for *Vangueria madagascariensis*, due to amplification difficulties and ambiguous sequence reads, respectively. As our primary interests concern the Afro-Madagascan element of *Ixora*, phylogenetic analyses from separate and combined nuclear DNA analyses were rooted using Asian and/or Pacific Ocean *Ixora* spp. Relationships in the Afro-Madagascan Clade were not affected by the root choice. The characteristics of the nuclear DNA data sets are listed in Table 4.

The topology of the ETS phylogenetic tree (Supplementary Data Fig. S1) is similar to that of the combined plastid tree, but there are some differences. Clades 1 and 2 are not fully recovered in the ETS topology, with the deepest nodes in the Afro-Madagascan group being unresolved. Furthermore, there is weak to moderate support (BS 78, PP 0·93) for the sister relationship of the Guineo-Congolian *I. nematopoda* with respect to Neotropical *Ixora* and the remaining Afro-Madagascan *Ixora* (discussed by Mouly *et al.*, 2009*b*). However, Clade 3 is strongly supported in the ETS topology (BS 94, PP 1·00). The ITS topology is poorly resolved and weakly supported, in particular in the MP strict consensus (Supplementary Data Fig. S2). Few clades are well supported in both MP and Bayesian analyses, and Clades 1–3 were not recovered.

Total combined data set. Despite some topological inconsistencies between the combined plastid and nuclear DNA data sets, results from the simultaneous partition homogeneity test did not reveal significant incongruence between data sets ($P = 0{\cdot}10$). The combined plastid–nuclear DNA data set comprised 4629 characters. In total, 212 of the 545 variable characters are potentially parsimony informative (Table 4).

The Mascarene clade is sister to the Neotropical and Afro-Madagascan species (BS 84, PP 1·00). The monophyly of the Afro-Madagascan group is supported (BS 65, PP 1·00), and in this group there is support for Clades 1–3 (Fig. 3). In the Bayesian analysis, *I. nematopoda* and *I. scheffleri* are sister to the rest of Clade 1 (PP 1·00). Although this relationship is recovered in the MP strict consensus, it is not supported by the bootstrap analysis. The phylogenetic position of *I. nematopoda* differs between the ETS and the plastid and ITS data sets (Fig. 2; Supplementary Data Figs S1 and S2). The monophyly of Clade 1 is supported in both the Bayesian and MP analyses (BS 73, PP 1·00) following the exclusion of *I. nematopoda* (not shown).

In Clade 1, the group of *I. foliosa* and *I. hippoperifera* is weakly supported (BS 57, PP 0·92), but two other groups of tropical West–Central African taxa are well supported: *I. guineensis*, *I. minutifolia*, *I. hiernii* and *I. batesii* (BS 92, PP 1·00) and *I. hartiana*, *I. macilenta* and *I. praetermissa* (BS 85, PP 1·00). Support for the sister relationships between these three West–Central African clades, and between the widespread *I. brachypoda*, is weak or lacking (Fig. 3). Branch lengths between these clades are short, which may be a contributing factor in the phylogenetic uncertainty between these West–Central African species (Supplementary Data Fig. S3).

Nested in Clade 1 is the strongly supported Clade 3 (BS 100, PP 1·00), comprised of East African and Madagascan species (Fig. 3). The branch length subtending Clade 3 is relatively long, with eight synapomorphies (Supplementary Data Fig. S3). In Clade 3, the East African *I. narcissodora* and *I. tanzaniensis* are sister to the Madagascan taxa (BS 66, PP 0·94). Phylogenetic relationships between these Madagascan taxa are poorly resolved (Fig. 3; Supplementary Data Fig. S3). However, there are a few species relationships that are well supported, such as *I. elliotii* and *I. rakotonasoloi* (BS 99, PP 1·00) and *I. crassipes* and *I. cremixora* (BS 98, PP 1·00). The accessions of *I. mangabensis* did not group together in our analyses, due to the presence of two distinct plastid haplotypes in the four accessions of this species.

The monophyly of Clade 2, comprised exclusively of Madagascan taxa, is supported in the combined plastid–nuclear DNA data set (BS 70, PP 1·00). There are two main subclades recovered in the MP strict consensus and Bayesian majority rule consensus trees, although support for these sub-clades is lacking (Fig. 3). Although the partition homogeneity test revealed no significant incongruence between individual data sets, the phylogenetic placement of certain taxa (e.g. *I. densithyrsa* and *I. regalis* 2) differed between plastid and nuclear data sets. In the plastid phylogenetic tree, *I. regalis* 2 is nested in the group containing *I. regalis* 1, *I. guillotii* and *I. amplidentata* (Fig. 2). In contrast, *I. regalis* 2 is unresolved in the ETS phylogenetic tree (Supplementary Data Fig. S1) and sister to *I. homolleae* and *I. quadrilocularis* in the ITS phylogenetic tree (Supplementary Data Fig. S2). Similarly, *I. densithyrsa* and *I. mocquerysii* demonstrate differing phylogenetic affinities in the plastid and nuclear data sets (Fig. 2;

