The LINNEAN SOCIETY of London BOTANICAL Journal of the Linnean Society

Botanical Journal of the Linnean Society, 2012, **169**, 284–296. With 2 figures

Phylogenetic inference of *Badula* (Primulaceae), a rare and threatened genus endemic to the Mascarene Archipelago

RUTH E. BONE[1]*, JOERI S. STRIJK[2,3], PETER W. FRITSCH[4], SVEN BUERKI[5], DOMINIQUE STRASBERG[6], CHRISTOPHE THÉBAUD[3] and TREVOR R. HODKINSON[1]

[1]*Botany Building, School of Natural Sciences, Trinity College Dublin, College Green, Dublin 2, Ireland*
[2]*Ecological Evolution Group, Xishuangbanna Tropical Botanic Garden, Chinese Academy of Sciences, Menglun, Mengla, 666303, China*
[3]*Laboratoire Evolution et Diversité Biologique (UMR 5174), Université Paul Sabatier & CNRS, F-31062 Toulouse, Cedex 9, France*
[4]*Department of Botany, California Academy of Sciences, 55 Music Concourse Drive, Golden Gate Park, San Francisco, CA 94118, USA*
[5]*Jodrell Laboratory, Royal Botanic Gardens, Kew, Richmond, Surrey, TW9 3AB, UK*
[6]*Université de la Réunion, 15 Avenue R. Cassin, BP 7151, 97 715 Saint-Denis Messag, Cedex 9, La Réunion, France*

Received 21 May 2011; revised 3 September 2011; accepted for publication 20 December 2011

With 14 species, *Badula* (Primulaceae) is the most species-rich endemic angiosperm genus of the Mascarene Archipelago. The relationship between *Badula* and its ally *Oncostemum* (*c*. 100 spp; Madagascar and the Comoros Islands) is uncertain, with implications for the circumscription of *Badula* as a Mascarene endemic. Within *Badula*, species rarity (several being critically endangered) and a paucity of herbarium specimens hamper proper species delimitations. Here, we estimate the phylogenetic relationships of *Badula* based on DNA sequence data from the nuclear ribosomal internal transcribed spacer (ITS) and plastid *trnS-trnG-trnG* regions with complete taxon sampling of the genus and three samples or more of each taxon. The results strongly supported the monophyly of *Badula*. Paraphyly of *Oncostemum* was inferred with weak support; explicit hypothesis testing did not favour this hypothesis over one that forced the monophyly of *Oncostemum*. Monophyly of several *Badula* spp. was supported, particularly for taxa from the older islands of Mauritius and Rodrigues. *Badula* is inferred to have reached the Mascarene Archipelago through a single colonization event. The majority of species segregated into island clades, implying that few, rather than multiple, colonization events have occurred in *Badula* among the islands of the archipelago. *Botanical Journal of the Linnean Society*, 2012, **169**, 284–296.

ADDITIONAL KEYWORDS: ITS – Mauritius – Myrsinoideae – *Oncostemum* – phylogenetics – Réunion – Rodrigues – *trnS-trnG-trnG*.

INTRODUCTION

The woody tropical genus *Badula* Juss. (Primulaceae *sensu* APG III, 2009; Myrsinoideae *sensu* Mez, 1902) comprises 14 species endemic to the Mascarene Archipelago in the Western Indian Ocean (Coode, 1981). This genus harbours more than twice the species richness of any of the other 34 Mascarene endemic genera and is one of only 16 other endemic genera that have successfully colonized all three islands of the archipelago (Mauritius, Réunion and Rodrigues; C. Baider, The Mauritius Herbarium, MSIRI and V. Florens, University of Mauritius, unpubl. data). *Badula* displays a striking diversity of habit, from low-growing decumbent or divaricate shrubs [*Badula decumbens* (Cordem.) Coode and

*Corresponding author. Current address: Herbarium Library Art and Archives, Royal Botanic Gardens, Kew, Richmond, Surrey, TW9 3AE, UK. E-mail: r.bone@kew.org

Badula platyphylla (A.DC.) Coode] to monocaulous treelets with large strap-shaped leaves (*Badula borbonica* A.DC.) and small multi-stemmed trees (e.g. *Badula insularis* A.DC.).

The genus was first described by Jussieu (1789) from specimens of '*bois pintade*' [*Badula barthesia* (Lam.) A.DC.] from Commerson's Réunion herbarium, and was characterized by the presence of axillary racemose-paniculate inflorescences, a corolla with a short tube and five lobes, subsessile anthers and a short style that bears a capitate stigma (Jussieu, 1789). In 1830, the myrsinoid genus *Oncostemum* Juss. was described from specimens from Madagascar, and was distinguished from *Badula* by the presence of stamens fused to form a tube surrounding the style, versus distinct anthers inserted simply onto the corolla (Jussieu, 1830). Such stamen characters have commonly been used to distinguish other genera of Myrsinoideae, such as *Amblyanthus* A.DC. versus *Amblyanthopsis* Mez, *Ctenardisia* Ducke versus *Yunckeria* Lundell and *Rapanea* Aubl. versus *Myrsine* L. (Ståhl & Anderberg, 2004).

Oncostemum was later delimited as containing *c*. 100 species endemic to Madagascar and the Comoros with various degrees of anther and/or filament fusion (Mez, 1902; Perrier de la Bâthie, 1952, 1953; Schatz, 2001). The most comprehensive and recent classification of this genus (Perrier de la Bâthie, 1953), however, is inadequate and largely obsolete, with many of the specimens collected in recent years irreconcilable with the descriptions and type material. In addition, the infrageneric ranks do not appear to reflect natural groups (L. Gautier, Conservatoire et Jardin Botaniques de la Ville de Geneve and P. B. Phillipson, Muséum national d'Histoire naturelle, Paris, pers. comm.).

As with other myrsinoid genera, the circumscription of *Badula* has changed several times (Table 1). For example, after describing many new species, De Candolle (1841) extended its distribution to include a broad area from Central and South America to the Philippines. All of these species were later treated as synonyms in other allied genera (e.g. *Ardisia* Sw., *Embelia* Burm.f and *Oncostemum*). Other changes have resulted from differing interpretations of the variable stamen characters. For example, those used to delimit *Oncostemum* from *Badula* not only vary among these genera, but have also been considered to be variable within *Badula* and, in some cases, even within individual species (Coode, 1976).

