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Maintenance of species integrity in the context of a recent radiation: the case of *Jamesbrittenia* (Scrophulariaceae: Limoselleae) in southern Africa

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Incomplete post-zygotic isolation poses challenges for the maintenance of species integrity in recently radiated lineages. An example is *Jamesbrittenia*, a southern African-centred genus, the species of which cross readily to produce viable offspring. We develop a dated phylogenetic hypothesis for *Jamesbrittenia* and used this to assess the evidence for recent radiation and to evaluate the roles of geography, relatedness and floral divergence in determining the incidence of wild hybridization. Phylogenetic inference is based on nuclear (*GScp*) and plastid (*rps16*, *psb–trnH*) loci, but uses morphological evidence to resolve instances of supported incongruence. Our data reveal four ecologically and biogeographically differentiated lineages in *Jamesbrittenia*. One of these, a widespread and predominantly shrubby lineage, reflects accelerated diversification, potentially triggered by environmental change, starting in the late Miocene epoch. In the widespread clade, strong range exclusivity indicates an important role for geography in maintaining species identity. Among species with overlapping ranges, however, differentiation in floral form is a powerful predictor of wild hybridization. The apparent importance of geography in maintaining species integrity in recently diverged lineages, like the widespread clade of *Jamesbrittenia*, needs to be considered when species are translocated, whether such translocation is horticulturally motivated or forms part of an 'assisted migration' exercise. © 2016 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2016, **182**, 115–139

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INTRODUCTION

Post-zygotic isolation facilitates the maintenance of species integrity in the face of secondary contact and its emergence represents a crucial phase in the process of speciation. As the strength of post-zygotic isolation between species is generally correlated with genetic divergence (e.g. Sasa, Chippindale & Johnson, 1998; Moyle, Olson & Tiffin, 2004; Bolnick & Near, 2005; Singhal & Moritz, 2013) and, by extension, divergence time, recently diverged species are more likely to be capable of interbreeding than are more anciently diverged species. Consequently, reproductive compatibility between species is a common feature of rapid, recent radiations (Wiens, Engstrom & Chippendale, 2006; Ackermann, Achatz & Weigend, 2008), with recently radiated species relying more heavily on geography and ethology to maintain their identities.

The roots of the modern flora of southern Africa are ancient, but much of its contemporary species richness is the product of recent radiation, possibly

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prompted by geomorphic and associated climatic events since the late Miocene epoch (Linder & Verboom, 2015; Neumann & Bamford, 2015). Of principal importance is an episode of significant tectonic uplift along the south-eastern margin of the subcontinent around the Miocene–Pliocene boundary (3–5 Mya: Partridge & Maud, 2000). Besides prompting major landscape rejuvenation and associated habitat diversification along the eastern escarpment, this event probably gave rise to the modern alpine zone of the Drakensberg and exaggerated the east–west aridity gradient in southern Africa (Linder, 2014; Linder & Verboom, 2015; Neumann & Bamford, 2015), producing accentuated aridity along the west coast and a semi-arid interior. In addition, recent uplift has been implicated in the appearance of greater substratum diversity in the Cape Floristic Region (Cowling, Procheş & Partridge, 2009).

Given the broad scale of these impacts, we expect Miocene–Pliocene uplift and potentially other major environmental transitions to have left their signature on the southern African biota, specifically by prompting the contraction of some lineages and stimulating the ecological expansion and radiation of others. Along the scarp edge, for example, the creation of novel alpine habitats paired with increased topographic complexity (Bentley, Verboom & Bergh, 2014) may explain the diversification of plant lineages that currently frequent these environments. Examples include the S2 clade of *Sebaea* Sol. ex R.Br. (Gentianaceae; Kissling *et al.*, 2009), *Zaluzianskya* F.W. Schmidt section *Nycterinia* (D. Don) Hilliard (Scrophulariaceae; Archibald, Mort & Wolfe, 2005), the southern African summer-rainfall-clade of *Gladiolus* L. (Iridaceae; Valente *et al.*, 2011), the *Hypoxis* L. clade (Hypoxidaceae; Kocyan *et al.*, 2011), *Macowania* Oliv. (Asteraceae; Bentley *et al.*, 2014), *Melianthus* L. (Melianthaceae; Linder *et al.*, 2006), *Nemesia* Vent. (Scrophulariaceae; Datson, Murray & Steiner, 2008) and *Kniphofia* Moench. (Asphodelaceae; Ramdhani, Barker & Baijnath, 2009). Importantly, as an early Pliocene origin would identify these radiations as being similar in age to those triggered by Andean uplift (Hughes & Atchison, 2015), the weak post-zygotic isolation, which is a common feature of Andean plant lineages (e.g. Smith & Baum, 2007; Ackermann *et al.*, 2008), should be a feature of this system also.

Here we examine factors that underpin the genesis and maintenance of taxonomic diversity in *Jamesbrittenia* Kuntze (Scrophulariaceae; Limoselleae), a species-rich (84 species) and florally heterogeneous genus having its centre of diversity in southern Africa. The distribution of high concentrations of species along the margins of the central South African escarpment identifies *Jamesbrittenia* diversity as a likely product of recent, uplift-triggered radiation. Consistent with this idea, post-zygotic isolation is weakly developed in *Jamesbrittenia*, with interspecific hand-crosses typically yielding copious viable seed. On the basis of extensive crossing trials geared towards the development of horticulturally marketable hybrids, Adam Harrower (horticulturist, South African National Biodiversity Institute; pers. comm.) writes, "We have found almost all species to hybridize with other species except *J. grandiflora* and *J. macrantha* (which cross with each other but nothing else!). We have also found that F1s seem to cross fairly well to produce viable F2 seed... . We are only just starting with crossing the F2s and are finding them to cross also. Seemingly there are very few genetic breeding barriers. The only wild barriers seem to be flower shape and pollinator." In this context, the numerous instances of wild interspecific hybridization reported by Hilliard (1994) is unsurprising, and she argues that "the distinctions between a good many species of Manuleae (≈ Limoselleae) appear to have been blurred by hybridization (introgression) in areas of sympatry", particularly in *Jamesbrittenia*, *Manulea* L., *Polycarena* Benth. and *Sutera* Roth.

The specific objective of this paper, therefore, is to evaluate the relative importance of geography, phylogenetic relatedness and floral form in limiting hybridization and thus in maintaining species integrity, in a recently radiated clade of *Jamesbrittenia*. To do this, we first develop a molecular phylogenetic hypothesis for the genus and provide context by exploring the diversification history of the group, testing the hypothesis that its radiation was triggered by environmental events taking place near the Miocene–Pliocene boundary. Given the generally localized distributions of *Jamesbrittenia* spp., we predict an important role for geography in keeping species distinct. Among species with range overlap, however, we predict a key role for floral divergence. Strong divergence in floral morphology has been shown to confer complete or partial isolation (floral isolation) between several recently diverged pairs of sister species (e.g. Fulton & Hodges, 1999; Ramsey, Bradshaw & Schemske, 2003; Kay, 2006), but the broader importance and role of floral isolation in speciation remains contentious (Kay & Sargent, 2009). Further, we know of few studies that explore its effectiveness as an isolating mechanism at a genus-wide scale.

Phylogenetic inference in *Jamesbrittenia* is complicated by the presence of supported gene tree incongruence. Although much effort has been invested in the development of 'species tree' methods, which address gene tree heterogeneity explicitly within a model-based framework (e.g. Liu & Pearl, 2007; Liu, 2008; Liu *et al.*, 2009; Heled & Drummond, 2010;

Liu, Yu & Edwards, 2010), the coalescent basis of these methods renders them inappropriate for addressing incongruence resulting from hybrid-mediated lateral gene transfer (Knowles, 2009; Leaché *et al.*, 2014). As supported incongruence in *Jamesbrittenia* is plausibly, perhaps even probably, a product of hybridization (see Discussion) and because the accuracy of species tree methods depends on more independently segregating loci being sampled than are available to us (Takahata, 1989; Maddison & Knowles, 2006; McCormack, Huang & Knowles, 2009), we employ an alternative approach to address incongruence. We adopt what is essentially a concatenation approach, but one that first identifies the taxa responsible for incongruence and then assesses, for each of these, which of the contradictory loci yields a placement that is most consistent with a broader genomic signal, as embodied by an independent morphological data set. For these taxa, the sequences of loci that suggest alternative, sub-optimal, placements are excluded from the data matrix prior to a combined analysis of the data.

We argue that, as an integrated product of the entire genome, morphology is an appropriate arbiter of incongruence between individual genes where the broader objective is to estimate a tree reflecting genome-wide character variation. Although the utility of morphology in phylogenetic inference undoubtedly has its limitations (e.g. Givnish & Sytsma, 1997; Scotland, Olmstead & Bennett, 2003), as an integrated product of genome-wide sequence variation morphology is less prone to be misled by the specific processes (e.g. incomplete lineages sorting, hybridization) that are most likely to mislead single-locus phylogenetic inference (Doyle, 1992; Hillis & Wiens, 2000). Although our study is the first to use morphology formally to choose between alternative placements of conflict taxa, we note that the use of morphology to choose between alternative molecular phylogenetic hypotheses or to argue for one placement of a species over another is not unprecedented (e.g. Järvinen *et al.*, 2004; Giribet & Edgecombe, 2006; Barber *et al.*, 2007; Linder *et al.*, 2010).

MATERIAL AND METHODS

Phylogenetic Taxon Sampling

To estimate phylogenetic relationships in *Jamesbrittenia*, we sampled 68 of the 84 currently recognized species (Table 1). Except for *J. canescens* (Benth.) Hilliard, for which we included accessions representing vars. *canescens*, *laevior* (Dinter) Hilliard and *seineri* (Pilg.) Hilliard, all species were represented by single accessions. In addition, we included an accession of a putative new species, collected from the plateau of the Erongo Mountains (*G. A. Verboom 1108*). An accession of *Teedia pubescens* Burch. was included as outgroup.

DNA Methods

Total DNAs were extracted by pulverizing ~40 mg of silica-dried leaf material in liquid nitrogen, followed by CTAB isolation (Doyle & Doyle, 1987). Three loci were sampled using PCR, two from the plastid genome and one from the nuclear genome. Marker choice was dictated primarily by the presence of appropriate variability and ease of amplification and sequencing. The two plastid markers (*rps16* intron, *psbA-trnH* intergenic spacer region) were amplified using primers rpsF and rpsR2 (Oxelman, Liden & Bergland, 1997) and psbAF and trnHR (Sang, Crawford & Stuessy, 1997), respectively. In choosing a nuclear marker, the glutamine synthetase locus (*GScp*; Emshwiller & Doyle, 1999) was preferred over the more widely used internal and external transcribed spacer regions (ITS, ETS) because the complex and unpredictable evolutionary behaviour of the latter compromises their phylogenetic utility (Álvarez & Wendel, 2003) and efforts to sequence them suggested the presence of multiple copies. As the standard *GScp* primers (Emshwiller & Doyle, 1999) were effective in amplifying only a few *Jamesbrittenia* spp. and *T. pubescens*, we designed three *Jamesbrittenia*-specific primers (GS38F: 5′-TGA GCC (C/T)TT CTT GTT TCG TG-3′; GS681R: 5′-AGC TTG TTC TGT TAT TCT CTG-3′; and GS784R: 5′-ATA CTT GTT A(A/G)T GAT TTT GCC-3′) which proved effective for most *Jamesbrittenia* spp.