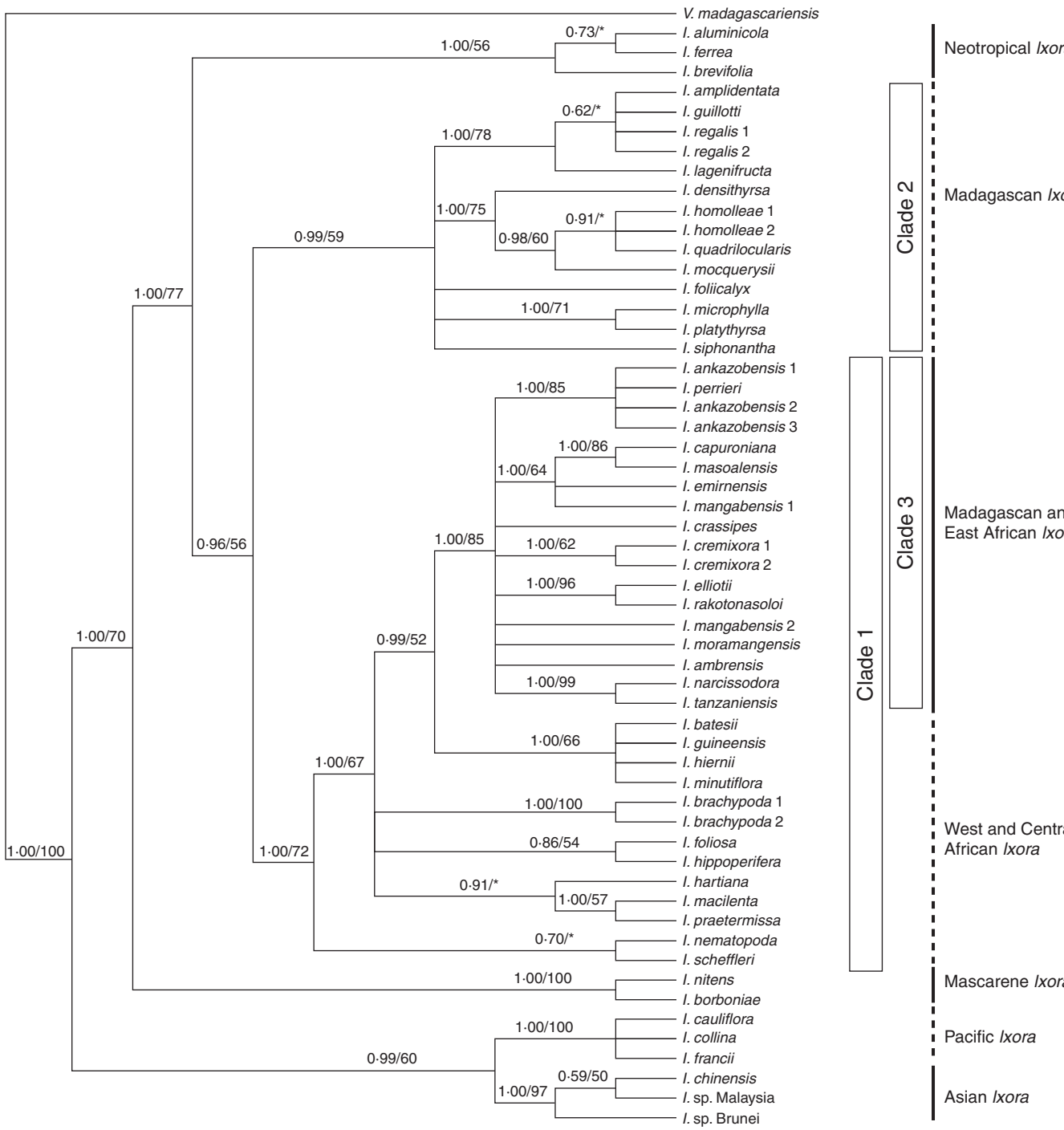

FIG. 2. Combined plastid Bayesian majority rule consensus tree. Bayesian posterior probabilities >0·5 and bootstrap values >50 % are indicated above branches (PP/BS). Asterisks (*) denote nodes that have bootstrap support <50 in the MP analysis. Clade 1, 'Afro-Madagascan clade'; Clade 2, 'Madagascan clade'; Clade 3, 'East African–Madagascan clade'.

Supplementary Data Figs S1 and S2). Exclusion of *I. regalis* 2 and *I. densithyrsa* (data not shown) from the Bayesian analyses results in increased support for the two sub-clades of Clade 2, with both sub-clades supported by a PP of 1·00. In the first subclade, *I. foliicalyx* is sister to *I. homolleae* and *I. quadrilocularis* (PP 1·00; not shown). In the second sub-clade, *I. microphylla* and *I. platythyrsa* are sister to the group of *I. regalis* 1, *I. guillotii*, *I. amplidentata*, *I. lagenifructa*, *I. siphonantha* and *I. mocquerysii* (PP 0·95; not shown).

Distribution of key morphological characters

The phylogenetic distribution of key morphological characters is illustrated in Fig. 4. Pedunculate and sessile inflorescences

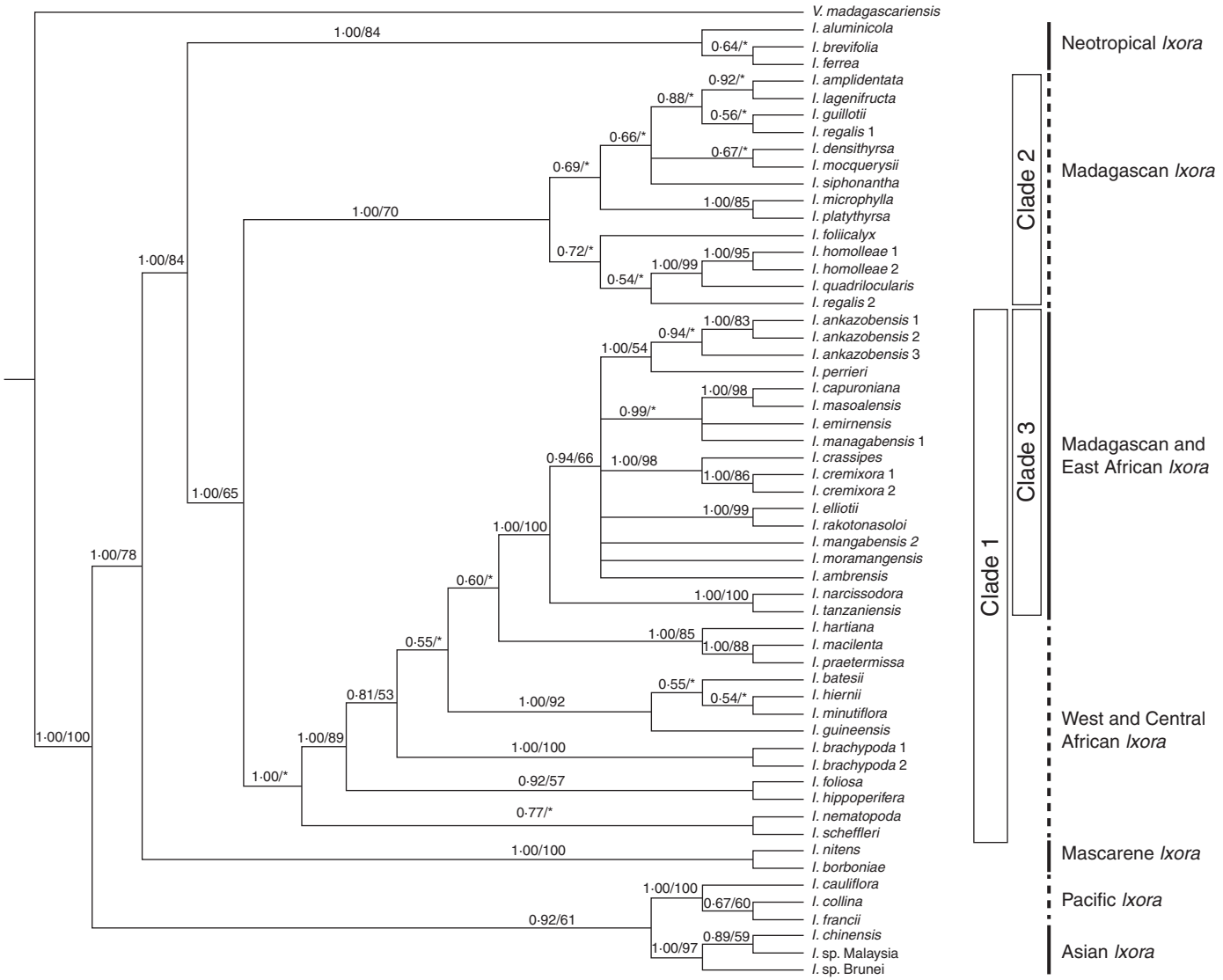

FIG. 3. Combined plastid–nuclear Bayesian majority rule consensus. Bayesian posterior probabilities >0·5 and bootstrap values >50 % are indicated above branches. Asterisks (*) denote nodes that have bootstrap support <50 in the MP analysis. Clade 1, 'Afro-Madagascan clade'; Clade 2, 'Madagascan clade'; Clade 3, 'East African–Madagascan clade'.

occur in African taxa and both clades of Madagascan taxa (Fig. 4A). All taxa from Clade 2 and the African *I. nematopoda* and the Madagascan *I. masoalensis* (Clade3) possess calyx lobes >1 mm long (Fig. 4B). Calyx tubes >1 mm long are found in all but two species from Clade 2, in addition to the African *I. narcissodora* and the Madagascan *I. masoalensis* and *I. crassipes* (Fig. 4C). Species with uniflorous inflorescences or corolla tubes >15 cm long occur in both Madagascan clades, but species with four-locular ovaries are only found in Clade 2 (Fig. 4D–F).