The current circumscription of *Badula* as endemic to the Mascarene Archipelago arose with its treatment for the *Flore des Mascareignes* by Coode (1981). Despite noting that *Badula* spp. share several characters (e.g. presence of thick stems often densely covered with leaf scars, leaves clustered towards the tips of branches and often red-tinged at the base and along the petioles, caducous and ciliate bracteoles subtending the pedicels and densely spotted or lined corolla lobes), Coode (1981) was unable to carry out a full species assessment of *Oncostemum* from Madagascar, and therefore could not establish whether any of these characters were diagnostic for *Badula*. For the purposes of the *Flore des Mascareignes*, all 11 *Badula* spp. and three *Oncostemum* spp. in the Mascarenes were treated as *Badula*, and the genus was circumscribed as a Mascarene endemic comprising 14 species (Coode, 1976, 1981; Table 1). Consequently, a new combination was published for *O. platyphyllum* (A.DC.) Mez, becoming *Badula platyphylla* (Coode, 1976), and *O. reticulatum* (A.DC.) Mez was listed as a synonym of *Badula reticulata* A.DC. (Coode, 1981). *Oncostemum latifolium* (Sieb.) Mez was treated as a synonym of *B. sieberi* A.DC. (Coode, 1981). In Madagascar, three *Badula* spp. were recognized by Perrier de la Bâthie (1952): *B. leandriana* H.Perrier, *B. pervilleana* H.Perrier and *B. richardiana* H.Perrier. These species are known only from their type specimens and were neither treated nor discussed by Coode (1981).

In the genus, several factors have caused taxonomic problems. The presence of intermediate forms on Réunion was noted among some of the more widespread *Badula* spp., where they grow sympatrically, particularly between *B. barthesia*, *B. borbonica* and *B. grammisticta* (Cordem.) Coode, making the delimitation of these species problematic (Coode, 1981). In Mauritius and Rodrigues, the combined effects of habitat loss, alien species invasion and predation by exotic fauna (e.g. Strahm, 1996; Cheke & Hume, 2008; Thébaud *et al*., 2009; Caujapé-Castells *et al*., 2010) have undoubtedly reduced the population sizes of several *Badula* spp. (Coode, 1981) and, in many cases, have considerably reduced the likelihood of any natural regeneration. Today, most species from Rodrigues and Mauritius are known from less than ten wild plants that survive in relict patches of once more extensive native vegetation. The extreme rarity of these species has resulted in their representation by few specimens in herbaria or by repeated collections from the same individuals. Indeed, over-collecting is thought to have increased the rarity of some species (Florens, Baider & Bosser, 2008).

The cryptic nature of some characters on herbarium specimens of Myrsinoideae compounds the problem. Specimens often lack detailed notes describing plant habit, in many cases are sterile, incomplete or damaged, and species rarity has lowered the chances of better quality specimens becoming available. Thus, the examination of living material is essential for adequate description (e.g. Coode, 1981; Pipoly, 1981, 1982).

Table 1. A summarized taxonomic history of *Badula* in the Mascarene Archipelago

Author, year and geographical scope of taxonomic treatment	Changes to the geographical distribution of the genus *Badula*	*Badula* species of the Mascarene Archipelago recognized by each author (currently accepted names in bold)
Jussieu (1789) Global	Réunion	First description of the genus *Badula* [based on specimens of 'Bois Pintade' = ***Badula barthesia*** **(Lam.) A.DC**.]
De Candolle (1834) Global	Réunion and Mauritius	*B. angustifolia* = ***Embelia angustifolia*** **(A.DC) A.DC.** ***B. barthesia*** **(Lam.) A.DC.** (the type species of *Badula*) ***B. crassa*** **A.DC.** ***B. insularis*** **A.DC.** *B. micrantha* A.DC. = ***Embelia micrantha*** **(A.DC) A.DC.** ***B. ovalifolia*** **A.DC**. ***B. sieberi*** **A.DC.**
De Candolle (1841) Global	Pan-tropical	***B. borbonica*** **A.DC.** ***B. multiflora*** **A.DC.** ***B. reticulata*** **A.DC.**
Baker (1877) Local (flora of Mauritius and the Seychelles)	–	*Ardisia insularis* Baker = ***B. insularis*** **A.DC.** *Ardisia sieberi* Baker = ***B. sieberi*** **A.DC.**
Cordemoy (1895) Local (flora of Réunion)	–	*Icacorea barthesia* Cordem. = ***B. barthesia*** **(Lam.) A.DC**. *Icacorea borbonica* Cordem. = ***B. borbonica*** **A.DC.** *Icacorea crassa* Cordem. = ***B. crassa*** **A.DC.** *Icacorea decumbens* Cordem. = ***B. decumbens*** **(Cordem.) Coode** *Icacorea grammisticta* Cordem. = ***B. grammisticta*** **(Cordem.) Coode** *Icacorea insularis* Cordem. = ***B. insularis*** **A.DC.** *Icacorea ovalifolia* Cordem. = ***B. ovalifolia*** **A.DC.**
Mez (1902) Global	Mascarene Archipelago endemic (Mauritius, Rodrigues & Réunion)	***B. balfouriana*** **(O. Kuntze) Mez** ***B. barthesia*** **(Lam.) A.DC**. ***B. borbonica*** **A.DC.** *B. candolleana* Mez = ***B. barthesia*** **(Lam.) A.DC**. *B. commersoniana Mez* = ***B. sieberi*** **A.DC.** ***B. crassa*** **A.DC.** ***B. insularis*** **A.DC.** *B. maculata* Mez = ***B. grammisticta*** **(Cordem.) Coode** ***B. multiflora*** **A.DC.** ***B. ovalifolia*** **A.DC**. ***B. richeana*** **Mez** [not treated by Coode, 1981]
Coode (1981) Local (flora of the Mascarene Islands)	Mascarene Archipelago endemic (Mauritius, Rodrigues & Réunion)	***B. balfouriana*** **(O. Kuntze) Mez** ***B. barthesia*** **(Lam.) A.DC**. ***B. borbonica*** **A.DC.** ***B. crassa*** **A.DC.** ***B. decumbens*** **(Cordem.) Coode** ***B. fragilis*** **Bosser & Coode** ***B. grammisticta*** **(Cordem.) Coode** ***B. insularis*** **A.DC.** ***B. multiflora*** **A.DC.** ***B. nitida*** **(Coode) Coode** ***B. ovalifolia*** **A.DC**. ***B. platyphylla*** **(A.DC.) Coode** ***B. reticulata*** **A.DC.** ***B. sieberi*** **A.DC.**

Although several myrsinoid genera have been included in phylogenetic studies, they have been used only to resolve relationships among families (e.g. Anderberg & Ståhl, 1995; Anderberg, Ståhl & Källersjö, 1998; Källersjö, Bergqvist & Anderberg, 2000; Anderberg, Rydin & Källersjö, 2002) or as outgroups in studies of herbaceous members of Primulaceae, such as *Cyclamen* L. (Yesson, Toomey & Culham, 2009) and *Lysimachia* L. (Hao *et al*., 2004). As such, evolutionary relationships are largely unresolved throughout Myrsinoideae and, especially, among putative allied pairs of genera. Furthermore, taxonomic concepts among such allies have been reported as vague and unclear (Ståhl, 1996; Ståhl & Anderberg, 2004). Detailed phylogenetic studies of these putative myrsinoid allies would help to clarify the overall phylogeny and taxonomy of Myrsinoideae.