PCR mixes were prepared on ice in 50 μL volumes, each comprising 33.5 μL sterile water, 5.0 μL SuperTherm 10× DNA polymerase buffer (Bioline, London, UK), 5.0 μL $MgCl_2$ (50 mM), 1.0 μL each primer (10 μM), 2.0 μL dNTP (10 mM), 2.5 units SuperTherm *Taq* DNA polymerase (5 units μL^{-1}; Bioline, London, UK) and 2.0 μL template DNA (or an aqueous dilution thereof). For *GScp* amplification, 0.6 μL of dimethyl sulphoxide was added to the reaction mix (in place of 0.6 μL water), to facilitate primer annealing (Buckler, Ippolito & Holtsford, 1997). Reactions were run on an Applied Biosystems GeneAmp 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA) as follows: initial denaturation of 2 min at 94 °C; 30 cycles of 60 sec at 94 °C, 60 sec at 52 °C and 2 min at 72 °C; final extension of 7 min at 72 °C. Amplification products were checked on 1% agarose, cleaned using a Qiaquick PCR purification kit (Qiagen GmBH, Hilden, Germany) and cycle sequenced using the same primers as used for PCR. Cycle sequencing products were visualized on an ABI 3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Table 1. Taxon and marker sampling, indicating voucher specimens (deposited at BOL), collection localities and GenBank accession numbers for sampled sequences

Taxon	Voucher	Collection locality	*GScp*	*psbA-trnH*	*rps16*
Diascia longicornis (Thunb.) Druce	*G.A. Verboom 888*	South Africa: Nieuwoudtville		KX057182	KX057263
Hemimeris racemosa (Houtt.) Merr.	*G.A. Verboom 803*	South Africa: Klawer		KX057181	KX057262
Lyperia violacea (Jarosz) Benth.	*M. L. Herron 32*	South Africa: George		KX057183	KX057264
Lyperia tristis (L.f.) Benth.	*G.A. Verboom 875*	South Africa: Kamieskroon		KX057186	KX057267
Manulea schaeferi Pilg.	*G.A. Verboom 822*	Namibia: Klein Karas		KX057189	KX057270
Manulea adenocalyx Hilliard	*G.A. Verboom 800*	South Africa: Klawer		KX057190	KX057271
Oftia africana (L.) Bocq.	*M. L. Herron 40*	South Africa: Cape Peninsula		KX057185	KX057266
Sutera hispida (Thunb.) Druce	*M. L. Herron 37*	South Africa: Cape Peninsula		KX057187	KX057268
Sutera subsessilis Hilliard	*M. L. Herron 33*	South Africa: Koue Bokkeveld		KX057188	KX057269
Teedia pubescens Burch.	*M. L. Herron 34*	South Africa: Koue Bokkeveld	KX057117	KX057184	KX057265
Jamesbrittenia accrescens (Hiern) Hilliard	*G.A. Verboom 1057*	South Africa: Lydenburg	KX057172	KX057253	
Jamesbrittenia acutiloba (Pilg.) Hilliard	*G.A. Verboom 1132*	Namibia: Waterberg	KX057147	KX057220	KX057301
Jamesbrittenia adpressa (Dinter) Hilliard	*G.A. Verboom 829*	Namibia: Seeheim	KX057149	KX057222	KX057303
Jamesbrittenia albanensis Hilliard	*G.A. Verboom 1008*	South Africa: Grahamstown	KX057160	KX057233	KX057314
Jamesbrittenia albiflora (Verdoorn) Hilliard	*G.A. Verboom 1066*	South Africa: Jagersfontein	KX057164	KX057237	KX057318
Jamesbrittenia albomarginata Hilliard	*M. L. Herron 36*	South Africa: Pearly Beach		KX057252	KX057333
Jamesbrittenia amplexicaulis (Benth.) Hilliard	*G.A. Verboom 870*	South Africa: Okiep	KX057120	KX057193	KX057274
Jamesbrittenia argentea (L.f.) Hilliard	*M. L. Herron 50*	South Africa: George		KX057247	KX057328
Jamesbrittenia aridicola Hilliard	*G.A. Verboom 806*	South Africa: Aggeneys	KX057119	KX057192	KX057273
Jamesbrittenia aspalathoides (Benth.) Hilliard	*A. Harrower 1695.*	Cultivation: NBG, Kirstenbosch	KX057157	KX057230	KX057311
Jamesbrittenia aspleniifolia Hilliard	*G.A. Verboom 1018*	South Africa: Barkly East	KX057170	KX057243	KX057324
Jamesbrittenia atropurpurea (Benth.) Hilliard	*G.A. Verboom 825*	Namibia: Groot Karas	KX057155	KX057228	KX057309
Jamesbrittenia aurantiaca (Burchell) Hilliard	*G.A. Verboom 1045*	South Africa: Amersfoort		KX057249	KX057330
Jamesbrittenia barbata Hilliard	*G.A. Verboom 1112*	Namibia: Bloedkoppie	KX057141	KX057214	KX057295
Jamesbrittenia bergae Lemmer	*G.A. Verboom 1065*	South Africa: Thabazimbi	KX057171	KX057244	KX057325
Jamesbrittenia bicolor (Dinter) Hilliard	*G.A. Verboom 856*	Namibia: Witputz	KX057124	KX057197	KX057278
Jamesbrittenia breviflora (Schltr.) Hilliard	*G.A. Verboom 776*	South Africa: Cathedral Peak	KX057177	KX057258	KX057338
Jamesbrittenia burkeana (Benth.) Hilliard	*G.A. Verboom 1059*	South Africa: Lydenburg	KX057179	KX057260	KX057340
Jamesbrittenia calciphila Hilliard	*A. Harrower 1679.*	Cultivation: NBG, Kirstenbosch	KX057154	KX057227	KX057308
Jamesbrittenia canescens (Benth.) Hilliard var. *canescens*	*G.A. Verboom 817*	Namibia: Ai-Ais	KX057143	KX057216	KX057297
Jamesbrittenia canescens (Benth.) Hilliard var. *laevior* (Dinter) Hilliard	*G.A. Verboom 1128*	Namibia: Otavifontein	KX057142	KX057215	KX057296
Jamesbrittenia canescens (Benth.) Hilliard var. *seineri* (Pilg.) Hilliard	*G.A. Verboom 833*	Namibia: Gaub Pass	KX057144	KX057217	KX057298
Jamesbrittenia chenopodioides Hilliard	*G.A. Verboom 1115*	Namibia: Brandberg	KX057145	KX057218	KX057299
Jamesbrittenia concinna (Hiern) Hilliard	*G.A. Verboom 1122*	Namibia: Tsumeb	KX057165	KX057238	KX057319
Jamesbrittenia crassicaulis (Benth.) Hilliard	*G.A. Verboom 1019*	South Africa: Elliot	KX057175	KX057256	KX057336

Table 1. *Continued*

Taxon	Voucher	Collection locality	*GScp*	*psbA-trnH*	*rps16*
Jamesbrittenia dentatisepala Hilliard	*G.A. Verboom 1030*	South Africa: Garden Castle	KX057167	KX057240	KX057321
Jamesbrittenia dolomitica Hilliard	*G.A. Verboom 1125*	Namibia: Otavi	KX057146	KX057219	KX057300
Jamesbrittenia elegantissima (Schinz) Hilliard	*G.A. Verboom 1124*	Namibia: Popa Falls	KX057166	KX057239	KX057320
Jamesbrittenia filicaulis (Benth.) Hilliard	*G.A. Verboom 1012*	South Africa: Cathcart		KX057250	KX057331
Jamesbrittenia fimbriata Hilliard	*G.A. Verboom 847*	Namibia: Sossusvlei	KX057131	KX057204	KX057285
Jamesbrittenia fleckii (Thell.) Hilliard	*G.A. Verboom 835*	Namibia: Gaub Pass	KX057139	KX057212	KX057293
Jamesbrittenia foliolosa (Benth.) Hilliard	*A. Harrower 552*	Cultivation: NBG, Kirstenbosch	KX057159	KX057232	KX057313
Jamesbrittenia fragilis (Pilg.) Hilliard	*G.A. Verboom 1126*	Namibia: Otavi	KX057180	KX057261	KX057341
Jamesbrittenia fruticosa (Benth.) Hilliard	*G.A. Verboom 864*	South Africa: Steinkopf	KX057126	KX057199	KX057280
Jamesbrittenia glutinosa (Benth.) Hilliard	*G.A. Verboom 814*	Namibia: Ai-Ais	KX057135	KX057208	KX057289
Jamesbrittenia grandiflora (Galpin) Hilliard	*G.A. Verboom 1048*	South Africa: Barberton	KX057150	KX057223	KX057304
Jamesbrittenia hereroensis (Engl.) Hilliard	*G.A. Verboom 1109*	Namibia: Bloedkoppie	KX057134	KX057207	KX057288
Jamesbrittenia heucherifolia (Diels) Hilliard	*G.A. Verboom 1120*	Namibia: Epupa Falls	KX057148	KX057221	KX057302
Jamesbrittenia huillana (Diels) Hilliard	*G.A. Verboom 1056*	South Africa: Barberton	KX057158	KX057231	KX057312
Jamesbrittenia incisa (Thunb.) Hilliard	*G.A. Verboom 885*	South Africa: Calvinia	KX057153	KX057226	KX057307
Jamesbrittenia integerrima (Benth.) Hilliard	*G.A. Verboom 851*	Namibia: Aus	KX057137	KX057210	KX057291
Jamesbrittenia jurassica (Hilliard & Burtt) Hilliard	*A. Harrower KB3*	Cultivation: NBG, Kirstenbosch	KX057178	KX057259	KX057339
Jamesbrittenia kraussiana (Bernh.) Hilliard	*A. Harrower W126*	South Africa: Oribi Gorge	KX057163	KX057236	KX057317
Jamesbrittenia lyperioides (Engl.) Hilliard	*G.A. Verboom 1101*	Namibia: Auasberg	KX057138	KX057211	KX057292
Jamesbrittenia macrantha (Codd) Hilliard	*G.A. Verboom 1062*	South Africa: Burgersfort	KX057169	KX057242	KX057323
Jamesbrittenia major (Pilg.) Hilliard	*G.A. Verboom 815*	Namibia: Ai-Ais	KX057125	KX057198	KX057279
Jamesbrittenia maritima (Hiern) Hilliard	*G.A. Verboom 1002*	South Africa: Alexandria	KX057162	KX057235	KX057316
Jamesbrittenia maxii (Hiern) Hilliard	*G.A. Verboom 805*	South Africa: Aggeneys	KX057128	KX057201	KX057282
Jamesbrittenia megadenia Hilliard	*G.A. Verboom 823*	Namibia: Ai-Ais	KX057136	KX057209	KX057290
Jamesbrittenia megaphylla Hilliard	*G.A. Verboom 859*	South Africa: Vioolsdrif	KX057127	KX057200	KX057281
Jamesbrittenia merxmuelleri (Roessler) Hilliard	*G.A. Verboom 866*	South Africa: Alexander Bay	KX057151	KX057224	KX057305
Jamesbrittenia microphylla (L.f.) Hilliard	*N. G. Bergh 1453*	South Africa: Colchester		KX057251	KX057332
Jamesbrittenia montana (Diels) Hilliard	*G.A. Verboom 1039*	South Africa: Dundee		KX057248	KX057329
Jamesbrittenia multisecta Hilliard	*G.A. Verboom 1022*	South Africa: Engcobo	KX057168	KX057241	KX057322
Jamesbrittenia pallida (Pilg.) Hilliard	*G.A. Verboom 1106*	Namibia: Erongo	KX057140	KX057213	KX057294
Jamesbrittenia pedunculosa (Benth.) Hilliard	*G.A. Verboom 874*	South Africa: Kamieskroon	KX057121	KX057194	KX057275
Jamesbrittenia phlogiflora (Benth.) Hilliard	*G.A. Verboom 1011*	South Africa: Peddie	KX057161	KX057234	KX057315
Jamesbrittenia primuliflora (Thell.) Hilliard	*G.A. Verboom 830*	Namibia: Seeheim	KX057130	KX057203	KX057284
Jamesbrittenia pristisepala (Hiern) Hilliard	*G.A. Verboom 1034*	South Africa: Sani Pass	KX057176	KX057257	KX057337
Jamesbrittenia racemosa (Benth.) Hilliard	*G.A. Verboom 878*	South Africa: Kamieskroon	KX057122	KX057195	KX057276
Jamesbrittenia ramosissima (Hiern) Hilliard	*G.A. Verboom 808*	South Africa: Pella	KX057118	KX057191	KX057272
Jamesbrittenia sessilifolia (Diels) Hilliard	*G.A. Verboom 854*	Namibia: Witputs	KX057129	KX057202	KX057283
Jamesbrittenia silenoides (Hilliard) Hilliard	*G.A. Verboom 1046*	South Africa: Amersfoort	KX057174	KX057255	KX057335
Jamesbrittenia sp. nov.	*G.A. Verboom 1108*	Namibia: Erongo	KX057133	KX057206	KX057287