Divergence time estimation

The estimated divergence times for nodes of interest are summarized in Fig. 5 and Supplementary Data Fig. S4, Tables S2 and S3. The onset of diversification of the genus started during the mid Miocene, with the emergence of most of the lineages at the Miocene–Pliocene boundary. Based on the three-gene Rubiaceae-wide data set (Supplementary Data Fig. S4, Table S2), the crown age for Ixoreae (node 152) is estimated at 16·67 million years old (9·67–27·55, 95 % highest posterior density; hereafter HPD). The subsequent age estimates (discussed below) are based on the results of the secondary dating analysis (Fig. 5; Supplementary Data Table S3). The onset of divergence between the Asian-Pacific Ocean *Ixora* (node 59) and the rest of the genus is estimated at 15·37 Ma (7·39–22·89, 95 % HPD). The estimated age of divergence between the Neotropical and Afro-Madagascan taxa (node 66) is 9·51 Ma (4·47–14·94, 95 % HPD), with the crown age for the Afro-Madagascan group (node 67) estimated at 7·95 million years old (3·71–12·52, 95 % HPD). The crown age of Clade 1 (node 68) is estimated at 7·22 million years old (3·36–11·46, 95 % HPD). The crown age of Clade 2 (node 98) is estimated at 6·24 million years old (2·88–10·03, 95 % HPD), with two separate periods of

FIG. 4. Optimization of selected morphological character states on the Bayesian majority rule consensus tree. Grey boxes denote Madagascan taxa from Clade 2; white boxes denote Madagascan taxa from Clade 3; black boxes denote African taxa. (A) Inflorescence type: pedunculate inflorescence (grey); sessile inflorescence (black); pedunculate or sessile inflorescence (stippled grey); equivocal state (stippled black). (B) Calyx lobe length: ≥1 mm (black); <1 mm (grey). (C). Calyx tube length: ≥1 mm (black); <1 mm (grey). (D) Uniflorous taxa (black); multiflorous taxa (grey). (E) Four-locular ovaries (black); bilocular ovaries (grey). (F) Corolla tube length: ≥15 cm (black); <15 cm (grey).

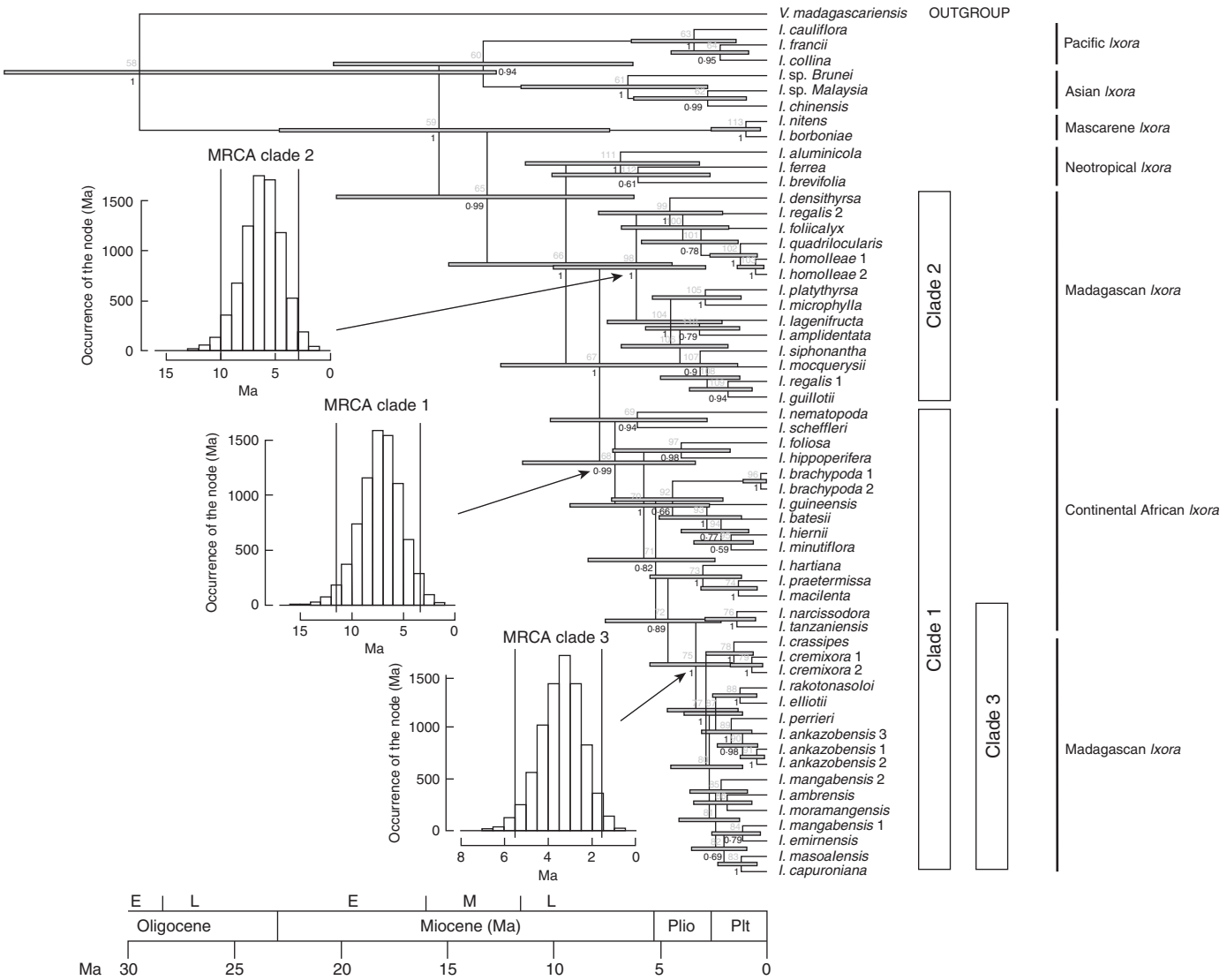

FIG. 5. Combined plastid–nuclear BEAST maximum clade credibility tree for *Ixora*. The 95 % highest posterior density intervals on time divergence estimates and posterior probabilities assigned to each node are indicated. Posterior distributions on node age estimations are also provided. Clade 1, 'Afro-Madagascan clade'; Clade 2, 'Madagascan clade'; Clade 3, 'East Africa–Madagascar clade'.

cladogenesis commencing 4·69 Ma (node 99) and 4·61 Ma (node104). The estimated crown age of Clade 3 (node 75) is 3·4 million years old (1·56–5·49, 95 % HPD), and divergence in the Madagascan lineage (node 77) began 2·92 Ma (1·34–4·69, 95 % HPD).

DISCUSSION

We observed topological incongruence (albeit weakly supported) in the phylogenetic placement of several taxa between the plastid and nuclear DNA data sets. Furthermore, we also observed two instances in which multiple accessions of species did not group together in the phylogenetic reconstruction (i.e. *I. mangabensis* and *I. regalis*). Phylogenetic incongruence between independent data sets can be indicative of hybridization (Linder and Rieseberg, 2004), although there are also several other processes such as incomplete lineage sorting, orthology–paralogy conflation and recombination that can produce the same pattern (e.g. Small *et al.*, 2004; Mort *et al.*, 2007).