As a contribution towards this goal, we present here the first nuclear and plastid phylogenetic analysis of *Badula*. We use complete species sampling to: (1) test the monophyly of the genus in relation to its close relative *Oncostemum*; (2) investigate species delimitations as outlined in the most recent taxonomic treatment; and (3) assess its biogeographical history. This study is also among the first to address phylogenetic relationships in one of the 16 plant genera endemic to all three islands of the Mascarene Archipelago [with studies of *Hyophorbe* Gaertn. (Cuenca, Asmussen-Lange & Borchsenius, 2008) and *Trochetia* DC. (Le Pechon *et al*., 2009)].

MATERIAL AND METHODS

Ingroup sampling

All 16 *Badula* taxa recognized by Coode (1981), i.e. 14 species and two varieties, were sampled. Multiple accessions per taxon (≥ three) were collected throughout the Mascarene Archipelago (Fig. 1), which, in several cases, represents > 50% of the total population of wild plants. In all, 62 accessions of *Badula* were sampled. The three *Badula* spp. from Madagascar recognized by Perrier de la Bâthie (1952) could not be included in this study. These species are represented only by their type specimens in the Paris Herbarium and are poorly known. The types were, for many years, considered to be lost (Schatz, 2001; R. Bone, personal observation, 2008; P. B. Phillipson, MNHN Paris, personal communication), although the type specimens of *B. leandriana* and *B. pervilleana* have recently been relocated.

Specimen collection is dissuaded for the critically rare species, particularly of the fertile material that is essential for regeneration and for the monocaulous species, where the collection of more than a single leaf would require removal of the entire apex of the plant.

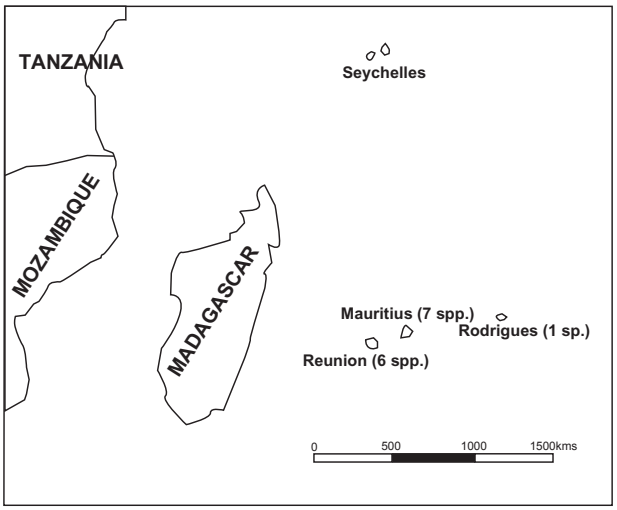

Figure 1. Map of the islands of the western Indian Ocean, showing the number of *Badula* (*sensu* Coode, 1981) species sampled from each of the Mascarene islands: Réunion, Mauritius and Rodrigues.

Vouchers were therefore collected as follows. In Rodrigues and Mauritius, herbarium vouchers were made when possible and deposited at the Mauritius Herbarium (MAU). Alternatively, detailed photographs of habit and plant parts were taken, mounted and labelled as a specimen and deposited in the Trinity College Dublin Herbarium (TCD). In Réunion, samples were collected from specific localities on the basis of detailed herbarium records, field observations and the taxonomic expertise of two of us (DS and CT). In these cases, existing specimens previously collected from the same populations (e.g. those of Cadet) served as vouchers for our samples (Table 2).

Outgroup sampling

J. S. Strijk *et al*. (unpubl. data) included internal transcribed spacer (ITS) sequences of *Badula* and *Oncostemum* in a phylogenetic analysis of several herbaceous-temperate and woody-tropical genera of Primulaceae, i.e. *Ardisia*, *Lysimachia* L., *Monoporus* A.DC., *Myrsine*, *Primula* L., *Rapanea* Aubl. and *Stylogyne* A.DC. In that analysis, the Malagasy endemic genus *Monoporus* was sister to a clade of *Ardisia*, *Badula* and *Oncostemum*. In the latter, *Badula* and *Oncostemum* formed a clade (J. S. Strijk *et al*., unpubl. data). Although the sister relationship of *Ardisia* with *Badula* and *Oncostemum* was not strongly supported (posterior probability, 0.4; J. S. Strijk *et al*., unpubl. data), the data are congruent with the results of previous phylogenetic analyses of several DNA markers that have provided evidence for the placement of *Ardisia* with *Oncostemum* (Källersjö

Table 2. Voucher specimens and EMBL-Bank accession numbers of the samples sequenced for internal transcribed spacer (ITS) and *trnS-trnG-trnG* regions

