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Phylogeny of the holly grevilleas (Proteaceae) based on nuclear ribosomal and chloroplast DNA

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Abstract. The holly grevilleas are an informal grouping of 15 species (19 taxa) of woody shrubs from south-eastern Australia, with a centre of distribution in central to western Victoria. Many of the species are narrowly endemic. The present study is the first molecular-phylogenetic analysis of the group, with the aim of providing an evolutionary framework for assessing species-level taxonomy and conservation priorities. Analyses using the nrDNA internal transcribed spacer (ITS) regions were complicated by the presence of divergent paralogues, including inferred pseudogenes; analyses restricted to presumed orthologous, functional ITS sequences were uninformative. Combined analyses of three chloroplast intergenic spacers (*trn*Q–5′*rps*16, *trn*L–*trn*F and *rpo*B–*trn*C) strongly support the monophyly of a core group of 16 taxa (the 'southern holly grevilleas') from Victoria and South Australia. However, nodes outside this group are poorly resolved and poorly supported, and the relationships of taxa from New South Wales and eastern Victoria (the 'northern holly grevilleas') are unclear. Among the southern holly grevilleas, the following four distinct and partly sympatric cpDNA clades are identified: the '*Grevillea ilicifolia*', '*G. aquifolium*', '*G. dryophylla*' and '*G. repens*' clades, among which the earliest and most strongly supported divergence is that of the western-most '*G. ilicifolia*' clade. Variation in cpDNA is incongruent with current species-level taxonomy, especially for *G. aquifolium* (polyphyletic), *G. montis-cole* (polyphyletic, but the two subspecies each monophyletic) and *G. microstegia* (nested in *G. aquifolium*). The effects of incomplete chloroplast lineage sorting, gene flow through hybridisation or introgression, and inappropriate taxonomy are possible explanations for this incongruence. The formal conservation listing for some species within the holly grevillea group requires re-evaluation.

Additional keywords: *Grevillea aquifolium* group, holly-leaved grevilleas, ITS, molecular phylogeny, Proteaceae, rDNA pseudogenes, *rpo*B–*trn*C, taxonomy, *trn*L–*trn*F, *trn*Q–5′*rps*16.

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Introduction

Grevillea R.Br. ex Knight (Proteaceae) is a genus in which there is considerable taxonomic uncertainty. The latest treatment lists 362 recognised species, 357 of which are endemic to Australia (Makinson 2000), and a small number endemic to (or also found in) New Caledonia, Papua New Guinea and Sulawesi. Several new taxa have also been described since that publication (e.g. Downing *et al*. 2004). As currently defined, *Grevillea* is the third largest genus of flowering plants in Australia, after *Acacia* and *Eucalyptus* (George 1998).

The size of the genus *Grevillea* and the range of forms found within it complicate the application of formal infrageneric taxonomy and the delineation of some taxa using traditional taxonomic methods. Two informal treatments (McGillivray and Makinson 1993; Makinson 2000) and an extensive, horticulturally oriented taxonomic reworking with narrower species treatments (Olde and Marriott 1994, 1995*a*, 1995*b*) have been applied in recent times after the last formal treatment over a century ago (Bentham 1870). Despite the extensive work of these taxonomists, the relationships among species and the taxonomy of some sections of the group are still contentious. The current classification is predominantly based on comparative morphology, with some phylogenetic considerations, and divides the genus into 33 groups

(Makinson 2000). However, there have been no phylogenetic analyses for the entire genus.

The holly-leaved grevilleas (or *G. aquifolium* group of species) are one group that warrants closer phylogenetic attention. This informal group of small woody shrubs is placed, along with 33 other species, in the Aspleniifolia–Hookeriana subgroup of the Pteridifolia group (Makinson 2000). It corresponds to Species 13–26 in Makinson (2000), Group 1.2.1.5 in the classification of McGillivray and Makinson (1993), part of Group 35 as described by Olde and Marriott (1994), and also closely to series *Hybegynae* of Bentham's (1870) classification. The group has been defined as including 'species with holly-like leaves and 'toothbrush' inflorescences, and…their close relatives' (McGillivray and Makinson 1993). These features are potentially apomorphic within the genus, but have not been tested by phylogenetic analysis.