Table 1. *Continued*

Taxon	Voucher	Collection locality	*GScp*	*psbA-trnH*	*rps16*
Jamesbrittenia stellata Hilliard	*A. Harrower 1702*	Cultivation: NBG, Kirstenbosch	KX057152	KX057225	KX057306
Jamesbrittenia stricta (Benth.) Hilliard	*G.A. Verboom 1047*	South Africa: Amersfoort	KX057173	KX057254	KX057334
Jamesbrittenia tenella (Hiern) Hilliard	*G.A. Verboom 1102*	Namibia: Auasberg	KX057132	KX057205	KX057286
Jamesbrittenia tenuifolia (Bernh.) Hilliard	*G.A. Verboom 915*	South Africa: Sedgefield	KX057156	KX057229	KX057310
Jamesbrittenia thunbergii (G.Don) Hilliard	*G.A. Verboom 882*	South Africa: Vanrhynsdorp	KX057123	KX057196	KX057277
Jamesbrittenia tortuosa (Benth.) Hilliard	*G.A. Verboom 785*	South Africa: Swartberg Pass		KX057245	KX057326
Jamesbrittenia tysonii (Hiern) Hilliard	*G.A. Verboom 1071*	South Africa: Britstown		KX057246	KX057327

Forward and reverse sequences were assembled and edited using ChromasPro version 1.34 (www.technelysium.com.au/ChromasPro.html) and manually aligned with BioEdit version 5.0.9 (Hall, 1999). The *GScp* traces were largely unambiguous (1.45 ambiguities per thousand bases, on average) but sites which were clearly ambiguous were scored as such using the IUPAC ambiguity codes. Sequences produced as part of this study have been submitted to GenBank (Table 1) and the final alignments submitted to TreeBase (Study accession no. S19152). In the final alignments, some stretches of *GScp* and *psbA-trnH* in the outgroup and in *J. ramosissima* (Hiern) Hilliard could not be meaningfully aligned to the rest of *Jamesbrittenia* and were accordingly scored as unknown for these taxa (see also Supporting Information, Figs S1–S3: white portions).

Assessment and Resolution of Incongruence Between Loci

To identify topological incongruence between the sampled loci, separate bootstrap (Felsenstein, 1985) majority rule consensus topologies were generated for the plastid (*psbA-trnH* and *rps16* were concatenated as they yielded congruent topologies; data not shown) and *GScp* partitions using parsimony as implemented in PAUP* version 4.0b10 (Swofford, 2003). Only accessions (n = 64) common to both data sets were included in these analyses. Each analysis sampled 500 bootstrap replicates with searches done heuristically using starting trees derived by simple-addition, and operating under a MAXTREES = 500 limit. Incompatible nodes having reciprocal bootstrap support $\geq$ 70% were taken as reflecting supported incongruence. NeighbourNet analysis, as implemented in SplitsTree4 version 4.14 (Huson & Bryant, 2006), was applied to the full *GScp* and plastid data sets in order to corroborate the patterns produced by parsimony inference.

Incongruence was resolved as follows. First, a minimum set of conflict taxa (the minimum set of taxa which, when pruned from both consensus trees, yields congruence) was identified. Following Pirie *et al.* (2008) each conflict taxon was then decomposed into its plastid and *GScp* sequences, and these were entered as separate accessions in a combined matrix. Taxa for which only plastid or *GScp* sequences were available were also included. The combined matrix was then subjected to mixed-model Bayesian inference, as implemented in MrBayes version 3 (Ronquist & Huelsenbeck, 2003), the optimal model structure for each locus (*psbA-trnH*: GTR+G; *rps16*: GTR+G; *GScp*: HKY+G) being identified under the Akaike information criterion (AIC) using MrModeltest version 2.3 (Nylander, 2004). Four independent Metropolis-coupled Monte Carlo Markov samplers

(comprising one unheated and three heated chains; temperature = 0.2) were run, each running for 10^7 generations and sampling every 100th generation. Tracer version 1.5 (Rambaut & Drummond, 2009) was used to confirm that individual runs had attained stationarity, to check for convergence across runs and to assess the adequacy of the effective sample sizes underpinning each parameter estimate. The posterior tree set was summarized as a maximum clade credibility (MCC) tree, with posterior probabilities on nodes being based on the post burn-in samples (i.e. the last 90 000 samples from each run).

The tree obtained from this analysis had conflict taxa (eight species) represented twice, their plastid and *GScp* accessions being resolved separately. To obtain a tree in which each conflict taxon was included just once, we used an independent morphological character matrix (Table 2; see also Supporting Information, Table S1) to assess which of the alternative placements of conflict taxa were most consistent with morphology. For this purpose, we first used tree pruning to derive, from the MCC tree containing both plastid and *GScp* accessions of conflict taxa, the full set of $2^8 = 256$ topological arrangements having each conflict taxon placed in either of its possible positions. The morphological length score of each topology was then determined in the context of a character matrix comprising 14 morphological characters (Table 2), scored largely on the basis of Hilliard's (1994) descriptive accounts. Tree pruning and morphological length assessment were achieved using the ape version 3.3 (Paradis, Claude & Strimmer, 2004) and phangorn version 1.99 (Schliep, 2011) packages, as implemented in R version 3.0.1 (R Development Core Team, 2008).

Dated Phylogenetic Tree

For the purpose of generating a dated hypothesis of species relationships in *Jamesbrittenia*, we made use of a combined data matrix in which conflict taxa were represented only by the sequences of those loci for which suggested placements were most consistent with morphology. Dating analyses were performed in BEAST version 1.8.3 (Drummond & Rambaut, 2007), using a lognormal relaxed Bayesian clock (Drummond *et al.*, 2006). In the absence of appropriate fossil data, we were compelled to use secondary node age estimates, derived from a higher-level analysis, to calibrate divergence times in *Jamesbrittenia*. For this purpose, we made use of the 111-taxon (adding their two rosid outgroups), six-gene (*matK*, *ndhF*, *rbcL*, *rps16*, *trnL-trnF*, *trnV*) alignment assembled by Bremer, Friis & Bremer (2004) to estimate divergence times in the asterid clade. To provide calibration points for the lower-level analysis, sequences representing *Diascia* Link & Otto, *Hemimeris* L., *Jamesbrittenia*, *Lyperia* Benth., *Oftia* Adans. and *Teedia* Rudolphi were added to this matrix, these being assembled as composite terminals from existing GenBank sequences (see also Supporting Information, Table S2).

BEAST input files were generated using BEAUTi version 1.8.3 (Drummond & Rambaut, 2007). For the higher-level analysis, separate models were applied to each locus, as selected by Mr Modeltest (all GTR+I+G), and priors were mostly applied using BEAUTi default settings. Branching times were estimated under a birth-death prior incorporating incomplete taxon sampling and molecular rate variation was modelled using a discretized lognormal

Table 2. List of morphological characters used to assess the alternative placements of conflict taxa, with state delimitation and state transition scheme indicated. Terminology follows Hilliard (1994)

Number	Character	Character states
1	Life history	0, annual; 1, perennial
2	Leaf margin	0, entire or subentire; 1, serrate or coarsely toothed; 2, shallowly lobed; 3, deeply dissected (unordered)
3	Leaf width	$0 \leq 3$ mm; $1 \geq 3$ mm
4	Corolla limb colour	0, white; 1, mauve-violet; 2, pink-red; 3, yellow-orange; 4, brown; 5, brown with white margins (unordered)
5	Markings on corolla limb	0, no markings; 1, one basal streak per lobe; 2, three basal streaks per lobe (unordered)
6	Corolla lobe margins	0, flat; 1, reflexed
7	Filament indumentum	0, glandular-puberulous, 1, bearded, 2, glabrous (unordered)
8	Glandular hairs on adaxial leaf surface	0, absent; 1, present
9, 10	Glistening glands on adaxial leaf surface	0,0, absent; 0, 1, sparse; 1,1, dense (ordered)
11	Glandular hairs on abaxial leaf surface	0, absent; 1, present
12, 13	Glistening glands on abaxial leaf surface	0,0, absent; 0,1, sparse; 1,1, dense (ordered)
14	Glistening glands	0, oozing; 1, not oozing

distribution. We used the fossil dates reported by Bremer *et al.* (2004) to date our asterid tree, implementing these as lognormal priors (Ho, 2007). In all cases, lognormal age priors were set to have a zero-offset of 0.95 times the estimated age of the fossil and, by adjusting the mean (the standard deviation was always set to one), the median was set to equal the fossil age (see also Supporting Information, Table S3). This treatment allows a lineage to be substantially older but also slightly younger than its reference fossil, thereby accommodating bi-directional error in fossil age estimation. In addition, based on a wide range of estimates from earlier dating studies (Wikström, Savolainen & Chase, 2001; Anderson, Bremer & Friis, 2005; Soltis *et al.*, 2008; Wang *et al.*, 2009; Bell, Soltis & Soltis, 2010; Smith, Beaulieu & Donoghue, 2010), the divergence of asterids and rosids (root node) was constrained (uniform prior) to lie between 100 and 125 Mya. Four separate Monte Carlo Markov chain (MCMC) runs were conducted, each running for 5×10^7 generations, with sampling every 10 000th generation. Tracer version 1.5 was used to confirm the attainment of stationarity of individual runs, to check convergence across runs and to evaluate the adequacy of effective sample sizes. Final posterior probabilities and node ages were based on the post-burn-in samples (i.e. the last 3800–4200 samples) drawn from each of the four runs, giving a total of 16 400 samples.

The lower-level analysis was done in a similar manner, with separate models being applied to each locus. In this analysis, however, calibration priors were set as normal, being specified to match as closely as possible the posterior age distributions derived from the higher-level analysis. Secondary calibrations were applied to all three nodes common to the higher-level and lower-level data sets. These are: (1) the root node (describing the divergence of Hemimerideae from Limoselleae); and (2) the node describing the divergence of Teedieae from Limoselleae; and (3) the Limoselleae crown node. We elected to apply secondary calibrations to all these nodes because this reduces reliance on any individual calibration, all of which may be erroneous, and thus dilutes the error associated with each. Although the application of secondary calibrations to three nodes, rather than one, has the effect of reducing the uncertainty associated with node ages in the lower-level analysis, we found that, compared to an analysis in which secondary calibration was applied only to the root node, this effect was slight (generally a 10–15% reduction in uncertainty). In addition, although the use of three calibrations yielded younger median node age estimates in *Jamesbrittenia*, this difference was also slight (generally 5–6% younger).

To ensure that the three calibration nodes were all represented in the lower-level tree, we included, in addition to *T. pubescens*, accessions (plastid only) of nine further outgroup taxa (*Diascia longicornis* Druce, *Hemimeris racemosa* (Houtt.) Merr., *Lyperia violacea* Benth., *L. tristis* Benth., *Manulea adenocalyx* Hilliard, *M. schaeferi* Pilg., *Oftia africana* Bocq. ex Baill., *Sutera hispida* (Thunb.) Druce and *S. subsessilis* Hilliard). In addition, we constrained Hemimerideae, Teedieae and Limoselleae to be monophyletic. Four separate MCMC runs were conducted, each running for 10^7 generations with sampling every 1000th generation. As before, the effectiveness of sampling was checked using Tracer and final posterior probabilities and node ages were based on the post-burn-in samples (i.e. the last 9000 samples) drawn from each of the four runs, giving a total of 36 000 samples.