Taxon	Voucher	ITS	*trnS-trnG-trnG*
Ardisia elliptica (Thunb.)	R. Bone 26; MAU	HE590595	HE599703
Badula balfouriana (O.Kuntze) Mez	R. Bone 51; TCD	HE590596	HE599704
Badula balfouriana (O.Kuntze) Mez	R. Bone 52; TCD	HE590597	HE599705
Badula balfouriana (O.Kuntze) Mez	R. Bone 54; TCD	HE590598	HE599706
Badula balfouriana (O.Kuntze) Mez	R. Bone 55; TCD	HE590599	HE599707
Badula balfouriana (O.Kuntze) Mez	R. Bone 56; TCD	HE590600	HE599708
Badula barthesia (Lam.) A.DC.	01 (DNA Bank No. 01, cf. D. Strasberg & B. Warren 333; REU)	HE590601	HE599709
Badula barthesia (Lam.) A.DC.	26 (DNA bank No. 26)	HE590602	HE599710
Badula barthesia (Lam.) A.DC	D. Strasberg 71	HE590603	HE599711
Badula barthesia (Lam.) A.DC.	134 (DNA Bank No. 134, cf. Cadet 3303; REU)	HE590604	HE599712
Badula barthesia (Lam.) A.DC.	163 (DNA Bank No. 163, cf. Cadet 5574; REU	HE590605	–
Badula barthesia (Lam.) A. DC	265 (DNA Bank No. 265)	HE590606	HE599713
Badula barthesia (Lam.) A.DC.	D. Strasberg & B. Warren 314	HE590607	HE599714
Badula barthesia (Lam.) A.DC.	D. Strasberg & B. Warren 333	HE590608	HE599715
Badula cf. *barthesia* (Lam.) A.DC.	LR 569; REU	HE590609	HE599716
Badula borbonica A.DC. var. *borbonica*	cf. R. Bone 68; REU	HE590615	HE599722
Badula borbonica A.DC. var. *borbonica*	113 (DNA Bank No. 113)	HE590612	HE599719
Badula borbonica A.DC. var. *borbonica*	D. Strasberg 194; REU	HE590613	HE599720
Badula borbonica A.DC. var. *borbonica*	D. Strasberg *et al*. 406; REU	HE590610	HE599717
Badula borbonica A.DC. var. *borbonica*	LR 540; REU	HE590611	HE599718
Badula borbonica A.DC. var. *borbonica*	545 (DNA Bank No. 545)	HE590614	HE599721
B. borbonica var. *macrophylla* (Cordem.) Coode	J. Dupont *s.n*. (collected 12/04/2008); REU	HE590618	HE599725
B. borbonica var. *macrophylla* (Cordem.) Coode	D. Strasberg 99	HE590616	HE599723
B. borbonica var. *macrophylla* (Cordem.) Coode	LR 497; REU	HE590617	HE599724
Badula crassa A.DC	R. Bone 22; TCD	HE590619	HE599726
Badula crassa A.DC	R. Bone 24; TCD	HE590620	HE599727
Badula crassa A.DC	R. Bone 58; TCD	HE590621	HE599728
Badula decumbens (Cordem.) Coode	LR 471; REU	HE590622	HE599729
Badula decumbens (Cordem.) Coode	LR 472; REU	HE590623	HE599730
Badula fragilis Bosser & Coode	12 (DNA Bank No. 12)	HE590624	HE599731
Badula fragilis Bosser & Coode	46 (DNA Bank No. 46, cf. R. Bone 69; REU)	HE590625	HE599732
Badula fragilis Bosser & Coode	R. Bone 69; REU	HE590628	HE599735
Badula fragilis Bosser & Coode	98 (DNA Bank No. 98, cf. Cadet 4078; REU)	HE590626	HE599733
Badula fragilis Bosser & Coode	212 (DNA Bank No. 212, cf. R. Bone 70; REU)	HE590627	HE599734
Badula grammisticta (Cordem.) Coode	D. Strasberg 55	HE590632	HE599739
Badula grammisticta (Cordem.) Coode	69 (DNA Bank No. 69, cf. Cadet 6606; REU)	HE590629	HE599736
Badula grammisticta (Cordem.) Coode	101 (DNA Bank No. 101, cf. Cadet 6606; REU)	HE590630	HE599737
Badula grammisticta (Cordem.) Coode	119 (DNA Bank No. 119)	HE590631	HE599738
Badula insularis A.DC.	R. Bone 04; MAU	HE590633	HE599740
Badula insularis A.DC.	R. Bone 11; MAU	HE590634	HE599741
Badula insularis A.DC.	R. Bone 39; MAU	HE590635	HE599742
Badula multiflora A.DC.	R. Bone 13; MAU	HE590636	HE599743

Table 2. *Continued*

Taxon	Voucher	ITS	*trnS-trnG-trnG*
Badula multiflora A.DC.	R. Bone 17; MAU	HE590637	HE599744
Badula multiflora A.DC.	R. Bone 19; MAU	HE590638	HE599745
Badula aff. *nitida* (Coode) Coode	D. Strasberg 48; REU	HE590640	HE599747
Badula aff. *nitida* (Coode) Coode	D. Strasberg 50; REU	HE590641	HE599748
Badula nitida (Coode) Coode	75 (DNA Bank No. 75, cf. LR 514; REU)	HE590639	HE599746
Badula nitida (Coode) Coode	LR 514; REU	HE590642	HE599749
Badula nitida (Coode) Coode	LR 515; REU	HE590643	HE599750
Badula ovalifolia A.DC.	R. Bone 28; TCD	HE590644	HE599751
Badula ovalifolia A.DC.	R. Bone 29; TCD	HE590645	HE599752
Badula ovalifolia A.DC.	R. Bone 31; TCD	HE590646	HE599753
Badula platyphylla (A.DC.) Coode	R. Bone 02; TCD	HE590647	HE599754
Badula platyphylla (A.DC.) Coode	R. Bone 03; TCD	HE590648	HE599755
Badula platyphylla (A.DC.) Coode	R. Bone 38; TCD	HE590649	HE599756
Badula reticulata A.DC.	R. Bone 09; TCD	HE590650	HE599757
Badula reticulata A.DC.	R. Bone 10; TCD	HE590651	HE599758
Badula reticulata A.DC.	R. Bone 21; TCD	HE590652	HE599759
Badula reticulata A.DC.	R. Bone 72; TCD	HE590653	HE599760
Badula sieberi A.DC.	R. Bone 08; MAU	HE590654	HE599761
Badula sieberi A.DC.	R. Bone 12; MAU	HE590655	HE599762
Badula sieberi A.DC.	R. Bone 44; MAU	HE590656	HE599763
Badula sieberi A.DC.	R. Bone 49; MAU	HE590657	HE599764
Oncostemum acuminatum Mez	G. de Nevers 11611; CAS	HE590659	HE599765
Oncostemum ankifiense Mez	P. Fritsch 1504; CAS	HE590662	HE599768
Oncostemum ankifiense Mez	P. Fritsch 1696; CAS	HE590663	HE599769
Oncostemum cf. *denticulatum* H. Perrier	P. Fritsch 1601; CAS	HE590664	HE599770
Oncostemum cf. *denticulatum* H. Perrier	P. Fritsch 1610; CAS	HE590665	HE599771
Oncostemum elephantipes H. Perrier	G. de Nevers 11557; CAS	HE590667	HE599773
Oncostemum evonymoides Mez	F. Almeda 8088; CAS	HE590673	HE599779
Oncostemum evonymoides Mez	P. Fritsch 1628; CAS	HE590672	HE599778
Oncostemum forsythii Mez	F. Almeda 7913; CAS	HE590674	HE599780
Oncostemum forsythii Mez	P. Fritsch 1553; CAS	HE590676	HE599782
Oncostemum forsythii Mez	P. Fritsch 1640; CAS	HE590675	HE599781
Oncostemum gracile Mez	P. Fritsch 1736; CAS	HE590671	HE599777
Oncostemum cf. *neriifolium* Baker	P. Fritsch 1727; CAS	HE590661	HE599767
Oncostemum nervosum Baker	P. Fritsch 1505; CAS	HE590677	HE599783
Oncostemum ovato-acuminatum H.Perrier	P. Fritsch 1641; CAS	HE590670	HE599776
Oncostemum ovato-acuminatum H.Perrier	P. Fritsch 1669; CAS	HE590669	HE599775
Oncostemum pachybotrys Mez	P. Fritsch 1488; CAS	HE590660	HE599766
Oncostemum palmiformae H.Perrier	P. Fritsch 1697; CAS	HE590668	HE599774
Oncostemum seyrigii H.Perrier	P. Fritsch 1742; CAS	HE590666	HE599772
Oncostemum sp.	C. Thébaud 97; TL	HE590658	–
Monoporus sp.	F. Almeda 8208; CAS	HE590594	HE599702