The conflicting placements of the Madagascan *I. densithyrsa*, *I. mocquerysii* and *I. regalis* 2 in the ETS, ITS and combined plastid data sets (Fig. 2; Supplementary Data Figs S1 and S2) contributed to a reduction in the overall resolution and node support in Clade 2. The ETS sequence data (Supplementary Data Fig. S1) support a close association between *I. densithyrsa*, *I. mocquerysii* and *I. siphonantha*. This is consistent with De Block (2013) who indicates that these three species are morphologically similar. However, the plastid haplotypes of *I. densithyrsa* and *I. mocquerysii* are similar to those of *I. homolleae* and *I. quadrilocularis*. The geographical distribution and habitat range of *I. densithyrsa* and *I. mocquerysii* overlap with those of *I. homolleae* and *I. quadrilocularis*; all four species occur either in littoral (i.e. *I. homolleae*) or in lowland humid forest in the eastern province of Toamasina.

Although further research is needed to elucidate fully the evolutionary relationships between these taxa, one explanation consistent with the observed phylogenetic incongruence involves hybridization followed by repeated backcrossing. Mouly *et al.* (2009*b*) invoked interspecific hybridization to account for topological incongruence between nuclear and plastid data sets, particularly among Asian species of *Ixora*.

Multiple accessions of *I. regalis* (Fig. 3) and *I. mangabensis* (Figs 2 and 3) did not form monophyletic species groups in our investigation. In both instances, collections from the Anjanaharibe-Sud Reserve (*I. regalis* 1, De Block 2081 and 2083; *I. mangabensis* 1, De Block 2040 and 2053) differed from collections made elsewhere (*I. regalis* 2, De Block 835, collected 12 km from Moramanga; *I. mangabensis* 2, Tosh 128 and 130, collected near Soanierano-Ivongo). The fact that populations of *I. regalis* and *I. mangabensis* in the Anjanaharibe-Sud Reserve in northern Madagascar are different from those further south in Moramanga and Sonierano-Ivongo is not strange since these localities represent different bioclimates. Populations of the same species may then be genetically isolated from other conspecific populations, resulting in the accumulation of genetic variation in the absence of morphological differentiation, as is the case for *I. regalis*. However, in the case of *I. mangabensis*, De Block (2013) observed some morphological differences between material collected from Anjanaharibe-Sud, and material collected from other localities throughout its geographical range. For example, *I. mangabensis* from Anjanaharibe-Sud is characterized by long and narrow bracts and bracteoles, and longer, narrowly triangular calyx lobes. The specimens also occur in different vegetation types and at different altitudes: lowland humid forest in Sonierano-Ivongo (altitude approx. 60 m) vs. montane humid forest (altitiude approx. 1000 m) in Anjanaharibe-Sud. The taxonomy of *I. mangabensis* may therefore require re-evaluation given the genetic differentiation and morphological variation observed between populations currently referred to as this species.

Placement of the Afro-Madagascan Ixora *spp. in the genus*

Our phylogenetic tree is consistent with that of Mouly *et al.* (2009*b*). In the genus, two main lineages are present, an Asian-Pacific lineage and an Afro-Indian Ocean-Neotropical lineage (Fig. 3). In the Asian-Pacific lineage, a clade of Asian species is sister to a clade of Pacific species. In the Afro-Indian Ocean-Neotropical lineage, a Mascarene clade is sister to the Afro-Madagascan and Neotropical species. The Neotropical clade is resolved as sister to the Afro-Madagascan species. Therefore, monophyletic groups recovered in this analysis are geographically delimited and correspond to tropical Asia, the Pacific regions, the Mascarenes, the Neotropics and continental Africa/Madagascar. In the Afro-Madagascan clade, the Madagascan taxa do not form a monophyletic group; instead they form two distinct clades (Fig. 3), one of which is nested in a group of African taxa. Mouly *et al.* (2009*b*) recovered this same general pattern, albeit with lower sampling of African and Madagascan species.

Mascarene Ixora *species*

The position of the Mascarene taxa in our phylogenetic inference, as sister to the Neotropical and Afro-Madagascan lineages, may appear somewhat incongruous given its geographical proximity to Africa and Madagascar. The time of divergence between the Mascarene and the Neotropical/Afro-Madagascan taxa (Fig. 5: node 65) is estimated at 13·14 Ma (6·26–20·23, 95 % HPD). It would seem that a single early colonization event of the Mascarenes occurred, after which the colonizing species adapted to new relatively extreme environments, resulting in markedly different morphological characters.

Until recently, the Mascarene species included in this analysis were considered to belong to the endemic genus *Myonima*. Both this genus and the monospecific Mascarene endemic *Doricera* [not sampled here, but sister of *Myonima* in the molecular study of Mouly *et al.* (2009*b*)], differ greatly from the Neotropical and Afro-Madagascan *Ixora* spp., to which they are the sister group (Fig. 3). Although having articulate petioles, free stigmatic lobes and a single ovule per locule (key characters for *Ixora*), they also have a number of aberrant characters. They are heterophyllous (juvenile foliage different), with coriacous leaves, short corolla tubes (<5 mm long), (2)-3–7-locular ovaries and relatively large fruits with stony pyrenes (De Block, 1997). Furthermore, the flowers of *Doricera* are reported to be dioecious (Verdcourt, 1983) and those of *Myonima* to be dioecious (Mouly *et al.*, 2009*b*) or polygamous (Bentham and Hooker, 1873; Baker, 1877). These differences certainly explain why in the past the genera were considered closely related to, but distinct from, *Ixora*. Recently, however, Mouly *et al.* (2009*b*) showed that *Ixora* is paraphyletic unless several small satellite genera, including *Doricera* and *Myonima*, are included.

Although the differences between the Mascarene and Afro-Madagascan/Neotropical *Ixora* species are considerable, they can all be explained as insular adaptations to browsing pressure and selection for outcrossing. There is a high incidence of coriaceous, tough foliage and developmental heterophylly in the Mascarenes (Friedmann and Cadet, 1976), probably evolved in response to browsing pressure from giant tortoises (Griffiths *et al.*, 2010). Several other Rubiaceae show a similar adaptation, e.g. *Coptosperma borbonicum* (Heine and Hallé, 1970). Many oceanic islands are particularly rich in dioecious species since selection for outcrossing in small, colonizing, hermaphroditic populations favours separation of the sexual functions (Anderson *et al.*, 2006). Many dioecious species have small, relatively inconspicuous, whitish or greenish flowers (pollinated by small generalist bees and flies) (Bawa, 1980; Baker, 1984), which is also the case in the species discussed here. As regards the differences in fruit morphology, these can be partly attributed as a defence against frugivory by giant tortoises (stony pyrenes) and an adaptation to dioecy. Dioecious species may have a reproductive disadvantage because not every individual in a population produces seeds. To compensate for this disadvantage, more seeds should be produced (Heilbuth *et al.*, 2001; Queenborough *et al.*, 2009). An increase in number of locules automatically increases the seed number since in *Ixora* a single seed per locule is produced. Furthermore, the increased mechanical protection of the seeds (stony pyrenes) may increase seed fitness.

Phylogenetic relationships of Afro-Madagascan Ixora *spp*

As exemplified by several studies (e.g. Malcomber, 2002; Maurin *et al.*, 2007; Tosh *et al.*, 2009), extremely low levels of

interspecific sequence divergence in woody Rubiaceae can confound attempts to retrieve fully resolved and well-supported phylogenetic inferences. However, in the current study, there are some well-supported relationships in the Afro-Madagascan clade that are recovered in our phylogenetic analyses of the plastid and nuclear combined data set.