The holly grevilleas are distributed in south-eastern mainland Australia, and include 15 species (19 taxa). The group's distribution is centred on central to western Victoria, but extends to the Eyre Peninsula, South Australia, to the west and to largely disjunct sites in southern New South Wales and near the Queensland–New South Wales border to the north-east (Fig. 1). Many taxa are narrowly endemic, especially in western Victoria (nine species, Fig. 1). Their evolutionary relationships and taxonomy are somewhat unclear because there is great phenotypic variability within some species, leading to several new species being recognised in recent decades (e.g. Molyneux 1975, 1985; Smith 1981, 1983). For example, *G. ilicifolia* was recently split into three species (two with subspecies) on the basis of leaf morphology (Downing *et al*. 2004), whereas *G. williamsonii* is no longer recognised, being considered a male-sterile morphological variant of *G. aquifolium* (James 2000, 2004; Walsh and Stasjic 2007). The species *G. aquifolium* has been the source of taxonomic contention, with many populations displaying distinct forms or ecological niches (e.g. Olde and Marriott 1995*a*). In all, 12 of the 19 recognised taxa were listed as *Rare*, *Vulnerable* or *Endangered* on the most recent 'Rare or Threatened Australian Plants' (ROTAP) list (Briggs and Leigh 1996) and six are currently considered vulnerable on the *EPBC Act* list of threatened flora (http://www.environment.gov.au/cgi-bin/sprat/public/publicthreatenedlist.pl?wanted=flora, accessed 17 July 2013). Several populations are known to be extinct or close to extinction (e.g. *G. infecunda* in south-eastern Melbourne, *G. ilicifolia* in New South Wales, *G. aquifolium* from near Portland) (Olde and Marriott 1995*a*). Two of the species in the group (*G. infecunda* and *G. renwickiana*) are thought to be sterile, displaying obligate clonal reproduction by 'root-suckering' (Kimpton *et al.* 2002; James and McDougall 2014).

The aim of the current study was to investigate relationships among the holly grevilleas by using DNA sequence data. The work was undertaken to provide an evolutionary framework for assessing species-level taxonomy and conservation priorities within the group. Given uncertainty surrounding higher-level relationships within *Grevillea*, and based on their morphological resemblance and informal classification, the holly grevilleas are considered *a priori* a cohesive group for phylogenetic study, even though their monophyly has not been tested by higher-level phylogenetic analysis.

Materials and methods

Taxon sampling

Leaf material (desiccated in silica gel for molecular analyses) and voucher herbarium specimens were obtained from each of the 15 recognised species (19 taxa) in the holly grevillea group. Where possible, more than one accession was collected for each ingroup taxon, particularly for those with disjunct or extensive geographical ranges and those with distinct morphological or ecological forms. In particular, sampling within *G. aquifolium* was targeted to ensure that the range of observed morphological variation across its geographic range was included in the analysis. Outgroup taxa included five species from the Pteridifolia group (Makinson 2000), namely three eastern members (*G. acanthifolia*, *G. laurifolia*, *G. willisii*) and one Western Australian member (*G. dryandroides*) of the Aspleniifolia–Hookeriana subgroup, and one Western Australian member of the Bipinnatifida subgroup (*G. bipinnatifida*). *G. alpina* (Linearifolia subgroup, Floribunda group *sensu* Makinson 2000), along with two species from the sister genus *Hakea*, were also included as outgroups for some analyses of internal transcribed spacer (ITS) sequences; these additional outgroups could not be used for analyses of cpDNA because sequence variation made alignment problematic. Samples were sourced from natural populations or from garden or nursery material grown from field-collected specimens (Table 1).

DNA isolation, polymerase chain reaction (PCR) and sequencing

DNA was isolated from ground, dried leaf tissue by using either a DNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA) following the manufacturer's protocol, or at the Australian Genome Research Facility (AGRF), Adelaide, by using a Nucleospin Plant L extraction kit (Machery–Nagel, Düren, Germany), following the PL1 method outlined in the product manual (J. Chambers, AGRF Adelaide, pers. comm. 2009). All DNA isolates were stored at −20°C.

The nuclear rDNA ITS regions (ITS-1, 5.8S, ITS-2) and small segments of the flanking 18S and 26S ribosomal subunits were successfully amplified for a limited number of taxa in the study group using the primer pairs ITS5m–ITS4 (ITS5m, Sang *et al*. 1995; ITS4, White *et al*. 1990) or the new primers PrF (5′-GCGAGAAGTCCACTGAACC-3′)–PrR (5′-CTGAGGACGCTTCTACAGAC-3′). However, because of amplification specificity problems, difficulties sequencing through ITS-1 in the majority of taxa and the presence of paralogous ITS copies, we designed a primer specific to the putative functional ITS copy that excluded ITS-1 (PriA; 5′-GAACATCACAACGGAACGGG-3′) to be coupled with the reverse primers PrR or Pr2R (5′-GCCCGATTCTCAAGCTGG-3′). Subsequently, only the 5.8S, ITS-2 of the ITS and partial 26S subunit (hereafter referred to as ITS2) was amplified with these primers. The complementary strands for this DNA fragment were subsequently sequenced with the forward primer PriA, and PrR, Pr2R or ITS4 as a reverse primer. A summary of approaches attempted for amplifying the ITS is presented in appendix 1 of Holmes (2008).