In the column labelled 'Voucher', herbarium acronyms follow *Index Herbariorum*; numbers with the prefix LR are specimen numbers, rather than collector numbers at the University of Réunion Herbarium (REU); the abbreviation 'cf.' indicates the existing herbarium specimen, from the same population as our sample, that serves as a voucher. Where listed, DNA Bank numbers correspond to the DNA Bank, Laboratoire Evolution et Diversité Biologique, Unité Mixte de Recherches 5174 CNRS-Université de Toulouse, F31062, Toulouse, Cedex 9. France.

et al., 2000; Yesson *et al*., 2009). These relationships support the traditional view of *Oncostemum* as closely allied to *Badula* (e.g. Jussieu, 1830; Mez, 1902; Perrier de la Bâthie, 1952, 1953; Coode, 1981). We therefore selected a sample of *Ardisia elliptica* Thunb. (sampled in Mauritius where it is a naturalized exotic) and a sample of *Monoporus* from Madagascar, as members of the outgroup, with the latter used to root the tree.

To test the monophyly of *Badula*, sampling of *Oncostemum* was more extensive than that for the other outgroup genera. Samples were from field surveys of *Oncostemum* in and around Ranamofana National Park in east-central Madagascar. The 14 species samples (20 accessions) encompass a wide array of morphological variation in the genus and were selected from each of the major groups (groups I and II) set out in the classification of Perrier de la Bâthie (1952). Unambiguous identification of the vouchers associated with our samples was not possible in some cases because of problems with the current classification of *Oncostemum* (Perrier de la Bâthie, 1953).

Species names, voucher information and EMBL-Bank accession numbers for all sequences are provided in Table 2.

DNA extraction, amplification and sequencing

Total genomic DNA was extracted from silica-gel-dried leaf material with DNeasy® Plant Mini kits (Qiagen Inc., Valencia, CA, USA) by following the manufacturer's protocol, or using the cetyltrimethylammonium bromide (CTAB) method of Doyle & Doyle (1987) as modified by Hodkinson *et al*. (2007). The nuclear ribosomal ITS region (ITS1, the 5.8S ribosomal gene and ITS2) and a plastid region (the *trnS-trnG* intergenic spacer and the *trnG* intron; hereafter *trnS-trnG-trnG*) were sequenced. Primers for the ITS region were from White *et al*. (1990) and Nickrent, Schuette & Starr (1994), and primers for the plastid region were from Shaw *et al*. (2005). The internal primer *5′trnG2S* was modified from Lu *et al*. (2010) to improve the sequencing of the *trnS-trnG* intergenic spacer. Other plastid regions sequenced during a pilot study [*rpoB-trnC*, *trnH-psbA*, *trnS-fM*, *trnT-trnL*, *trnD-trnT*; based on plastid regions defined by Shaw *et al*., (2005)] either failed to amplify or amplified regions showing no or inadequate levels of sequence variation among the *Badula* spp. sampled.

DNA regions were amplified by polymerase chain reaction (PCR) using an amplification mixture that contained 2–4 μL of template DNA (approximately 50–100 ng μL^{-1}), 10 μL of buffer (5 ×; Promega, Southampton, UK), 1 μL of deoxynucleoside triphosphates (dNTPs) (10 mM), 3 μL of $MgCl_2$ (25 mM), 0.5 μL of each primer (25 μM), 0.25 μL *Taq* polymerase (5 U μL^{-1}) and ultrapure water to bring the total reaction volume to 50 μL. The amplification parameters for the ITS region were as follows: initial denaturation of 96 °C for 5 min, followed by 40 cycles (each of 94 °C for 30 s, 46 °C for 30 s and 72 °C for 1 min), with a final extension of 72 °C for 7 min. Amplification of the *trnS-trnG-trnG* region followed protocol 1 of Shaw *et al*. (2005), with the annealing temperature raised from 66 °C to 68 °C if double-banded products were amplified. Sequencing was carried out with Big Dye Terminator v 1.1 or v 3.1 cycle sequencing kits (Applied Biosystems) by following the manufacturer's protocols. Cycle sequencing reactions were cleaned with ethanol precipitation and then run on Applied Biosystems automated capillary sequencers (AB3100 or AB3130xl).

Phylogenetic analyses

The programme Sequencher v. 4.7 (Gene Codes Corp., Ann Arbor, MI, USA) was used to assemble complementary strands and to verify software base-calling. The nuclear and plastid sequences were manually aligned in the programme Se-Al v. 2.0a11 (Rambaut, 2002).

Phylogenetic relationships in the ingroup were evaluated with Bayesian inference and maximum parsimony (MP) methods. Although separate nuclear and plastid phylogenetic trees provided different levels of resolution, no statistically supported incongruent clades were recovered (data not shown) that would otherwise suggest contrasting evolutionary history for this region (Wendel & Doyle, 1998). Therefore, a total-evidence approach was applied and data were combined for further analysis.

A partitioned Bayesian analysis was performed as in Buerki *et al*. (2011) and implemented in MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003). Best-fit models for each region were selected using MrModeltest v.2.3 (Nylander, 2004) based on the Akaike Information Criterion (Akaike, 1974). The Hasegawa Kishino and Yano model with a gamma distribution (HKY + G) was the best-fit model for the ITS region, and the general-time-reversible model with a proportion of invariant sites (GTR + I) was the best-fit model for the *trnS-trnG-trnG* region. Two Metropolis-coupled Markov chains (MCMCs) with an incremental heating temperature of 0.2 were run for 10 million generations and sampled every 100th generation. The analysis was repeated twice, starting from random trees. Convergence was accepted when the standard deviations of attained values fell below 0.1 and when the potential scale reduction factor index (Gelman & Rubin, 1992) approached 1.0. We considered the MCMC sampling to be sufficient when the

effective sample size was greater than 200 (checked on Tracer v.1.4; Rambaut & Drummond, 2007). After a burn-in period of one million generations, a half-compatible consensus tree with its associated Bayesian posterior probabilities (BPPs) was reconstructed in MrBayes v.3.1.2 (Ronquist & Huelsenbeck, 2003).