Ancestral Biome Reconstruction and Diversification

Ancestral biome associations of *Jamesbrittenia* were inferred using under an unconstrained dispersal–extinction–cladogenesis model (DEC model; Ree & Smith, 2008) as implemented in BioGeoBEARS version 0.2.1 (Matzke, 2013), in R version 3.2.1. Reconstructions were done both in the context of the MCC tree obtained from the lower-level BEAST analysis (non-*Jamesbrittenia* spp. pruned out) and, to account for phylogenetic uncertainty, in the context of a set of 100 trees randomly drawn from the posterior tree set generated by that analysis. The biome associations of extant *Jamesbrittenia* taxa were scored as a presence/absence matrix on the basis of herbarium record data (BOL, NBG and PRE), descriptive accounts (Hilliard, 1994) and field observations. As units of analysis, we used seven of the biome units defined by Mucina & Rutherford (2006), with extrapolation to Namibia using the vegetation classification of Giess (1971). Biome classes were as follows: desert and desert margin; succulent karoo; Nama karoo; savanna/woodland; grassland; fynbos; and Albany thicket. Analyses were run in the absence of constraints on dispersal or ancestral distribution.

To assess whether the occupation of novel biomes facilitated radiation in *Jamesbrittenia*, we employed a Bayesian approach, as implemented in BAMM version 2.5.0 (Rabosky, 2014), to assess the fit of alternative rate-shift scenarios to the MCC tree obtained from BEAST. To account for incomplete taxon sampling, unsampled taxa were assigned to major clades (i.e. *J. ramosissima*, Namaqualand, Namib or widespread clade) on the basis of morphology, primarily as embodied in the informal infrageneric classification of Hilliard (1994) and the sampling fractions for each clade specified as part of the analysis. The Poisson rate prior was set to 1.0, as recommended for small trees (< 500 taxa), and four MCMCs of length

5×10^6 generations were run, with a ΔT parameter of 0.01. Samples were drawn every 1000^{th} generation, giving a total of 5000 samples. The R package BAMMtools version 2.1.1 (Rabosky *et al.*, 2014) was used to check for convergence, to assess the adequacy of sampling and to summarize the analytical results. Following removal of the first 5% of samples as burn-in, support for alternative rate-shift scenarios was evaluated by visualizing the 95% credible set of rate-shift configurations. In addition to the BAMM analysis, laser version 2.4 (Rabosky, 2006) was used to estimate net diversification rates for the three principal clades of *Jamesbrittenia* using the method of Magallón & Sanderson (2001). To account for phylogenetic uncertainty, these estimates were derived in the context of both the BEAST MCC tree and a set of 100 trees drawn randomly from the BEAST posterior tree set. Analyses were run using crown node ages, with missing species again incorporated on the basis of morphology. Zero extinction (i.e. $\varepsilon = 0$) was assumed throughout.

Range Overlap, Floral Divergence and Wild Hybridization

Phylogenetic analyses suggest that the bulk of species diversity in *Jamesbrittenia* is the product of a single radiation (widespread clade) associated primarily with the central plateau of southern Africa. To assess the relative contributions of geography, floral morphology and phylogenetic relatedness in determining the distribution of wild hybridization between species in this clade, we derived, for the sampled South African species, a series of triangular matrices describing pairwise (species × species) variation in: (1) range overlap; (2) floral divergence; (3) floral morph identity; (4) phylogenetic distance; and (5) incidence of wild hybridization. The first of these matrices was used to determine the extent to which species in the radiated clade are isolated by geography. Then, focusing on species pairs showing non-zero range overlap, we used generalized linear models (GLM) with logit link functions (binary response variable) to assess the significance of floral dissimilarity and phylogenetic relatedness as predictors of wild hybridization. As the matrix structure of the input variables renders the individual observations (i.e. species pairs) non-independent, the significances of the observed variable coefficients were assessed against nulls derived by shuffling the response matrix 999 times. All analyses were conducted in R version 3.0.1, making use of the packages ape version 3.3, raster version 2.1 (Hijmans, 2013), dismo version 0.9 (Hijmans *et al.*, 2013) and picante version 1.6 (Kembel *et al.*, 2010). Although we would have preferred to include all widespread clade species in this analysis, a South African focus was necessitated by limitations to the availability of requisite data.

To determine pairwise range overlaps, the distribution range of each species was estimated by first geo-referencing as precisely as possible all *Jamesbrittenia* collection records at NBG and PRE, and then determining the minimum convex hull polygons enclosing the records of each species. Pairwise range overlaps were determined as the area of intersection divided by the range area of the more narrowly distributed species.

Pairwise dissimilarities in floral morphology were based on floral morphometric data reported by Hilliard (1994). Thirteen floral characters were considered, namely tube length, tube width, distance across the lateral corolla lobes, posticous corolla lobe length, posticous corolla lobe width, anticous corolla lobe length, anticous corolla lobe width, posterior filament length, posterior anther length, anticous filament length, anticous anther length, stigma length and style length. Where Hilliard (1994) reported a range for a character, the midpoint of that range was used. Dissimilarities were expressed both as simple Euclidean distances based on floral dimensions and as a binary score indicating whether the two species were identical (score = 0) or non-identical (score = 1) in terms of a categorically coded floral morph classification. For this purpose we defined a series of floral morph classes by first using principal components analysis (PCA) to identify clusters reflecting similarity in floral form and then mapping floral colour onto the resulting ordination scheme, the combination of flower shape and colour being used to delimit floral morphs.

Pairwise phylogenetic distances were determined as twice the divergence time (in Myr) inferred by the BEAST analysis. In the first instance, a matrix of distances was derived using the MCC tree, but, recognizing the substantial phylogenetic uncertainty that exists within the radiation clade, this was supplemented with a set of 1000 matrices based on 1000 trees randomly sampled from the posterior tree set.

Finally, a matrix describing the distribution of wild hybridization between species pairs was assembled on the basis of Hilliard's (1994) inferences relating to hybridization in *Jamesbrittenia*. The scoring was binary, reflecting the occurrence (score = 1) or non-occurrence (score = 0) of hybrids between each pair of species.

RESULTS

Incongruence Among Loci

Considering only accessions common to both data sets, the *GScp* and plastid sequences yielded, respectively, 631 and 1334 characters, of which 129 (20.4%)

and 77 (5.8%) were potentially parsimony informative. Respectively, the two partitions had consistency indices of 0.82 and 0.96. Owing to the low number of potentially parsimony-informative characters, the bootstrap consensus trees were poorly resolved and supported, with < 15 nodes in each receiving bootstrap support (BS) ≥ 70% (Fig. 1). Notwithstanding, there was an indication of broad congruence between the two data sets. Both consensus trees identified *J. ramosissima* as sister to the rest of *Jamesbrittenia* and, except for a set of eight conflict taxa (see below), both identified an assemblage of 11 herbaceous species from the succulent karoo [*J. amplexicaulis* (Benth.) Hilliard, *J. aridicola* Hilliard, *J. bicolor*

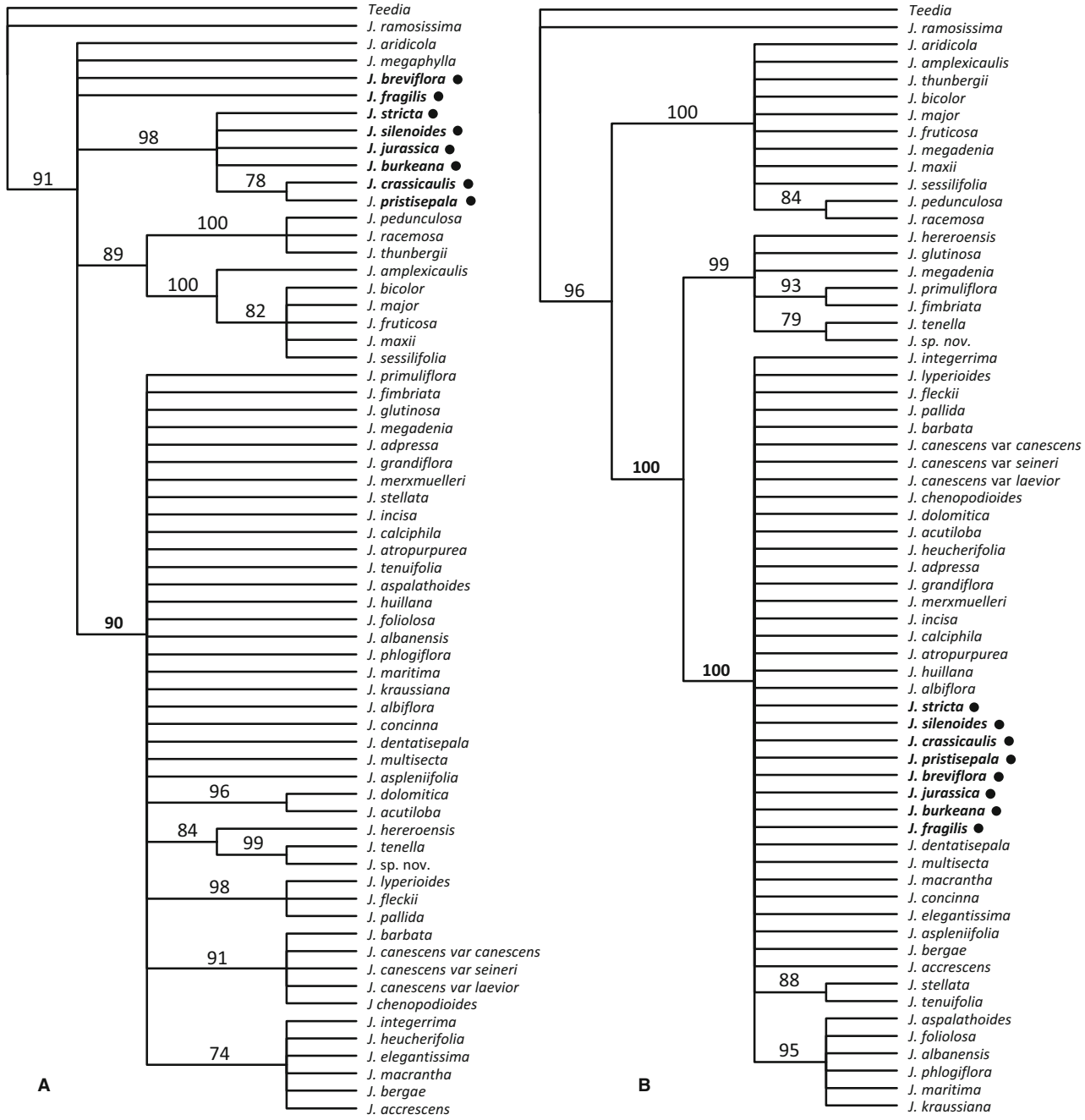

Figure 1. Seventy per cent bootstrap consensus topologies, based on (A) *GScp* and (B) plastid DNA sequence data. Conflict taxa are shown in bold type and marked with solid dots. Bootstrap percentiles are indicated, with values on contradictory nodes shown in bold type.