The West–Central African *I. nematopoda* and the East African Afromontane *I. scheffleri* are sister to all other Afro-Madagascan taxa in Clade 1 (Fig. 3). There is also strong support for the West–Central African species *I. foliosa* (Afromontane element) and *I. hippoperifera* being sister to all the remaining taxa in Clade 1 (Fig. 3). These four taxa all possess pedunculate inflorescences subtended by inflorescence-supporting leaves (De Block, 1998), though the inflorescences of *I. hippoperifera* may also be sessile (Fig. 4A). For the continental African taxa, De Block (1998; fig. 9, p. 25) postulated that many-flowered, pedunculate, lax, corymbose inflorescences constitute the basic type. Our results corroborate De Block's assumption (1998) that sessile inflorescences represent a derived condition in continental African *Ixora*. *Ixora brachypoda* and *I. hartiana* also have pedunculate inflorescences, whereas all other continental African species represented in this study have sessile inflorescences (Fig. 4A).

Some African species groups, thought to be related on the basis of their morphological similarity (De Block 1998), are recovered in our phylogenetic analyses. *Ixora guineensis*, *I. batesii* and *I. minutiflora* (*I. guineensis* complex) are largely endemic to the lower Guinea regional sub-centre of endemism (RSE) occupying lowland and gallery forest. These three species are recovered in a well-supported monophyletic group (Fig. 3), with the upper Guinea endemic *I. hiernii*. This group is supported by the presence of sessile, lax, more or less densely pubescent inflorescences (glabrous to densely covered with minute hairs in the case of *I. minutiflora*), and sessile to shortly pedicellate flowers with relatively short corolla tubes (0·5–3·0 cm).

Our results also provide support for a close relationship between *I. macilenta* and *I. praetermissa* (Fig. 3). These two species are endemic to the lower Guinea sub-centre of endemism, occupying both primary and secondary forest and occurring in riverine (gallery) forest. In addition, both of these species possess sessile, lax inflorescences. Sister to these two species in our phylogenetic analyses is *I. hartiana*, a species with disjunct distribution in Africa, which grows in gallery forest and open woodland. This species is known from only a small number of collections, three from Congo, one from Tanzania and one collection from Angola (De Block, 1998). The morphology of *I. hartiana* differs somewhat from that of both *I. macilenta* and *I. praetermissa*, most notably by the presence of pedunculate, erect and lax inflorescences and long pedicellate flowers.

There is strong support for the close relationship between the eastern African species *I. narcissodora* and *I. tanzaniensis*, although these two taxa differ in both morphology and habitat (De Block, 1998). *Ixora narcissodora* is widespread throughout the lowland evergreen and riverine forests from Kenya to Mozambique. In contrast, *I. tanzaniensis* is restricted to a small area of lowland forest in Tanzania. These two species differ most notably in inflorescence structure (*I. narcissodora*, sessile and lax; *I. tanzaniensis*, sessile and compact), corolla tube length (*I. narcissodora*, 30–75 mm; *I. tanzaniensis*, 22–32 mm) and pubescence (greater in *I. tanzaniensis* than in *I. narcissodora*).

However, there are also examples where species that are thought to be close relatives on the basis of morphological similarities are not grouped together in our phylogenetic tree(s). De Block (1998) noted the close resemblance between the Afromontane species *I. scheffleri* and *I. foliosa* and considered them to be close relatives. However, our results do not support a close relationship between these two species (Fig. 3). Although both have pedunculate, erect and compact inflorescences, there are key differences between them, notably in the morphology of bracts and bracteoles (typically absent in *I. scheffleri*) and in their geographical distribution (*I. scheffleri* from East Africa and *I. foliosa* from West Africa). Another example involves the Madagascan taxa *I. amplidentata*, *I. emirnensis*, *I. mangabensis* and *I. moramangensis*. De Block (2013) noted the similarity between these species as they all possess pedunculate, pendulous, moderately lax to lax inflorescences with a moderate number of flowers with relatively short corolla tubes. In our phylogenetic analyses (Figs 2 and 3), *I. emirnensis*, *I. mangabensis* and *I. moramangensis* are nested in Clade 3, although the exact relationship between these taxa is not fully resolved. In our individual and combined phylogenetic analyses, *I. amplidentata* is nested in Clade 2, with *I. regalis* and *I. guillotii* among others. Mouly *et al.* (2009*b*) also included sequence data of *I. amplidentata* and *I. emirnensis* (from different specimens) in their study of Ixoreae, and their analyses also indicated that *I. amplidentata* and *I. emirnensis* are not closely related, despite morphological similarities.

There are few resolved or well-supported relationships in either clade of Madagascan taxa. In Clade 2, there is strong support for the group of *I. homolleae* and *I. quadrilocularis* (discussed in more detail in the next section) and for the group of *I. platythyrsa* and *I. microphylla*. These last two species differ markedly in inflorescence structure (long pedunculate, pendulous inflorescences with numerous flowers in *I. platythyrsa* vs. sessile or shortly pedunculate, erect inflorescences with few flowers in *I. microphylla*), but other flower/inflorescence characters are similar, notably the well-developed triangular bracteoles and calyx lobes and the corolla lobes with acuminate tip. In Clade 3, *I. capuroniana* and *I. masoalensis* are strongly supported sister taxa (Fig. 3). These taxa have sessile inflorescences; coriaceous leaves that are pale yellow when dried; and small calyces, bracts and bracteoles (De Block, 2013). Finally, *I. crassipes* and *I. cremixora* form a strongly supported clade. These species occur in humid or sub-humid (semi-)deciduous forests in Western and Northern Madagascar. Most of the other species sampled in this study occur in the humid forests (littoral, lowland or altitudinal) on the eastern and North-Eastern coasts of Madagascar.

Key morphological traits in Madagascan Ixora

Important morphological features for species-level identification of Madagascan *Ixora* include inflorescence and flower characters (De Block, 2003). However, there are some morphological features that can be used to distinguish between taxa from the two Madagascan lineages. Taxa from Clade 2 possess comparatively long calyx tubes and calyx lobes, relative to taxa from Clade 3

(Fig. 4B, C). In Clade 2, calyx lobe length varies between 0·5 and 15·0 mm, compared with between 0·1 and 0·8 mm in Clade 3 (Table 3). With the exception of *I. amplidentata*, calyx tube length typically varies between 0·5 and 10·0 mm in Clade 2, and between 0·20 and 0·75 mm in Clade 3 (Table 3). Calyx tube and calyx lobe lengths are particularly long in the four-locular species and *I. foliicalyx*, the latter of which is sister to *I. homolleae* and *I. quadrilocularis* on our plastid and nuclear combined phylogenetic inference (Fig. 3).