The parsimony ratchet (Nixon, 1999) was performed with PAUPrat (Sikes & Lewis, 2001) for the MP analysis. Ten independent searches were performed with 200 iterations and 15% of the potentially parsimony-informative characters perturbed. A strict consensus tree was constructed from the shortest equally parsimonious trees. To assess the support at each node, nonparametric bootstrap analyses (Felsenstein, 1985) were performed using PAUP* version 4.0b10 (Swofford, 2002) with 400 replicates and applying tree bisection–reconnection (TBR) branch swapping, simple sequence addition and MULTREES, and including 10 trees per replicate.

Hypothesis testing

To examine the relationship between *Badula* and *Oncostemum* further and to perform tests of certain alternative topologies, constrained partitioned Bayesian analyses were performed. The analyses were conducted with the same parameters as those in the unconstrained partitioned Bayesian analysis (see above). The Shimodaira–Hasegawa test (SH test; Shimodaira & Hasegawa, 1999) was employed to determine whether the half-compatible consensus trees resulting from the constrained partitioned Bayesian analyses were statistically worse than the unconstrained topology. The SH test was carried out in PAUP* (Swofford, 2002) using the RELL method with 10 000 bootstrap replicates. Because only one set of model parameters can be implemented in a single analysis in PAUP*, the partitioned data were combined. The best-fit model for the combined dataset was estimated to be GTR + G + I with MrModeltest v2.3 (Nylander, 2004), and these estimated model parameters were used to set the SH tests.

RESULTS

The ITS sequences for the *Badula* taxa were either 570 or 572 bp in length. Sequence lengths for the *trnS-trnG-trnG* region for *Badula* were more variable and ranged from 1464 to 1536 bp, with an average length of 1513 bp across all *Badula* taxa. The combined aligned dataset contained 2404 characters (aligned lengths were 680 bp for the ITS region and 1724 bp for the *trnS-trnG-trnG* region). The ITS region provided more parsimony-informative sites (73) than did *trnS-trnG-trnG* (22).

The strict consensus tree was based on 2010 most parsimonious trees generated by PAUPrat (length, 279 steps; consistency index, 0.81; retention index, 0.91). Both MP and Bayesian inference methods provided highly congruent topologies, and only the Bayesian half-compatible consensus tree is presented and discussed hereafter because it contains the maximum amount of phylogenetic information (Fig. 2).

Badula was resolved as monophyletic with strong support (1.00 BPP; Fig. 2). *Badula* (Clade B) and *Oncostemum* Clade O2 (0.94 BPP) form a clade that is sister to *Oncostemum* clade O1. The two *Oncostemum* clades each comprise a mixture of taxa from the two major infrageneric groups of the classification (groups I and II, Fig. 2; Perrier de la Bâthie, 1952, 1953). In *Badula*, a clade of five accessions of *B. balfouriana* (0.83 BPP; Clade B1) was recovered as sister to a large clade that contained all other *Badula* taxa (0.9 BPP; Clade B2, Fig. 2). The latter was subdivided into a trichotomy: Clade B3, comprising three accessions of *B. multiflora* (1.0 BPP), Clade B4, comprising all other species from Mauritius (0.86 BPP) and Clade B5, comprising all taxa from Réunion (0.78 BPP; Fig. 2). The branches of several species in Clade B4 were longer than those of several taxa in Clade B5. The monophyly of several taxa was moderately to strongly supported, e.g. *B. ovalifolia* (1.0 BPP), *B. nitida* (0.85 BPP) and *B. sieberi* (0.99 BPP; Fig. 2, Table 3). Others were resolved as paraphyletic, including *B. reticulata* (1.00 BPP) and several taxa in Clade B5. Taxa such as *B. crassa*, *B. decumbens* and *B. fragilis* formed polytomies in Clades B4 and B5, probably because there were insufficient characters to provide resolution of all accessions. The outlying positions of accessions in *B. reticulata* (RB72) and *B. sieberi* (RB12) imply a higher genetic diversity within these taxa than between them and other taxa for the markers sequenced.

The SH test result comparing the topology with one that forced the monophyly of *Oncostemum* was not significant (Table 4). Test results comparing the topology with two alternative forced topologies that constrained *B. multiflora* with either Réunion or Mauritius taxa were also not significant (Table 4).

DISCUSSION

Monophyly of *Badula* and the relationship with *Oncostemum*

This study represents the first estimate of phylogenetic relationships in any sizeable group of myrsinoid Primulaceae. Both MP and Bayesian inference analyses strongly supported the monophyly of *Badula sensu* Coode (1981). This study therefore supports the circumscription of the genus in the most recent taxo-