The following PCR reagents and conditions proved successful for amplifying the putative functional ITS2 fragment:

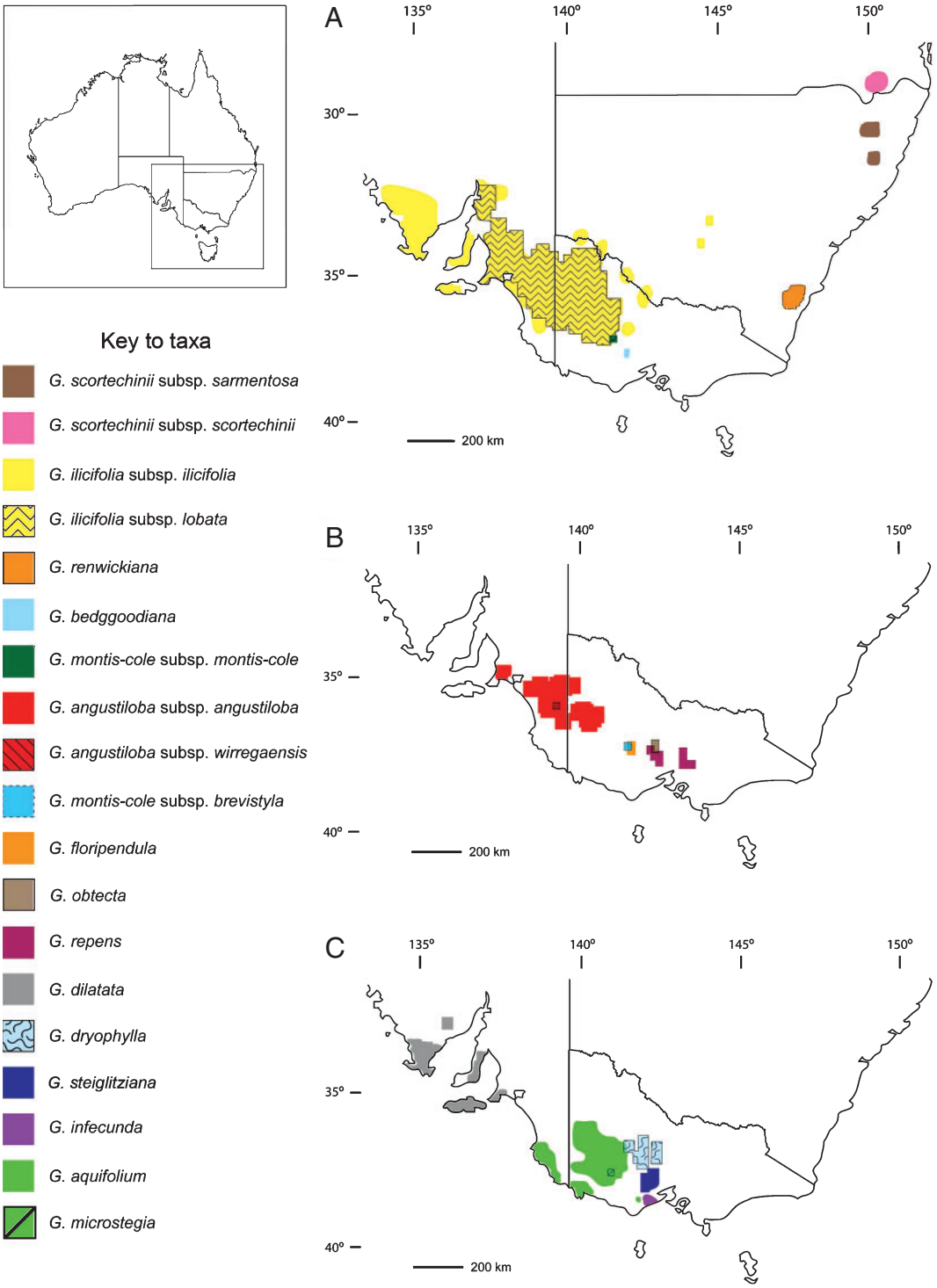

Fig. 1. Geographic distributions for the members of the holly grevilleas in south-eastern Australia.