There are three morphological traits occurring in Madagascan *Ixora* that are rare or absent throughout the rest of the genus. The taxonomic distribution of these traits can be interpreted in light of our phylogenetic results (Fig. 4D–F). The first of these is the extreme reduction in flower numbers towards uniflorous inflorescences (Fig. 4D). Most species in the genus have striking inflorescences containing large numbers of flowers. For example, Nilsson *et al.* (1990) reported up to 282 flowers in a single inflorescence of the Madagascan species *I. plathythyrsa*. Pauciflorous species, containing <15 flowers per inflorescence, are rare in *Ixora* (De Block, 2008). The occurrence of solitary-flowered inflorescences is exceptionally rare, and only a few uniflorous *Ixora* spp. have been described, such as *I. dzumacensis* from New Caledonia (Guillaumin, 1929). On Madagascar, there are six uniflorous species currently recognized, and about 30 % of Madagascan *Ixora* spp. are uniflorous or contain <15 flowers per inflorescence (De Block, 2008). Five of these uniflorous species have been accommodated in *Ixora* section *Microthamnus* (Guédès, 1986; De Block, 2008). A sixth uniflorous Madagascan species (*I. homolleae*) differs from members of section *Microthamnus* in a number of characters, most notably its large flowers, four-locular ovaries, and fruits and stigma with four stigmatic lobes. Members of section *Microthamnus* are poorly collected in the field, because of the inconspicuous nature of their inflorescences and their rarity (De Block, 2008). We were only able to obtain silica gel material from one representative of this section (*I. rakotonasoloi*) and, as a result, we were unable to test the monophyly of this seemingly natural group of uniflorous species. However, we were able to include multiple accessions of *I. homolleae*, and from our phylogenetic analyses it is evident that the evolution of uniflorous species has occurred at least twice in Madagascan *Ixora* (Fig. 4D).

A second morphological trait present in Madagascan, but not continental African, *Ixora* is four-locular ovaries (Fig. 4E). The genus as a whole is typically characterized by uniovulate bilocular ovaries, and four-locular ovaries are rare (De Block, 2013). Mouly *et al.* (2009*b*) favoured a broad circumscription of the genus, incorporating a number of taxa previously recognized as satellite genera of *Ixora*. Among these are the plurilocular Mascarene endemic genus *Myonima* and the four-locular *Ixora mooreensis*, an endemic to the Society Islands (French Polynesia, Pacific Ocean) that was formerly placed in the monotypic genus *Hitoa*. As such, the generic circumscription of *Ixora* now accommodates plurilocular (two- to seven-locular) ovaries. The uniflorous four-locular *I. homolleae*, endemic to the littoral forests of eastern Madagascar, was formerly classified in the monotypic genus *Thouarsiora*. De Block (2013) recognizes several other Madagascan *Ixora* spp. allied to *I. homolleae* that have four-locular ovaries and fruits, notably *I. lagenifructa*, *I. quadrilocularis* and *I. trimera*. These species also share a number of other characters, including large fruits with a thick fruit wall and stony pyrenes. The fact that *I. lagenifructa* is not placed with the other four-locular species (i.e. *I. homolleae* and *I. quadrilocularis*) in our separate or combined phylogenetic analyses could be considered surprising (Figs 2, 3 and 4E). Although most *Ixora* spp. differ from each other on the basis of minor and continuous characters, an increase in the number of locules and associated characters (e.g. thickened fruit wall, stony endocarp) represents a more profound shift in morphology. De Block (2013) regards these four-locular *Ixora* spp. as a natural group, and therefore additional independent material of *I. lagenifructa* is required to verify the results of our molecular analyses.

A third morphological trait unique to the Madagascan element of *Ixora* is an increase in corolla tube length (Table 3). Flower size, and in particular the length of the corolla tube, is extremely variable among the Madagascan representatives of *Ixora* (De Block, 2007). Typically the length of corolla tubes varies between 1 and 9 cm, although there are a number of species that possess corolla tubes up to 13 cm in length (De Block, 2007). Furthermore, there are four species in which corolla tubes exceed 15 cm in length and can be as long as 23 cm. Corolla tubes of this size are rare in Rubiaceae as a whole, and the range in corolla tube lengths among Madagascan *Ixora* spp. (0·4–23·0 cm) is remarkable (De Block, 2007). In comparison, corolla tube length varies between 0·5 and 11·0 cm in continental African species (Table 3; De Block, 1998). We were able to incorporate sequence data from three of the four *Ixora* spp. that have extremely long corolla tubes. As postulated by De Block (2007), the increase in corolla tube length has occurred several times (Fig. 4F). *Ixora densithyrsa* and *I. siphonantha*, thought to be close relatives due to the possession of identical bracts, bracteoles and calyces (De Block, 2007), are recovered in the exclusively Madagascan Clade 2. *Ixora crassipes* is nested in Clade 3 in our phylogenetic analyses, and is clearly distinct from the other large flowered *Ixora* sampled in this investigation notably because of the reduced bracts, bracteoles and calyx lobes and the yellowish colour of the dried specimens. Therefore, with the exception of the four-locular ovaries that are exclusive to Clade 2, both lineages of Madagascan taxa contain pauciflorous and uniflorous species, and species with corolla tubes >15 cm in length.

Historical biogeography

Our molecular study dates the crown age of *Ixora* sometime during the mid Miocene (16·67 Ma). This date is similar to that in the studies of Mouly (2007) and Bremer and Eriksson (2009), who estimated the crown age of *Ixora* at 14–15 Ma. Similarly, the age estimate for the crown age of the Afro-Madagascan group during the Late Miocene (7·95 Ma) is consistent with Mouly (2007). Divergence in the Afro-Madagascan group began within the last 8 Ma during the late Miocene, with extensive cladogenesis occurring throughout the Pliocene. The two separate lineages of Madagascan taxa are of different ages. The exclusively Madagascan Clade 2 started to diversify during the Miocene–Pliocene boundary, whereas Clade 3 had its origin in the late Pliocene and underwent a period of rapid speciation that continued into the Pleistocene.

The results of our phylogenetic investigations for *Ixora* are in keeping with the general observations from the growing

literature on the historical biogeography of the flora and fauna of Madagascar (see below). Despite its prolonged geographical isolation, the current biota of Madagascar is seemingly comprised primarily of recently evolved endemics, evolving *in situ* following Cenozoic trans-oceanic dispersal (Yoder and Nowak, 2006). Other studies focusing both on Rubiaceae (e.g. Malcomber, 2002; Maurin *et al.*, 2007; Groeninckx, 2009; Tosh *et al.* 2009; Wikström *et al.*, 2010) and on other angiosperm families (e.g. Davis *et al.*, 2002 on Malpighiaceae; Meve and Liede, 2002 on Apocynaceae; Plana, 2003 on Begoniaceae; Renner, 2004 on Melastomataceae; Yuan *et al.*, 2005 on Gentianaceae; Trénel *et al.*, 2007 on Arecaceae; Schaefer *et al.*, 2009 on Cucurbitaceae; Buerki *et al.*, 2013 on Sapindaceae and other families; Strijk *et al.*, 2012 on Asteraceae) have revealed multiple independent dispersal events across the Mozambique Channel during the Cenozoic era. The most commonly observed pattern thus far is limited dispersal (one of few events) per genus from East Africa into Madagascar, followed by speciation in Madagascar.

Possible mechanisms driving the radiation of Madagascan Ixora *spp*

Dispersal of *Ixora* into Africa and Madagascar, and the species diversification that followed, occurred in the late Miocene (i.e. approx. 8 Ma). Palynological and macrofossil data (reviewed in Maley, 1996; Jacobs, 2004) indicate that grass-dominated savannahs began to expand throughout sub-Saharan Africa at the expense of humid forest in the mid Miocene (16 Ma) and were widespread by the late Miocene (8 Ma). Rain forest taxa would have been restricted to small patches of humid forest in upland areas or along lowland river systems (Robbrecht, 1996; Plana, 2004) at the time when *Ixora* began to diverge in continental Africa. The Cenozoic climate history of Madagascar and the chronology of the development of its biomes remain largely unknown (Wells, 2003). Nevertheless, Wells (2003) surmised that humid forest conditions would probably have been present in the eastern watershed of Madagascar from the Oligocene onwards. Although several lineages of Afro-Madagascan *Ixora* were already established at the Pliocene–Pleistocene boundary, a number of Pleistocene speciation events appear to have occurred.