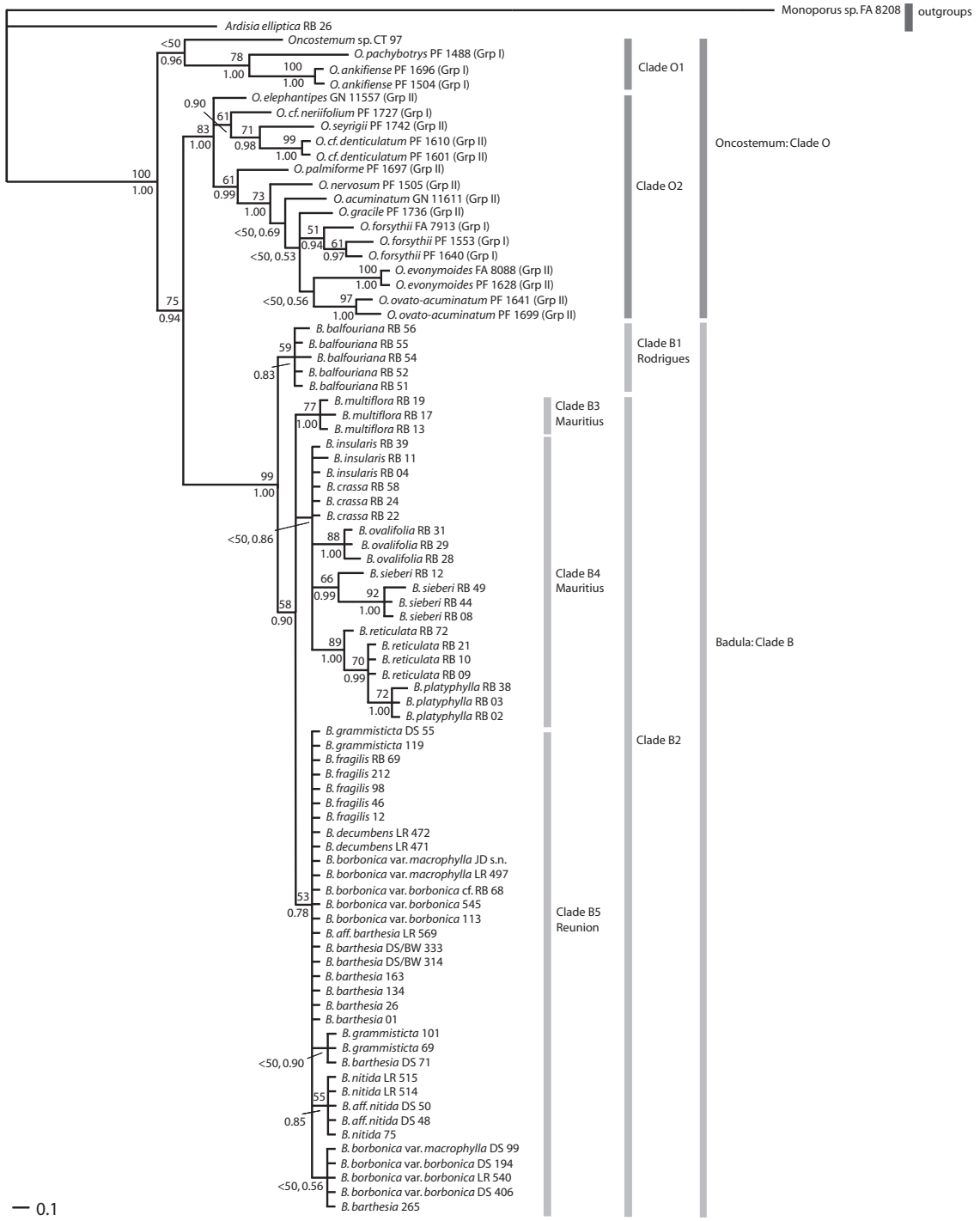

Figure 2. The Bayesian half-compatible consensus tree based on the combined data. Values below the branches are the Bayesian posterior probability (BPP) support values. Values above the branches indicate bootstrap support (where space is limited, bootstrap values precede BPP, and are on the same line). Vertical bars indicate clades referred to in the text.

nomic account for the *Flore des Mascareignes* (Coode, 1981). Our study suggests that *Oncostemum* is paraphyletic and indicates that the two major infrageneric groups (groups I and II; Perrier de la Bâthie, 1952, 1953) are not monophyletic. These results are consistent with the complex taxonomic history and high species richness of this genus (> 100 species with many new species to be described; Schatz, 2001), and

Table 3. Summary of the phylogenetic status of each *Badula* taxon, as revealed by this study

Badula taxa (*sensu* Coode, 1981)	Clade	Distribution	Phylogenetic status (support BPP)
Badula balfouriana	B1	Rodrigues	Monophyletic (0.83)
Badula multiflora	B3	Mauritius	Monophyletic (1.00)
Badula crassa	B4	Mauritius	Unresolved (< 0.5)
Badula insularis	B4	Mauritius	Unresolved (< 0.5)
Badula ovalifolia	B4	Mauritius	Monophyletic (1.00)
Badula platyphylla	B4	Mauritius	Monophyletic (1.00)
Badula reticulata	B4	Mauritius	Paraphyletic (1.0)
Badula sieberi	B4	Mauritius	Monophyletic (0.99)
Badula barthesia	B5	Réunion	Unresolved (< 0.6)*
Badula borbonica var. *borbonica*	B5	Réunion	Unresolved (< 0.6)
Badula borbonica var. *macrophylla*	B5	Réunion	Unresolved (< 0.6)
Badula decumbens	B5	Réunion	Unresolved (< 0.5)
Badula fragilis	B5	Réunion	Unresolved (< 0.5)
Badula grammisticta	B5	Réunion	Unresolved (< 0.5)*
Badula nitida	B5	Réunion	Monophyletic (0.85)

*One accession of *B. barthesia* and two accessions of *B. grammisticta* formed a clade with moderate support (0.9 BPP), but a lack of resolution for other accessions of these taxa prevents inferences being made about the relationship between them.

Table 4. Results of the Shimodaira–Hasegawa (SH) tests for the half-compatible Bayesian consensus tree

Hypothesis 1 (H1)	Hypothesis 2 (H2)	–ln L H1	–ln L H2	Diff in –ln L	*P* value
Unconstrained	Constrained MAU	5074.41713	**5074.41149**	0.00564	0.3541
Unconstrained	Constrained REU	5074.41713	**5074.41149**	0.00564	0.3541
Unconstrained	Constrained Onco	**5074.41713**	5076.05087	1.63374	0.2106
Constrained MAU	Constrained REU	5074.41149	5074.41149	0.00000	0.0585

The best hypothesis is given in bold type.
An example of the 'Unconstrained' topology is shown in Figure 2. 'Constrained MAU ' and 'Constrained REU' refer to the two alternative hypotheses in which *Badula multiflora* was forced into a clade with all other *Badula* taxa from Mauritius or from Réunion, respectively. 'Constrained Onco' refers to the constraint placed on *Oncostemum* to force the monophyly of this genus.

indicate that much taxonomic work remains before a comprehensive understanding of *Oncostemum* can be attained.

When *Oncostemum* was first described by Jussieu (1830), the presence of fused filaments was used to distinguish it from *Badula*. This character was variously modified by successive workers to accommodate filaments that were partly fused in *Oncostemum* (Mez, 1902; Perrier de la Bâthie, 1952), such as those seen in *O. platyphyllum* and *O. reticulatum*. These taxa were subsequently included in *Badula* (Coode, 1976, 1981), and our molecular data support this treatment, as these two species are embedded in a well-supported clade among other *Badula* taxa from Mauritius. This highlights the inadequacy of the stamen characters traditionally used to distinguish these genera. The molecular data presented here are suitable as a framework for further investigation into the phylogenetics and evolution of *Oncostemum*, with or without the inclusion of *Badula*.