(Dinter) Hilliard, *J. fruticosa* (Benth.) Hilliard, *J. major* (Pilg.) Hilliard, *J. maxii* (Hiern) Hilliard, *J. megaphylla* Hilliard, *J. pedunculosa* (Benth.) Hilliard, *J. racemosa* (Benth.) Hilliard, *J. sessilifolia* (Diels) Hilliard and *J. thunbergii* (G.Don) Hilliard] as the next earliest-diverging element. However, the two data sets disagreed strongly with regard to the placement of eight, mostly Drakensberg-endemic taxa [conflict taxa: *J. breviflora* (Schltr.) Hilliard, *J. burkeana* (Benth.) Hilliard, *J. crassicaulis* (Benth.) Hilliard, *J. fragilis* (Pilg.) Hilliard, *J. jurassica* (Hilliard & B.L.Burtt) Hilliard, *J. pristisepala* (Hiern) Hilliard, *J. silenoides* (Hilliard) Hilliard and *J. stricta* (Benth.) Hilliard)], the contradictory placement of which is also evident in the NeighbourNet networks produced by the two data partitions (see also Supporting Information, Fig. S4). Whereas *GScp* placed these taxa in a basal polytomy in *Jamesbrittenia*, plastid DNA embedded them in the large crown clade.

The contradictory placement of the conflict taxa was also apparent when the *GScp* and plastid accessions for these taxa were included as separate elements in a combined analysis (Fig. 2). Like the separate analyses, this analysis retrieved *J. ramosissima* as sister to the remainder of *Jamesbrittenia* and, ignoring the conflict taxa, identified three well supported clades of *Jamesbrittenia*: (1) a clade of annual and herbaceous species from the Namaqualand area (Namaqualand clade); (2) a clade of annual species from the semi-arid fringe of the Namib Desert (Namib clade); and (3) a clade of mostly shrubby, perennial species occurring in a wide range of environments throughout southern Africa (widespread clade). In this context, the *GScp* accessions of the conflict taxa were resolved in the Namaqualand clade, whereas their plastid accessions were placed in the widespread clade.

Morphological evaluation of the alternative placements of the conflict taxa identified the plastid-suggested placements as being consistently optimal. The topology with all conflict taxa in their plastid-suggested positions was strongly favoured over all other arrangements, having a morphological length of 128 steps, compared with an overall mean (± standard deviation) length of 133.73 ± 1.67 steps (Fig. 3). On the basis of greater morphological consistency, the conflict taxa were represented in the BEAST analysis only by their plastid accessions, the product of which was used to examine biome evolution and the incidence of hybridity in *Jamesbrittenia*.

Dated Phylogenetic Tree

With the sole exception of the Icacinaceae–Rubiaceae split, the age priors on the calibration nodes closely matched the posterior estimates inferred by the higher-level (asterid) dating analysis (see also Supporting Information, Table S3), indicating high chronological consistency among the various fossils used. The ages of the Hemimerideae–Limoselleae split, the Teedieae–Limoselleae split and the Limoselleae crown node were estimated at 71.64 [60.75, 81.36], 59.28 [45.09, 72.70] and 51.45 [35.26, 64.91] Myr (see also Supporting Information, Fig. S5), respectively (median and 95% HPD interval), these values being used to specify normal calibration priors for the lower-level (*Jamesbrittenia*) dating analysis. On this basis, the latter analysis identified *Jamesbrittenia* (Limoselleae crown node) as originating at 55.11 [46.37, 63.85] Mya and having a crown age of 45.13 [34.49, 55.97] Myr (Fig. 4). Respectively, the Namaqualand, Namib and widespread clades originated (stem nodes) 29.21 [20.11, 38.29], 15.37 [9.96, 21.77] and 15.37 [9.96, 21.77] Mya, with crown ages of 16.66 [10.76, 23.27], 8.40 [4.73, 12.79] and 8.98 [5.97, 12.61] Mya.

The sister relationship of *J. ramosissima* to the rest of *Jamesbrittenia* was well supported by the BEAST analysis (posterior probability [PP] = 1.00), as were the monophyly and interrelationships of the Namaqualand, Namib and widespread clades (Fig. 4). With the exception of a few nodes, species relationships in the Namaqualand and Namib clades were also supported (PP ≥ 0.95), but this was not true of the widespread clade, in which support for internal relationships was generally weak. This lack of support is at least partly attributable to missing data, as evidenced by an improvement in support at several nodes when species with incomplete data were omitted from the analysis (see also Supporting Information, Fig. S6).

Historical Biome Shifts and Diversification

Application of a DEC model to biome association data in the context of the BEAST MCC tree identified an association with desert environments as ancestral in *Jamesbrittenia* (Fig. 4). Reconstructions on a random sample of trees drawn from the BEAST posterior distribution (Fig. 4: nodal values) provided corroboration, with desert being identified most frequently (91%) as the highest probability state on the root node. From an ancestral association with desert environments, *Jamesbrittenia* is inferred to have undergone a transition, near the base of the widespread clade, to more mesic biomes. Although the precise timing of this shift is unclear, a transition in the interval 7.50 [5.07, 10.30]–15.37 [9.96, 21.77] is most likely, with grassland and woodland/savanna habitats having been occupied by 7.50 [5.07, 10.30] Mya and 6.20 [4.32, 8.50] Mya, respectively.

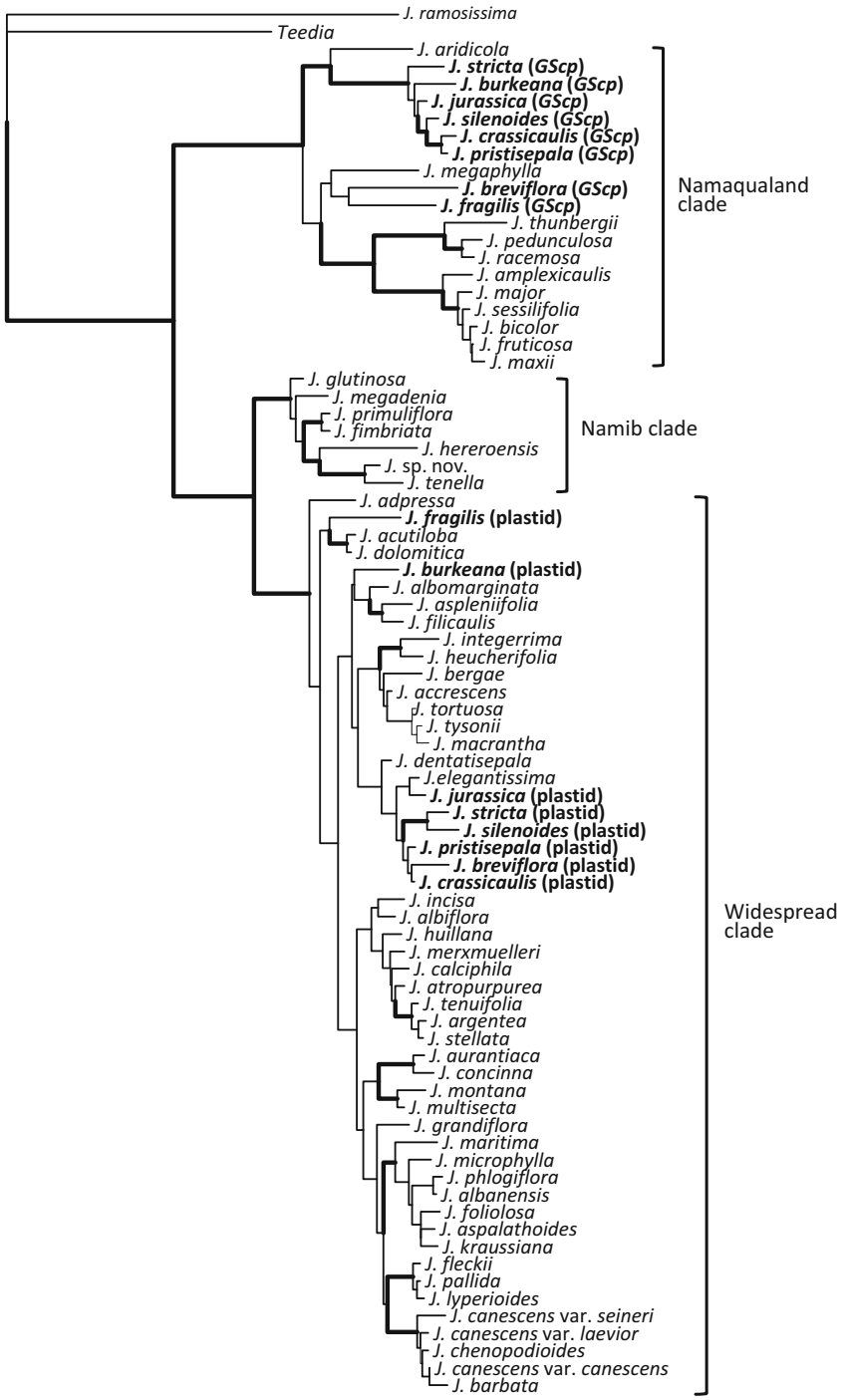

Figure 2. Maximum clade credibility tree based on the posterior tree sample generated by MrBayes using the combined matrix with conflict taxa decomposed into their *GScp* and plastid accessions (shown in bold type). Heavy branches have posterior probability > 0.95. The principal *Jamesbrittenia* clades are indicated on the right.

The morphological assignment of unsampled taxa to major clades was relatively straightforward since the widespread clade is morphologically distinct from the other three lineages, in terms of growth form and foliar and floral characters. This situation is reflected in Hilliard's (1994) treatment which, with a single exception (*J. fragilis*), assigns widespread clade species to a different set of species groups (Fig. 4: '1a, 1', '1a, 2', '1a, 3','2, 1', '2, 2', '2, 3' and '2, 5') than those from the *J. ramosissima*, Namaqualand and Namib

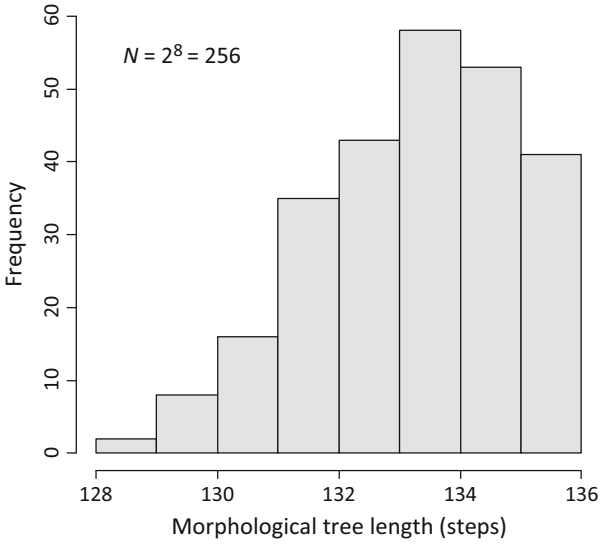

Figure 3. Distribution of morphological tree lengths across the full set of topological combinations in which each conflict taxon is retrieved in one of its alternative positions. The alternative topologies were derived by pruning the MrBayes maximum clade credibility tree having conflict taxa decomposed into their *GScp* and plastid accessions (Fig. 2).

lineages (Fig. 4: '1b, 1', '1b, 2' and '2, 4'). On the basis of this classification, all unsampled taxa could be assigned to the widespread clade, giving this clade a sampling fraction of 0.75 (50/67). Incorporating this information and in the context of the MCC tree, net diversification rates in the Namaqualand, Namib and widespread clade were estimated at 0.10, 0.15 and 0.40 spp Myr^{-1}, respectively. This implies that the widespread clade diversified four times as quickly as the Namaqualand clade and 2.7 times as quickly as the Namib clade. The use of a random posterior tree sample corroborated this result, yielding median rate estimates of 0.10, 0.15 and 0.39 spp Myr^{-1}, and revealing limited overlap between the widespread clade and the other two clades in terms of the rate ranges suggested by alternative trees (Fig. 5). Consistent with these patterns, BAMM diversification analysis, done in the context of the BEAST MCC tree, provides evidence of accelerated diversification in the widespread clade. The 95% credible set of rate configurations (Fig. 6) is dominated by configurations involving a single rate increase either at 8.98 [5.97, 12.61] Mya on the crown node of the widespread clade (51%), or at 7.50 [5.07, 10.3] Mya on the node subtending the *J. filicaulis* (Benth.) Hilliard-*J. atropurpurea* (Benth.) Hilliard clade (23%). Zero-rate-shift scenarios account for the balance (26%) of the credible configuration set.