On continental Africa, the Pliocene and Pleistocene epochs were characterized by a mosaic of fragmented humid forest, interspersed by savannah (Plana, 2004). Expansion and subsequent contraction of each biome type was mediated by the climatic conditions of the time (Plana, 2004). In Madagascar there is considerable habitat heterogeneity resulting from a number of factors, such as a wide variety of geology, significant topographic relief and the orographic rainfall in the east (Wells, 2003; Dewar and Richard, 2007). There is also evidence of considerable displacement of vegetation zones as a result of Pleistocene and Holocene climatic fluctuations (Burney, 1996; Straka, 1996). This habitat heterogeneity, coupled with the cyclical expansion and retraction of biome ranges during periods of climate oscillation in the Pliocene and Pleistocene, may have led to formerly contiguous populations of conspecific taxa becoming geographically isolated in temporal forest refugia (Janssen *et al.*, 2008). One of the main driving forces of rapid radiations is thought to be the development of new ecological opportunities in the absence of competition in largely unoccupied, depauperate environments (e.g. following orogenic uplift or post-glacial re-expansion) (Hughes and Eastwood, 2006; Janssens *et al.*, 2009).

In addition to novel ecological opportunities afforded by repeated habitat fragmentation during periods of climatic perturbation, pollination syndromes are thought to be a major factor in the reproductive isolation of species (Hodges and Arnold, 1994). Malcomber (2002) postulated that the rapid diversification of *Gaertnera* spp. (also Rubiaceae) is correlated with a change in corolla morphology, and that the elongated tubular corolla morphology (relative to the sister genus *Pagamea*) represented a key innovation in the group. Perhaps the most conspicuous feature of Madagascan *Ixora* spp. is the extreme variability in corolla tube length, which would indicate that speciation is at least partly pollinator driven (De Block, 2007). Neal *et al.* (1998) stated that narrow and/or long corolla tubes could preclude a wide range of potential pollinators from gaining access to the reward, thereby increasing both pollination precision and pollinator fidelity. *Ixora* spp. also exhibit secondary pollen presentation, whereby pollen from the protandrous anthers is deposited on the non-receptive abaxial sides of the stigmatic lobes and/or the upper part of the style prior to anthesis (Puff *et al.*, 1996). The stigmatic lobes (and style) therefore provide the dual function of the pollen-presenting organ, and then, at maturity, the pollen recipient (Nilsson *et al.*, 1990). In *Ixora*, the narrow tubular corolla provides a mechanical guide to ensure precise transfer of pollen between the style and the pollination vector (Nilsson *et al.*, 1990). The variation in corolla tube length in Madagascan *Ixora* has clearly resulted in some pollinator specialization. For example, the flowers of *I. densithyrsa* and *I. siphonantha* reach lengths >20 cm long, and must be pollinated by members of the guild of long tongue Madagascan hawkmoths (Nilsson, 1998). Nilsson *et al.* (1990) reported that pollination in *I. platythyrsa* is carried out by nocturnal moths, and many other Madagascan *Ixora* have delicately scented, white corollas typical of moth pollination. Changes in corolla tube length over time may preclude generalist pollinators from successfully affecting their pollinator services and ultimately lead to pollinator specificity.

Given the evidence from other studies (e.g. Wilmé *et al.*, 2006; Janssen *et al.* 2008; Pearson and Raxworthy, 2009; Strijk *et al.*, 2012), it is increasingly apparent that short-term climatic events and associated habitat fragmentation during the Pliocene and Pleistocene have facilitated the rapid accumulation of biodiversity of some taxonomic groups on Madagascar. However, as with other studies (e.g. Malcomber, 2002), it would appear likely that pollination biology (pollinator specificity and phenology) has played an equally vital role in the recent diversification of Madagascan *Ixora*.

Conclusions

Madagascan *Ixora* do not form a monophyletic group, but are represented by two lineages of different ages. Our results indicate at least one dispersal event from East Africa into Madagascar towards the end of the Pliocene. Both *Ixora* lineages on Madagascar exhibit morphological innovations that are rare in the rest of the genus, including a trend towards pauciflorous inflorescences and a trend towards extreme corolla tube length.

This suggests that the same ecological and selective pressures are acting upon taxa from both Madagascan lineages. The recent radiation in Madagascan *Ixora* is likely to have been driven by increased ecological opportunities following periods of habitat contraction and expansion during the Pliocene/Pleistocene, coupled with increased pollinator specificity between *Ixora* spp. resulting from corolla tube length diversification in the genus.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: ETS Bayesian majority rule consensus tree. Figure S2: ITS Bayesian majority rule consensus tree. Figure S3: one of the 5362 most-parsimonious trees generated in the maximum parsimony analysis of the combined plastid–ETS–ITS data set. Figure S4: BEAST maximum clade credibility tree for Rubiaceae based on three plastid regions. Table S1: DNA substitution models used in Bayesian analyses. Table S2: BEAST node age estimations–Rubiaceae wide analysis. Table S3: BEAST node age estimations–*Ixora* analysis.

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APPENDIX 1. *List of Rubiaceae and Gentianales taxa used in molecular dating analyses*