Support for species delimitations within *Badula*

Several species of *Badula* were resolved as monophyletic, particularly those from Rodrigues (*B. balfouriana*) and Mauritius. For example, all accessions of *B. ovalifolia* form a clade with strong support (1.0 BPP). Two other species clades with strong support are *B. sieberi* and *B. multiflora.*

Strong support was also found for the monophyly of *B. platyphylla*. This clade, however, is embedded within *B. reticulata*, thus rendering the latter paraphyletic. *Badula platyphylla* was first described as a variety of *B. reticulata* (*B. reticulata* var. *platyphylla* A.DC.). It was later transferred to *Oncostemum* by

Mez (1902), and was subsequently treated as *Badula* by Coode (1976), who maintained this specific rank. In the *Flore des Mascareignes*, *B. platyphylla* is distinguished from *B. reticulata* by wider, thicker leaves with reticulate venation, smaller lobes of the calyx and corolla, and a shorter inflorescence (Coode, 1981). These species grow in different, but adjacent, habitats, with *B. platyphylla* on lateritic heathland and *B. reticulata* restricted to mesic forest. From the description by Coode (1981), *B. platyphylla* could be interpreted as a xerophytic form of *B. reticulata*. Both species are critically endangered and of high conservation priority in Mauritius. For a decision to be reached on an appropriate taxonomic rank for *B. platyphylla*, increased sampling of *B. reticulata* should be included in future phylogenetic analysis and the morphology of these taxa should also be investigated in more detail. For example, fruiting material was not seen by Coode (1976, 1981; when this species was considered to be extinct) and the morphology of the fruits remains unknown. Moreover, no detailed micromorphological or anatomical studies have been carried out for this species or *B. reticulata*. As a result of the rarity of both species, this work would require the establishment of living collections to provide suitable material for study, whilst also securing an *ex situ* conservation resource.

Intermediate morphological forms have been noted among several taxa from Réunion (Coode, 1981), and relationships among these taxa remain largely unresolved through the lack of sequence variation. Among the resolved regions of the topology involving species from Réunion is the grouping of *B. fragilis* with other taxa from Réunion. The description of this species was first published with some reluctance because information was lacking at the time regarding the morphologically similar Mauritian species *B. crassa* (Coode, 1979), then thought to be extinct. Although accessions representing these two species do not each form clades, they are segregated along geographical lines, with accessions of *B. crassa* grouping with other taxa from Mauritius, and *B. fragilis* grouping with other taxa from Réunion. Our phylogenetic analysis thus provides a measure of support for the distinction of these species from one another.

Biogeographical patterns in *Badula*

From our data, a single colonization event to the Mascarene Archipelago can be inferred in *Badula* from Madagascar. This scenario is based on the acceptance of the paraphyly of *Oncostemum*, however, and further investigation of this relationship is required before the origins of *Badula* and *Oncostemum* can be assessed with confidence. For example, if evidence was found for a monophyletic *Oncostemum*, a Mascarene origin for the *Oncostemum–Badula* clade could also be hypothesized.

Within the *Badula* clade, the genus is composed predominantly of taxa that appear to be single-island endemics, and 13 of the 14 *Badula* spp. recognized by Coode (1981) are segregated into clades that reflect their distribution throughout the archipelago. The lack of phylogenetic resolution in our results precludes an assessment of the number and direction of colonization scenarios within the archipelago; however, the high clustering of intra-island endemics suggests that ancestral *Badula* became established on each of the Mascarene islands through few colonization events, followed by intra-island speciation. Rodrigues, the easternmost of the islands, has been dated to *c.* 10 Myr (Giorgi & Borchellini, 1998, in Cheke & Hume, 2008), Mauritius, centrally located, to 8–10 Myr (McDougall & Chamalaun, 1969), and Réunion, the westernmost of the islands, to 2.1 Myr (McDougall, 1971); the latter is the only island that is volcanically active. Although it has been shown that Mascarene endemic taxa can be older than the islands on which they grow (Renner, 2004; Cuenca *et al.*, 2008; Renner *et al.*, 2010), the general pattern revealed by our data of more phylogenetic resolution on the two older islands than on the youngest island suggests that island age has played a significant role in *Badula* speciation. *Badula multiflora* from Mauritius is the notable exception in the formation of these geographical clades. Despite explicit hypothesis testing, this species was not reconciled with a clear geographical pattern and its position requires further investigation.

Altitudinal variation and the availability of suitable habitat may also have played a part in *Badula* speciation. Réunion has the most varied topography and largest altitudinal range of the Mascarene islands, reaching 3069 m asl (the highest point in the Indian Ocean; Thébaud *et al.*, 2009), whereas mountain ranges in Mauritius and Rodrigues do not exceed 828 and 393 m asl, respectively. Furthermore, Réunion has retained a comparatively large amount of pristine native vegetation (30%; Strasberg *et al.*, 2005), particularly at higher elevations, where four of the 19 habitat types are > 80% intact (Strasberg *et al.*, 2005). On Mauritius, < 2% of the remaining fragments of native vegetation are considered to be rich in native species (Atkinson & Sevathian, 2005) and, on Rodrigues, no primary vegetation is intact (Strahm, 1996).

A lack of morphological differentiation among taxa from Réunion has been attributed to hybridization, where species grow sympatrically (Coode, 1981). The results of this study indicate a lack of sequence divergence among many of these taxa, which could have

resulted from hybridization or, alternatively, recent or incomplete speciation on this geologically young and topographically diverse island. Any potentially evolving *Badula* lineages may not only be affected by the differing geological histories of the Mascarene islands, but also by the various levels of habitat destruction and 'ecological ruin' among the islands (Cheke & Hume, 2008), compounding differences in the availability of habitats that can be occupied by this genus.

ACKNOWLEDGEMENTS

The authors thank the Mauritius Sugar Industry Research Institute (MSIRI) for logistical support; the National Parks and Conservation Service (NPCS; Mauritius) for collecting permits; Laurent Gautier (Conservatoire et Jardin Botaniques de la Ville de Gèneve) and Régine Verlaque (Laboratoire de Botanique, Université de Provence, Marseille) for access to herbarium specimens; the following people for field assistance: Claudia Baider and Kersley Pynee (the Mauritius Herbarium, MSIRI); Jean-Claude Sevathian and Gabriel D'Argent (the Mauritian Wildlife Foundation); Mario Allett (NPCS); Vincent Florens (University of Mauritius); Richard Payandee; and, for assistance with climbing le Morne Mountain, Frederic Robert and his team (Incentive Partners Ltd) and Yan de Maroussem (YanNature); Laurent Vencatachellum for accommodation for REB during fieldwork in Mauritius; Boni Cruz (California Academy of Sciences, CAS) for help and guidance with molecular work; and two anonymous reviewers for their helpful comments on the manuscript. Part of the laboratory work was carried out in the Center for Comparative Genomics at CAS. This research was supported by a Trinity College Dublin School of Natural Sciences Studentship (Ireland) and the Eden Foundation (UK) (to REB), the EU Marie Curie Early Stage Research Training HOTSPOTS MEST-2005–020561 (to JSS) and the BIOTAS project funded by the Agence Nationale de la Recherche (to CT and DS).

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