Range Overlap, Floral Divergence and Wild Hybridization

Jamesbrittenia spp. typically show strong habitat-specificity, with the consequence that most South African species are locally distributed. With the exception of *J. atropurpurea* (1493 km) and *J. aurantiaca* (Burch.) Hilliard (1108 km) all South African widespread clade species have distributions the maximum extent of which (the maximum distance between collection localities) is 820 km or less. Overall, species in the widespread clade have a mean ($\pm$ standard deviation) maximum extent of 407 ± 301 km. Consequently, there is a high level of geographical separation between species, with 576 of the 741 pairwise comparisons between species in the widespread clade (77.7%) showing zero range overlap. The remaining 165 pairs showed a mean ($\pm$ standard deviation) range overlap of $53.5 \pm 34.8\%$.

For species pairs with non-zero range overlap, full GLMs including floral dissimilarity and phylogenetic distance (based on the BEAST MCC tree) and their interaction as predictors of wild hybridization identified none of these terms as significant (data not shown; all $P > 0.05$). This situation was the case whether floral dissimilarity was expressed as Euclidean distance or in terms of floral morph identity, where morphs were delimited on the basis of a PCA overlaid with floral colour (Fig. 7; the phylogenetic distribution of morphs also indicated in Fig. 4). Following the approach of Crawley (2007), both models were simplified by first dropping the non-significant interaction terms. Both resulting models, which had improved AIC scores relative to the full models (Euclidean distance: 109.50 vs. 110.27; floral morph identity: 108.69 vs. 110.07), identified floral dissimilarity as having a highly significant, negative effect on wild hybridization (Table 3a, c). Phylogenetic distance, however, was non-significant. Evaluation of significance against nulls generated by shuffling the response matrix broadly confirmed these results (Table 3a, c), although phylogenetic distance was found to be marginally significant when floral dissimilarity was expressed as Euclidean distance (Table 3a). Simplifying the models further, by dropping phylogenetic distance as a predictor, further improved model fit (AIC; Euclidean distance: 108.62; floral morph identity: 107.43), the resulting models both identifying the effect of floral dissimilarity as highly significant (Table 3b, d). Once again, significance was corroborated using a null obtained by matrix shuffling.

The preceding results are based on phylogenetic distances derived from the MCC tree and as such ignore the considerable phylogenetic uncertainty that exists in the widespread clade. However, repeating

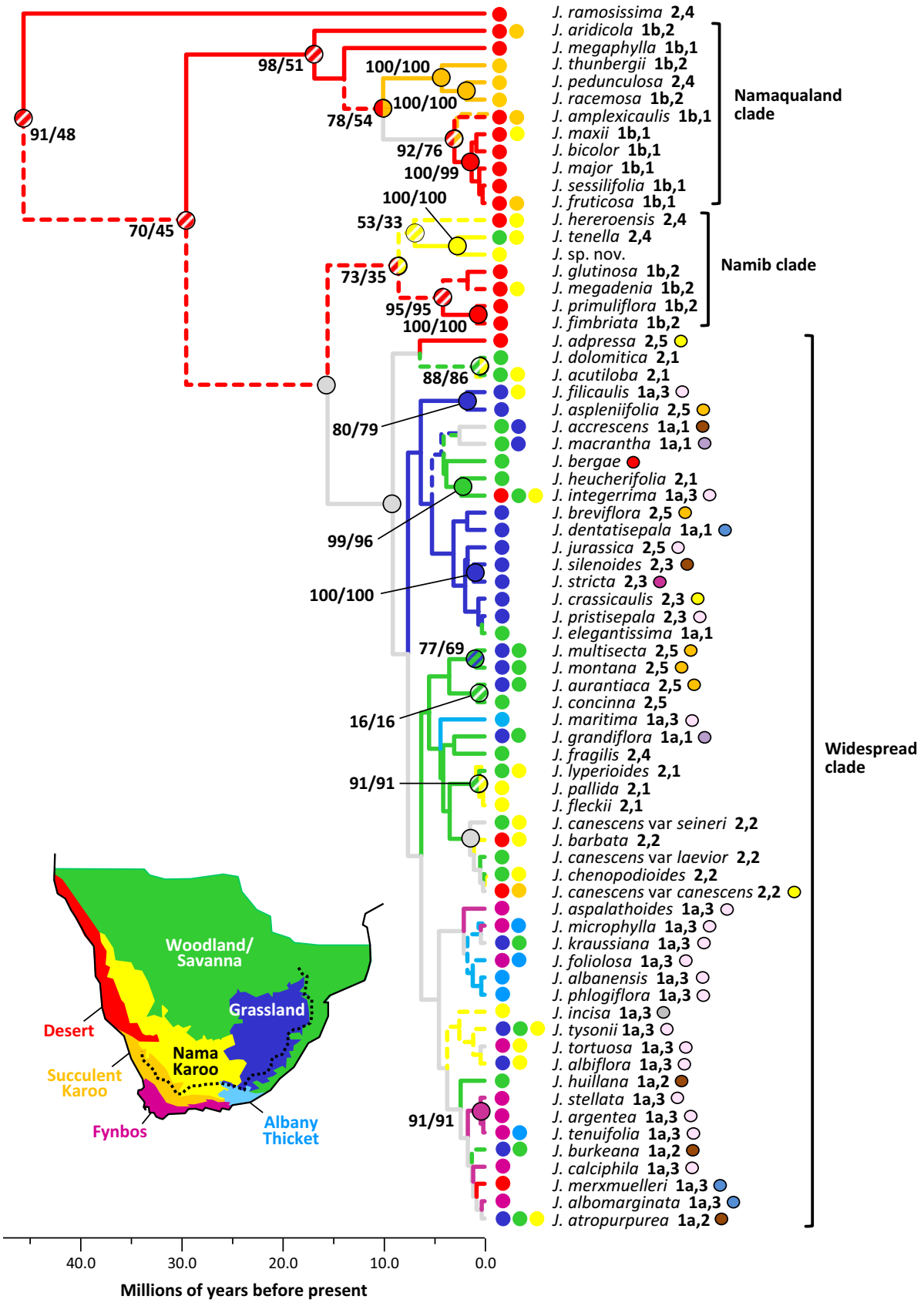

Figure 4. Maximum clade credibility (MCC) tree obtained by BEAST using the combined matrix but including for conflict taxa only those accessions (i.e. *GScp* or plastid), the suggested placements of which are more consistent with morphology. A timescale is provided below and species names are followed (in bold type) by an indication of the species groups (Hilliard, 1994) to which they belong, and of their floral morphotype (filled circles; colours set to match the polygons in Fig. 7). Contemporary biome occupancy patterns are indicated using filled coloured circles at the terminals (colours as in the biome map, inset) and ancestral biome reconstructions, inferred under a dispersal–extinction–cladogenesis model, are indicated using coloured branches and nodes (only provided for nodes having posterior probability > 0.95). Reconstructions on branches reflect the states of daughter lineages immediately following cladogenesis, whereas nodal reconstructions reflect states immediately prior to cladogenesis. Reconstructions having probability > 0.75 are indicated by solid branches (but branches may be two-coloured, indicating polymorphism) and solid-filled circles on nodes, whereas reconstructions having probability > 0.50 but < 0.75 are indicated by dashed branches and hatch-filled circles on nodes. Branches or nodes having no state with probability > 0.50 (i.e. uncertain) are coloured grey. Numbers in bold type beside supported nodes reflect the sensitivity of nodal reconstructions to phylogenetic uncertainty, as determined on the basis of reconstructions done on a set of 100 trees drawn randomly from the BEAST posterior tree set. In each instance, the first number indicates the percentage of trees identifying the MCC tree-based reconstruction as the highest probability state and the second indicates the percentage of trees in which the probability of this state exceeded 0.5.

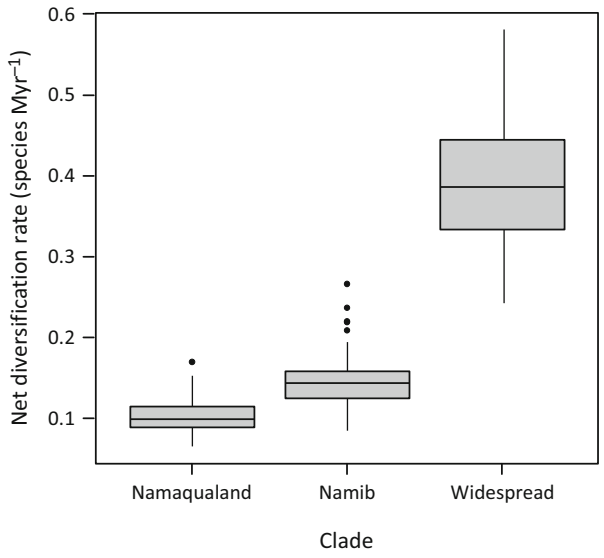

Figure 5. Box-and-whisker plot comparing net diversification rates in the Namaqualand, Namib and widespread clades, on the basis of a sample of 100 trees drawn randomly from the BEAST posterior tree set. Diversification rates were estimated using the method of Magallón & Sanderson (2001) under the assumption of zero extinction ($\varepsilon = 0$).

these analyses using a set of 1000 trees drawn randomly from the BEAST posterior tree set, with significance assessed using nulls generated by matrix shuffling, supports the basic patterns obtained. For the two-variable models (no interaction term), all 1000 trees identified the effect of floral dissimilarity as negative and significant, whether floral dissimilarity was expressed as Euclidean distance or in terms of floral morph identity. In contrast, only 56 trees identified the effect of phylogenetic distance as significant (effect negative in 55 trees; positive in one) when floral dissimilarity was expressed as Euclidean distance and only 19 trees (effect negative in 18 trees, positive in one) when it was expressed in terms of floral morph identity.

DISCUSSION

Taken together, morphological evidence (Hilliard, 1994) and the observations of plant breeders (A. Harrower, pers. comm.) suggest that post-zygotic barriers to interspecific hybridization are weakly developed in *Jamesbrittenia*. In this context, supported gene tree incongruence spanning the major lineages of *Jamesbrittenia* is plausibly a product of historical hybridization. Moreover, that this incongruence spans several well supported nodes (Fig. 2) and traverses divergence times > 10 Myr, identifies incomplete lineage sorting as an unlikely explanation of incongruence (Rosenberg, 2003), especially in the context of the short generation times (typically 1 year) and localized distribution ranges (implying smaller population sizes) which typify *Jamesbrittenia*. Although Hilliard's (1994) treatment of the genus associates most contemporary hybridization with recently diverged (< 5 Mya) species complexes, evidence of contemporary hybridization between *J. canescens* and *J. primuliflora* (Thell.) Hilliard, bridging a divergence time of 15.37 [9.96, 21.77] Myr, indicates that hybridization between more deeply diverged species is possible. Although precise determination of the number of hybridization events involved in generating deep gene tree incongruence in *Jamesbrittenia* is prohibited by poor resolution of the plastid and *GScp* trees, the clustered placement of the decoupled plastid and *GScp* accessions of conflict taxa (Fig. 2) suggests it is small,