	Species	Voucher information (kept at BR) and accession origin
Outgroup	*Ceropegia linearis* E.May	Van Caekenberghe 347 (Kenya)
	Strychnos potatorum L.f.	Van Caekenberghe 346 (Zimbabwe)
Ingroup	*Luculia pinceana* Hook.	Van Caekenberghe 14 (China)
Rubiidinae	*Anthospermum palustre* Homolle ex Puff	De Block 1922 (Madagascar)
	Coprosma repens A.Rich.	Van Caekenberghe 280 (New Zealand)
	Danais sp.	De Block 2011 (Madagascar)
	Galium mollugo L.	Billiet 571 (Reunion)
	Mycetia malayana (G.Don) Craib	Van Caekenberghe 5 (Thailand)
	Paederia foetida L.	Van Caekenberghe 98 (China)
	Rubia fruticosa Aiton	Van Caekenberghe 256 (Spain)
	Serissa japonica (Thunb.) Thunb.	Van Caekenberghe 178 (China)
	Triainolepis sp.	De Block 1958 (Madagascar)
Psychotriidinae	*Colletoecema magna* Sonké & Dessein	Dessein 1608 (Cameroon)
	Coussarea hydrangeifolia (Benth.) Benth. & Hook.f. ex Müll.Arg	Sequences obtained from GenBank
	Faramea trinervia K.Schum. & Donn.Sm.	Sequences obtained from GenBank
	Gaertnera sp. nov.	De Block 1795 (Madagascar)
	Geophila repens (L.) I.M.Johnst.	De Block 402 (Kenya)
	Ophiorrhiza mungos L.	Van Caekenberghe 15 (China)
	Myrmecodia tuberosa Jack	Van Caekenberghe 343 (Borneo)
	Psychotria kirkii Hiern	Van Caekenberghe 25 (DRCongo)
	Stelechantha makakana N.Hallé	Dessein 1483 (Cameroon)
Cinchonidinae	*Antirhea borbonica* J.F.Gmel.	De Block 2004 (Madagascar)
	Breonadia salicina (Vahl) Hepper & J.R.I.Wood	De Block 1151 (Madagascar)
	Chiococca alba (L.) Hitchc.	Van Caekenberghe 80 (Costa Rica)
	Cinchona pubescens Vahl	De Block 932 (Madagascar)
	Cubanola domingensis (Britton) Aiello	Van Caekenberghe 168 (Dominican Republic)
	Exostema longiflorum (Lamb.) Schult.	Van Caekenberghe 95 (Dominican Republic)
	Guettarda uruguensis Cham. & Schltdl.	Sequences obtained from GenBank
	Hamelia patens Jacq.	Van Caekenberghe 85 (Mexico)
	Hoffmannia refulgens (Hook.) Hemsl.	Van Caekenberghe 67 (Mexico)
	Hymenodictyon biafranum Hiern.	Van Caekenberghe 350 (Cameroon)
	Rondeletia odorata Jacq.	De Block 1407 (Cuba)
	Uncaria rhynchophylla (Miq.) Miq. ex Havil.	Van Caekenberghe 50 (Japan)
Ixoridinae	*Razafimandimbisonia humblotii* (Drake) Kainul. & B.Bremer	Tosh 263 (Madagascar)
	Aulacocalyx caudata (Hiern) Keay	Dessein 1510 (Cameroon)
	Calycophyllum spruceanum (Benth.) Hook.f. ex K.Schum.	Van Caekenberghe 318 (Ecuador)
	Canthium sp.	De Block 691 (Madagascar)
	Coffea mangoroensis Portères	Rakotonasolo 41 (Madagascar)
	Coffea moratii J.-F.Leroy ex A.P.Davis & Rakotonas.	Davis 2326 (Madagascar)
	Coffea stenophylla G.Don	Billiet 3034 (DRCongo)
	Coptosperma nigrescens Hook.f.	Van Caekenberghe 52 (Madagascar)
	Cremaspora triflora (Thonn.) K.Schum.	Van Caekenberghe 17 (Nigeria)
	Cuviera cf. *leniochlamys* K.Schum.	Dessein 1448 (Cameroon)
	Didymosalpinx norae (Swynn.) Keay	Van Caekenberghe 62 (Zimbabwe)
	Diplospora dubia (Lindl.) Masam.	Van Caekenberghe 49 (China)
	Emmenopterys henryi Oliv.	Van Caekenberghe 100 (cultivated at Kalmthout)
	Empogona concolor (N. Hallé) J.Tosh & Robbr.	Degreef 95 (Gabon)
	Empogona kirkii Hook.f.	Van Caekenberghe 79 (Zimbabwe)
	Empogona ovalifolia (Hiern) J.Tosh & Robbr.	De Block 1072 (Madagascar)
	Euclinia longiflora Salisb.	Van Caekenberghe 348 (Sierra Leone)
	Fadogia sp.	Van Caekenberghe 349 (Zambia)
	Fernelia buxifolia Lam.	Sequences obtained from GenBank
	Gardenia jasminoides J.Ellis	Van Caekenberghe 57 (Japan)
	Genipa americana L.	Van Caekenberghe 317 (Ecuador)
	Ixora ankazobensis De Block sp. nov ined.	Tosh 30 (Madagascar)

Continued

Appendix 1. *Continued*

Species	Voucher information (kept at BR) and accession origin
Ixora batesii Wernham	Dessein 1455 (Cameroon)
Ixora borboniae Mouly & B.Bremer	Van Caekenberghe 42 (Mauritius)
Ixora brachypoda DC.	Walters 1437 (Gabon)
Ixora brevifolia Benth.	Delprete (Brazil)
Ixora capuroniana De Block	Tosh 400 (Madagascar)
Ixora chinensis Lam.	Van Caekenberghe 316 (China)
Ixora collina (Montrouz.) Beauvis.	Mouly 236 (New Caledonia)
Ixora crassipes Boivin ex De Block	Groeninckx 80 (Madagascar)
Ixora densithyrsa De Block	De Block 1773 (Madagascar)
Ixora elliotii Drake ex De Block	De Block 1977 (Madagascar)
Ixora emirnensis Baker	De Block 1788 (Madagascar)
Ixora ferrea (Jacq.) Benth.	Merello 1716 (Caribbean)
Ixora francii Schltr.	Mouly 241 (New Caledonia)
Ixora guillotii Hoch.	Tosh 408B (Madagascar)
Ixora homolleae De Block & Govaerts	Tosh 107 (Madagascar)
Ixora macilenta De Block	Dessein 1404 (Cameroon)
Ixora nematopoda K.Schum.	Dessein 1449 (Cameroon)
Ixora nitens (Poir.) Mouly & B.Bremer	Friedmann 2631 (Mauritius)
Ixora perrieri De Block	Tosh 232 (Madagascar)
Ixora platythyrsa Baker	De Block 773 (Madagascar)
Ixora quadrilocularis De Block	Tosh 85 (Madagascar)
Ixora siphonantha Oliv.	Tosh 389 (Madagascar)
Ixora sp.	Billiet 7327 (Malaysia)
Ixora tanzaniensis Bridson	Luke 9304 (Tanzania)
Kraussia floribunda Harv.	Van Caekenberghe 193 (Mozambique)
Mitriostigma axillare Hochst.	Van Caekenberghe 44 (Mozambique)
Mussaenda pubescens Dryand.	Van Caekenberghe 74 (China)
Oxyanthus unilocularis Hiern	Van Caekenberghe 198 (Cameroon)
Pentagonia tinajita Seem.	Van Caekenberghe 252 (Costa Rica)
Petitiocodon parviflora (Keay) Robbr.	Dessein 1597 (Cameroon)
Petitiocodon parviflora (Keay) Robbr.	Dessein 1612 (Cameroon)
Pinckneya bracteata (Bartram) Raf.	Sequences obtained from GenBank
Posoqueria latifolia (Rudge) Schult.	Van Caekenberghe 166 (Costa Rica)
Pseudomussaenda sp.	Dessein 1422 (Cameroon)
Psilanthus ebracteolatus Hiern	Billiet 53054 (Ivory Coast)
Psilanthus mannii Hook.f.	De Block 1409 (Ghana)
Rothmannia longiflora Salisb.	Van Caekenberghe 279 (Gabon)
Rosenbergiodendron formosum (Jacq.) Fagerl.	Van Caekenberghe 344 (Venezuela)
Sabicea venosa Benth.	Van Caekenberghe 58 (Zambia)
Tarenna gracilipes (Hayata) Ohwi	Van Caekenberghe 7 (Japan)
Tricalysia ambrensis Randriamb. & De Block	De Block 1313 (Madagascar)
Tricalysia analamazaotrensis Homolle ex Randriamb. & De Block	Tosh 11 (Madagascar)
Tricalysia coriacea (Benth.) Hiern	Dessein 1283 (Zambia)
Tricalysia cryptocalyx Baker	Tosh 322 (Madagascar)
Tricalysia dauphinensis Randriamb. & De Block	Tosh 349 (Madagascar)
Tricalysia leucocarpa (Baill.) Randriamb. & De Block	Tosh 398 (Madagascar)
Tricalysia microphylla Hiern	De Block 405 (Kenya)
Tricalysia pedunculosa (N.Hallé) Robbr.	Degreef 86 (Gabon)
Vangueria madagascariensis J.F.Gmel.	Van Caekenberghe 82 (Mozambique)
Virectaria procumbens (Sm.) Bremek.	Van Caekenberghe 212 (Cameroon)
Warszewiczia coccinea (Vahl) Klotzsch	Van Caekenberghe 165 (Hawaii)