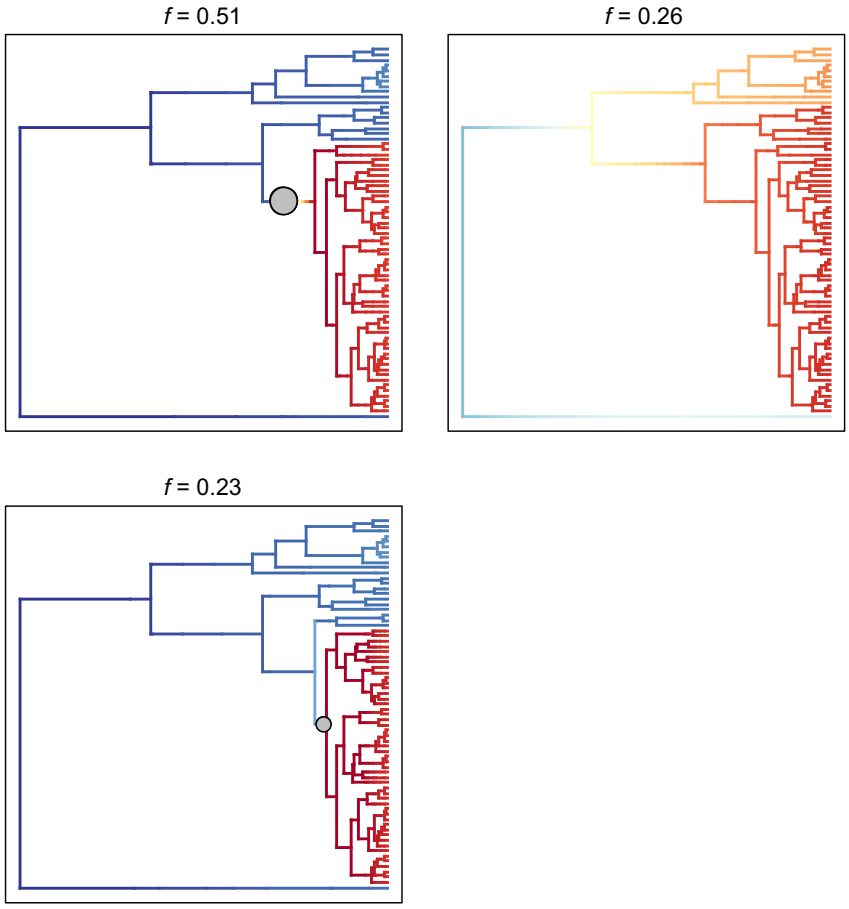

Figure 6. Rate shift scenarios included in the 95% credible posterior sample produced by the BAMM analysis. Rate shifts are indicated by circles, with higher rates indicated by warmer colours. The frequency (f) of each rate-shift scenario in the posterior sample is indicated.

probably between one and three events. Except possibly in the case of *J. fragilis*, the data consistently suggest that gene tree incongruence is the product of paternal (pollen-mediated) introgression from a Namaqualand clade species (*GScp* signal) into one or more Drakensberg-centred widespread clade taxa (plastid DNA and morphology).

Whatever its causes, the presence of supported conflict presents challenges for the accurate inference of phylogenetic relationships (e.g. Seelenan, Schnabel & Wendel, 1997; Wiens, 1998) and lineage divergence times (Pfeil, 2009). As the presence of such conflict in *Jamesbrittenia* is possibly, even probably, a consequence of hybrid-mediated lateral gene transfer rather than incomplete lineage sorting, we elected not to employ coalescent 'species tree' methods, applying instead a conditional concatenation approach in which conflict taxa were represented by either their *GScp* or plastid sequences, the choice of locus being determined by its consistency with an independent morphological data set. We justify our use of morphology as an arbiter of gene tree conflict on the grounds that: (1) morphology is a product of genome-wide sequence variation and is therefore less sensitive to processes which mislead single-gene phylogenetic inference; (2) the use of morphology to choose amongst alternative placements of conflict taxa and alternative phylogenetic hypotheses is not an uncommon practice (e.g. Järvinen *et al.*, 2004; Giribet & Edgecombe, 2006; Barber *et al.*, 2007; Linder *et al.*, 2010); and (3) the approach yields a phylogenetic hypothesis for *Jamesbrittenia* which is morphologically, ecologically and biogeographically sensible.

Our phylogenetic hypothesis provides strong evidence for the early differentiation of four principal lineages in *Jamesbrittenia* (Fig. 4): (1) the early-diverging *J. ramosissima*, the distinctiveness of which from the rest of *Jamesbrittenia* had already been noted by Hilliard (1994); (2) a clade of annual and herbaceous-perennial species from the lower Orange River and Namaqualand regions

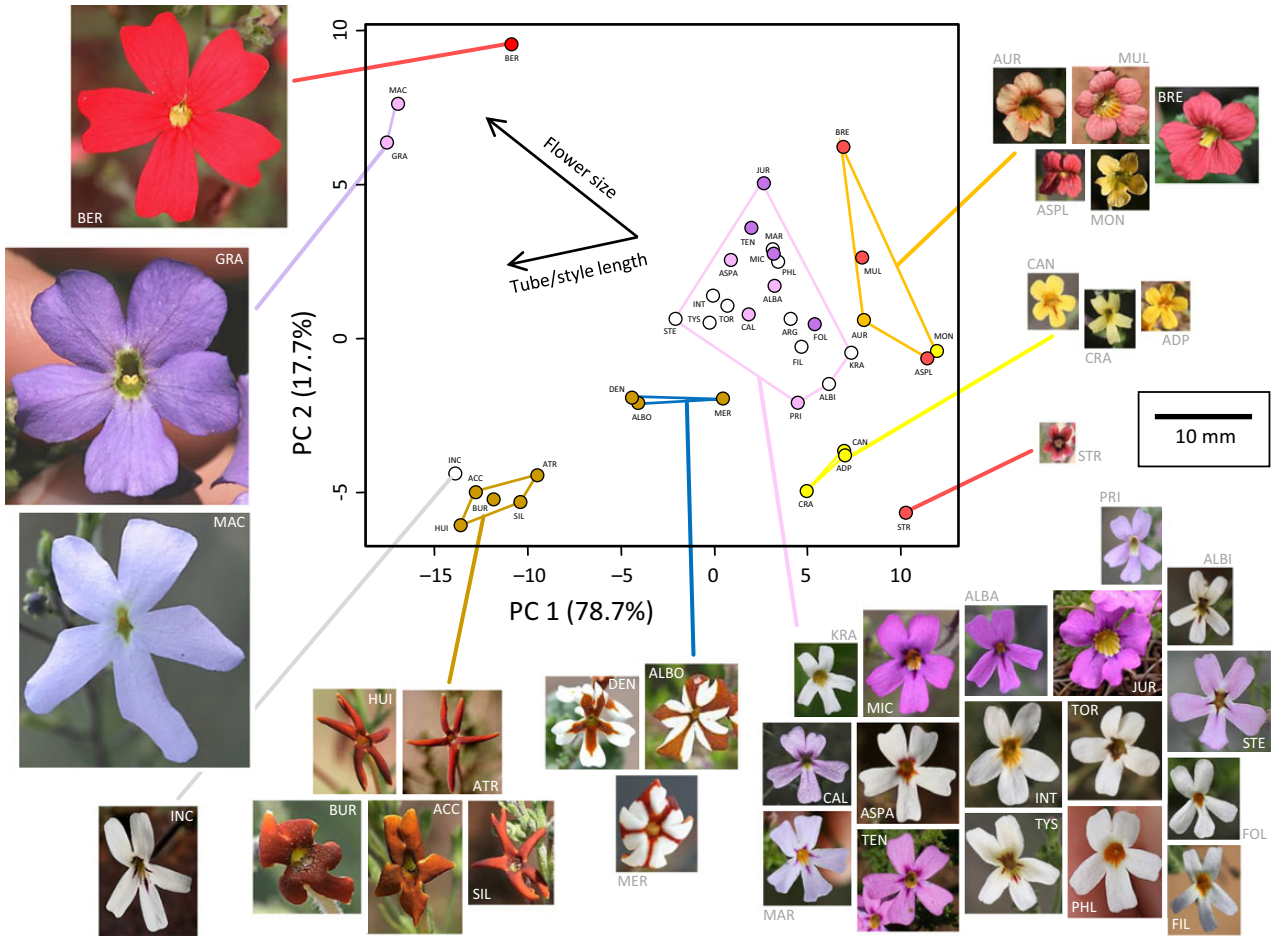

Figure 7. Delimitation of floral morphs among South African widespread clade species. Morphs were delimited by using principal components analysis (PCA) first to identify clusters reflecting similarity in floral form and then mapping floral colour onto the resulting ordination scheme. Morphs were defined on the basis of both floral form and colour. Species are represented by labelled (first three or four letters of the name of the species) coloured points (indicative of floral colour) and have been grouped into morphs as indicated by coloured polygons. The first two principal components account for > 96% of the variance in the 13 floral morphometric characters used, the latter describing two axes of variation, one reflecting variation in floral tube length and a second variation in the size of the floral face. Photographs (scale bar on right of figure) depicting the flowers of all species included in each morph are shown on the periphery.

(Namaqualand clade); (3) a clade comprising exclusively annual species from the arid to semi-arid fringes of the Namib Desert (Namib clade); and (4) a clade of shrubby, woody-perennial species inhabiting a wide range of environments throughout southern Africa (widespread clade). Although relationships among these clades are robust, as are those in the Namaqualand and Namib clades, relationships in the widespread clade are generally weak and should be regarded with caution. Overall, phylogenetic relationships in *Jamesbrittenia* show broad correspondence to Hilliard's (1994) system of informal morphological groups (Fig. 4), with species in the *J. ramosissima*, Namaqualand and Namib clades falling into Hilliard's '1b, 1' '1b, 2' and '2, 4' species groups and the widespread clade containing species included in her remaining species groups. Moreover, although the patterns are somewhat fuzzy, some correspondence is also evident in the widespread clade, with species from Hilliard's groups '1a, 2' plus '1a, 3', '2, 2' and '2, 5' tending to form clades.

Phylogenetic uncertainty in the widespread clade is partly attributable to the effect of missing data, as evidenced by a slight strengthening of relationships when taxa with missing data are omitted from the analysis. However, consistently low levels of sequence divergence suggest that the lack of strong phylogenetic signal in this clade is predominantly a consequence of the recentness and rapidity of its radiation. Our data show that the widespread clade

Table 3. Results of generalized linear models assessing the significance of phylogenetic distance (BEAST maximum clade credibility tree) and floral dissimilarity as predictors of wild hybridity in the widespread clade of *Jamesbrittenia*

Effect	Coeff. est.	SE	Z	*P*	*P* (shuff.)
(a) Two-variable model, floral dissimilarity expressed as Euclidean distance					
Intercept	0.059	0.653	0.090	0.928	NS
Phylogenetic distance	−0.054	0.051	−1.063	0.288	≈0.05
Floral dissimilarity	−0.213	0.072	−2.967	0.003	≤0.001
(b) One-variable model, floral dissimilarity expressed as Euclidean distance					
Intercept	−0.465	0.438	−1.064	0.288	NS
Floral dissimilarity	−0.214	0.070	−3.056	0.002	≤0.001
(c) Two-variable model, floral dissimilarity expressed as floral morph (non)identity					
Intercept	−0.487	0.561	−0.866	0.386	NS
Phylogenetic distance	−0.045	0.052	−0.867	0.386	NS
Floral dissimilarity	−2.094	0.558	−3.756	< 0.001	≤0.001
(d) One-variable model, floral dissimilarity expressed as floral morph (non)identity					
Intercept	−0.903	0.306	−2.950	0.003	NS
Floral dissimilarity	−2.170	0.550	−3.943	< 0.001	≤0.001

Non-significant interaction terms have been dropped from all models and non-significant main effects (i.e. phylogenetic distance) have additionally been dropped from the one-variable models (b, d). Floral dissimilarity between species was expressed either as (a, b) Euclidean distance based on a set of 13 floral morphometric characters or as (c, d) a binary score indicating whether the two species were identical (score = 0) or non-identical (score = 1) in terms of a categorically coded floral morph classification (Fig. 7). As the response variable was binary (0, no hybrids; 1, hybrids occur) all analyses were structured using a logit link function. The final column presents significance levels assessed against a null obtained by reshuffling the response matrix 999 times.

diversified four times as quickly as the Namaqualand clade and 2.7 times as quickly as the Namib clade, these differences reflecting accelerated diversification in the widespread clade starting 7.50 [5.07, 10.30]–8.98 [5.97, 12.61] Mya. Although the absolute rate of diversification reported here for the widespread clade (0.39–0.40 spp. Myr^{-1}) is modest compared with the highest rates reported from plants globally (e.g. Valente, Savolainen & Vargas, 2010; Hughes & Atchison, 2015), it is higher than the majority of rates reported for southern African plant lineages (e.g. Verboom *et al.*, 2014; Hoffmann, Verboom & Cotterill, 2015). This result may, however, reflect a bias in the sampling of southern African lineages, with most published rates from the subcontinent describing fynbos-centred Cape lineages, the low diversification rates of which may reflect the greater antiquity of their radiations (Linder, 2008), this in turn being linked to the long-term environmental stability of the fynbos zone (Linder, 2008; Verboom *et al.*, 2014). In this context, it seems likely that future research will reveal faster diversification rates to be more commonplace in the southern African flora, especially in lineages associating with recently perturbed environments. The explosive radiations of lineages such as Ruschioideae (Klak, Reeves & Hedderson, 2004), Heliophileae (Mummenhoff *et al.*, 2005) and *Babiana* Ker Gawl. ex Sims. (Schnitzler *et al.*, 2011) represent good examples, being linked to the dramatic appearance of strong summer aridity along the west coast of South Africa, towards the end of the Miocene epoch (Linder, 2008; Verboom *et al.*, 2009; Dupont *et al.*, 2011; Hoffmann *et al*. 2015).

Radiation of the widespread clade (7.50 [5.07, 10.30]–8.98 [5.97, 12.61] Mya) coincided with a transition from desert-like environments into a series of moister biomes, including grassland (7.50 [5.07–10.30] Mya) and woodland/savanna (6.20 [4.32, 8.50] Mya). Although this finding suggests a role for vegetation change in stimulating radiation in *Jamesbrittenia*, the idea that late Miocene–Pliocene uplift (3–5 Mya; Partridge & Maud, 2000) triggered radiation through its impact on the southern African landscape and vegetation appears unlikely, given our (early) estimate of the start time of radiation. In this context, it seems likely that the occupation of woodland and grassland habitats by *Jamesbrittenia* was, in the first instance, prompted by other events. One event that would almost certainly have been influential is the ecological expansion of C_4 grasses around 6–8 Mya (Cerling, Wang & Quade, 1993; Cerling *et al.*, 1997; Ségalen, Lee-Thorp & Cerling, 2007; Strömberg, 2011; Hoetzel *et al.*, 2013). Through their association with fire, C_4 grasses would have generated openings in the comparatively more closed woodlands and forests that characterized the early to mid Miocene epoch (Quade, Cerling & Bowman, 1989; Bond & Keeley, 2005; Bouchenak-Khelladi

et al., 2014), thereby creating opportunities for the occupation of such habitats by lineages of light-loving herbs and forbs (Peterson & Reich, 2008). In addition, and notwithstanding the possible prior existence of C_3 grasslands both in southern Africa (Linder & Bouchenak-Khelladi, 2015) and elsewhere (Edwards *et al.*, 2010), the overwhelmingly C_4 character of the modern grassland biome of southern Africa implies an important role for C_4 grass expansion in establishing its present extent and floristic composition. As such, it seems highly probable that the ecological expansion of C_4 grasses was instrumental in prompting the radiation of *Jamesbrittenia* in grassland and woodland/savanna habitats. This situation does not, of course, preclude a role for other events, such as late Miocene–Pliocene tectonic activity, as additional radiation stimuli. Diversification of the predominantly alpine *J. breviflora*–*J. stricta* clade (3.05 [1.66, 4.70] Mya), for example, would almost certainly have been linked to uplift, as it is this event which most likely produced the modern Drakensberg alpine zone (Linder, 2014; Linder & Verboom, 2015; Neumann & Bamford, 2015). Similarly, marine regression following a sea-level highstand around 5 Mya (Miller *et al.*, 2005) was probably instrumental in exposing the coastal limestone/calcretic substrata (Hoffmann *et al.*, 2015) that have served as an arena for radiation of the *J. stellata* Hilliard–*J. albomarginata* Hilliard clade (1.65 [0.78, 2.79] Mya). In summary, then, it appears that the Miocene–Pliocene radiation of the widespread clade was complex, being a consequence of multiple environmental phenomena operating at various stages over the past 7–9 Myr.

The major radiation of *Jamesbrittenia* was centred in non-desert environments, but our data clearly indicate a desert origin for the genus (Fig. 4). This result matches the pattern observed in other genera of Limoselleae (*Zaluzianskya*: Archibald *et al.*, 2005; *Nemesia*: Datson *et al.*, 2008) and in Scrophulariaceae as a whole (Linder & Verboom, 2015). Although an Eocene (45.13 [34.49, 55.97] Mya) origin in the desert zone, geographically centred in the southern Namib, appears at odds with suggestions based on palaeontological and sedimentary evidence that the Namib originated *c.* 17–16 Mya (Pickford & Senut, 1999, 2003; Pether, Roberts & Ward, 2000; Senut, Pickford & Ségalen, 2009), faunal deposits from the Sperrgebiet (southwestern Namibia) indicate semi-arid conditions in this area from the Eocene epoch (Pickford *et al.*, 2008, 2014). Biogeographically, most early-differentiating species in *Jamesbrittenia* (*J. ramosissima*, *J. aridicola*, *J. megaphylla* and the *J. glutinosa* (Benth.) Hilliard–*J. fimbriata* Hilliard clade) associate principally with rocky or alluvial situations surrounding the lowest reaches of the Orange River, identifying this (Gariep centre of endemism *sensu* Van Wyk & Smith, 2001) as the most likely centre of origin of the genus. This pattern is consistent with the assertion of Howis, Barker & Mucina (2009), based on a biogeographic analysis of *Gazania* Gaertn. (Asteraceae), that the 'broader region comprising Namaqualand, the Gariep region (lower Orange River valley) and the Namib Desert is an ancient centre of diversity, and possibly origin, that contains palaeo-endemics of many southern African plant lineages'. It also accords with interpretation of the arid zone of southwestern Africa as an important source of origin for the arid-land biota of Africa as a whole (Pickford, 2004; Pickford *et al.*, 2014).

Weak post-zygotic isolation, as observed in the widespread clade of *Jamesbrittenia*, appears to be a general feature of recently radiated lineages. In the Hawaiian silversword alliance (crown age = 5.2 Mya; Baldwin & Sanderson, 1998), for example, interspecific crosses typically yield highly viable offspring (Carr & Kyhos, 1986) and the same is true for the similarly aged (Willyard *et al.*, 2011) *Schiedea* Cham. & Schltdl. clade (Weller, Sakai & Wagner, 2001). Similar patterns are also observed in Andean plant radiations, with species in both the Iochrominae clade of Solanaceae (crown age = 4.56 Mya; Särkinen *et al.*, 2013) and the genus *Caiophora* C.Presl. (crown age unknown) crossing readily to produce viable offspring (Smith & Baum, 2007; Ackermann *et al.*, 2008). The implication of these patterns is that the development of strong post-zygotic isolation typically requires divergence intervals of at least 6 Myr, and that the question of whether plant species represent reproductively independent entities, corresponding to biological species (Rieseberg, Wood & Baack, 2006), depends on evolutionary context, including divergence time. When species divergence is recent, weak post-zygotic isolation forces a heavier reliance on geographical isolation and pollinator differentiation for the maintenance of species boundaries. In the widespread clade of *Jamesbrittenia* both factors are influential. As in *Schiedea* (Weller *et al.*, 2001), species in the South African widespread clade show strong range exclusivity, with 77.7% of pairwise range comparisons reflecting zero range overlap. Among pairs of species with overlapping ranges, however, the incidence of wild hybridization correlates strongly with the degree of differentiation in floral form, with florally similar species being most likely to hybridize (Table 3). This implies that florally differentiated species employ contrasting pollinators (i.e. contrasting pollinator guilds; Fenster *et al.*, 2004) and indicates an important role for floral isolation in maintaining species integrity in *Jamesbrittenia*. The importance of floral isolation has been

demonstrated previously, in studies involving a diverse range of taxa (e.g. Fulton & Hodges, 1999; Ramsey *et al.*, 2003; Kay, 2006). However, our study differs from these earlier studies in that, whereas the latter focus on single species pairs, we demonstrate the effectiveness of floral isolation across a large clade.

A general lack of post-zygotic isolation between species in the widespread clade of *Jamesbrittenia*, and in other recently radiated lineages, has important consequences for the management of floristic diversity. Although hybridization has the potential to facilitate diversification (Soltis & Soltis, 2009; Abbott *et al.*, 2013), it can also erode diversity when rare or localized species are assimilated into species with expanding ranges (e.g. introduced species) and when selection favours hybrids over their progenitors, leading to extinction of the latter ('reverse speciation': Taylor *et al.*, 2006; Sanders, Rasmussen & Guinea, 2014). Although concern over the biodiversity impacts of anthropogenic climate change has prompted serious consideration of 'assisted migration' exercises, involving the translocation of threatened species to future-suitable sites situated outside their current ranges (Milton *et al.*, 1999; Richardson *et al.*, 2009), the destructive consequences of introgression and hybridization (Rhymer & Simberloff, 1996; Allendorf *et al.*, 2001) need be borne in mind. The same is true for demographic augmentation programmes (Laikre *et al.*, 2010), revegetation of degraded landscapes (Byrne, Stone & Millar, 2011), agricultural translocations (Sampson & Byrne, 2008; Byrne & Stone, 2011) and the translocation of taxa which, like *Jamesbrittenia* (e.g. http://www.google.com/patents/USPP12574), have horticultural appeal. Particularly in the case of recently diverged lineages, in which species boundaries are maintained largely by geography, the implication is that the hybridization risk associated with any translocation needs to be thoroughly assessed before such translocation is put into effect.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1. Sequences used in the construction of composite terminals for inclusion in the higher-level dating analysis. For each sequence, the species sampled and the GenBank accession number are indicated.

Table S2. Morphological character matrix used to assess the alternative placements of conflict taxa. State numbering follows Table 2.

Table S3. Parameter values describing the calibration priors used in the higher-level (rows 1–7) and *Jamesbrittenia* (rows 8–10) molecular dating analyses.

Figure S1. Aligned *GScp* sequences employed in phylogenetic analyses. Nucleotide bases are colour-coded as follows: A, red; C, green; G, yellow; T, blue; ambiguous or missing, grey; gap, black; unalignable, white.

Figure S2. Aligned *psbA–trnH* sequences employed in phylogenetic analyses. Nucleotide bases are colour-coded as follows: A, red; C, green; G, yellow; T, blue; ambiguous or missing, grey; gap, black; unalignable, white.
Figure S3. Aligned *rps16* sequences employed in phylogenetic analyses. Nucleotide bases are colour-coded as follows: A, red; C, green; G, yellow; T, blue; ambiguous or missing, grey; gap, black; unalignable, white.
Figure S4. NeighbourNet networks derived using the (a) GScp and (b) plastid sequence data.
Figure S5. BEAST maximum clade credibility chronogram based on the higher-level data set. Open circles indicate primary calibration points. The major angiosperm lineages are labelled on the right.
Figure S6. Comparison of branch support (heavy branches have posterior probability > 0.95) suggested by BEAST analyses of the combined matrix (a) including all taxa but including for conflict taxa only those accessions (i.e. *GScp* or plastid) whose suggested placements are more consistent with morphology, and (b) excluding taxa with incomplete data (indicated in pale grey). To facilitate comparison, both sets of branch support are plotted on the same background tree, the maximum clade credibility tree produced by analysis (a).