30-μL reactions consisted of 1.0 U DNA polymerase (Immolase, Bioline, Alexandria, NSW), 200 μM dNTPs (Bioline), 2.0 mM $MgCl_2$, 1 × PCR buffer (Immobuffer, Bioline), 0.5 μM of each primer, 0.3 μL 100 × bovine serum albumin (BSA) (New England BioLabs, Ipswich, MA, USA), 5% (v/v) dimethyl sulfoxide (DMSO), 0.5–1.5 μL genomic DNA (~10–50 ng) and dH_20 to volume. PCR conditions consisted of an initial denaturation cycle at 95°C for 7 min, followed by 30 cycles of 30 s at 95°C, 45 s at 49°C and 45 s at 72°C. The last cycle was followed by an extension step of 3 min at 72°C. Reactions were performed using

Table 1. Voucher information for samples analysed in the study

Samples are listed alphabetically by species and identifier with outgroup species given first. Details of herbarium voucher numbers, collectors, sample-collection localities and GenBank numbers for four DNA regions are listed where applicable. Herbarium abbreviations are as follows: AD, State Herbarium of South Australia; BISH, Herbarium Pacificum; MEL, National Herbarium of Victoria; MELU, The University of Melbourne Herbarium; NE, NCW Beadle Herbarium. Collector abbreviations are as follows: AKG, A. Gibbs; CI, C. Imada; EAJ, E. James; GDH, G. Holmes; JJ, J. Jeanes; KM, K. McDougall; NM, N. Marriott; NW, N. Walsh; PO, Peter Olde; TD, T. Downing; TJC, T. Christensen; WM, W. McDowell. Locality abbreviations are as follows: Cult., Cultivated; NSW, New South Wales; Qld, Queensland; SA, South Australia; Vic., Victoria; WA, Western Australia; PNG, Papua New Guinea; RBGM, Royal Botanic Gardens Melbourne; RBGC, Royal Botanic Gardens Cranbourne; WBG, Wittunga Botanic Gardens; NFR, Native Forest Reserve; NHP, National Heritage Park; NP, National Park; SP, State Park; SF, State Forest. ITS accession numbers refer to the 5.8S ribosomal subunit, ITS-2 and partial 26S subunit, unless otherwise indicated

Taxon	Identifier	Herbarium voucher number	Voucher collector	Locality and reference	GenBank accession numbers			
					ITS	*trn*L–*trn*F	*rpo*B–*trn*C	*trn*Q–5′*rps*16
Outgroup taxa								
Grevillea acanthifolia A.Cunn. subsp. *acanthifolia*	ECNSW1	MELU D104195	GDH	NSW. Cult. RBGM nursery. Source unknown (east–central NSW).	FJ468564	FJ468645	FJ468632	KJ653411
G. alpina Lindl.	CASTLEMAINE-DIGGINGS	MELU D104193	GDH	Vic. Porcupine Ridge Road, Castlemaine Diggings NHP.	FJ468561[A]	–	–	–
G. bipinnatifida R. Br.	SWWA-1	MELU D104198	GDH	WA. Cult. RBGM nursery. Source unknown (south-western WA).	FJ468562	–	–	–
G. dryandroides C.A.Gardner subsp. *hirsuta* Olde & Marriott	SWWA-2	MELU D104188	GDH	WA. Cult. Maranoa Gardens, Melbourne. Source unknown (south-western WA).	FJ468563	–	–	–
G. laurifolia Sieber ex Spreng.	ECNSW2	MELU D104179	GDH	NSW. Cult. Vaughan's Nursery, Curlewis Vic. Source unknown (east-central NSW).	FJ468587	FJ468639	FJ468634	KJ653412
G. papuana Diels	PAPUA NEW GUINEA	No voucher reported		PNG. (Wright *et al.* 2006).	DQ499131	–	–	–
G. robusta A.Cunn. ex R.Br.	HAWAII	BISH 687877	CI/WM	Hawai'i. Naturalised population (Howarth *et al.* 2007).	AY864893	–	–	–
G. willisii R.V.Sm. & McGill.	EVIC	MELU D104186	GDH	Vic. Cult. Maranoa Gardens, Melbourne, Vic. Source unknown (Omeo region, eastern Vic.).	–	FJ468640	FJ468635	KJ653413
Hakea nodosa R.Br.	SVIC1	MELU D103888	GDH	Vic. RBGC, southern Vic.	FJ468560	–	–	–
H. ulicina R.Br.	SVIC2	MELU D103889	GDH	Vic. RBGC, southern Vic.	FJ468559	–	–	–
Ingroup taxa								
G. angustiloba (F.Muell.) Downing subsp. *angustiloba*	SETTLEMENT-RD	MEL 2280819	JJ/NW	Vic. Settlement Cooack Road, Little Desert NP.	FJ468571	FJ468648	FJ468597	KJ653435

(*continued next page*)

Table 1. (*continued*)

Taxon	Identifier	Herbarium voucher number	Voucher collector	Locality and reference	GenBank accession numbers			
					ITS	*trn*L–*trn*F	*rpo*B–*trn*C	*trn*Q–5′*rps*16
G. angustiloba (F.Muell.) Downing subsp. *wirregaensis* Downing	WIRREGA	MELU D110017	TD	SA. Between rail lines and Wurlyana Road north of Dukes Highway, 650 m from junction of Wurlyana Road and Wirrega Road North, near Wirrega.	–	KJ653401	KJ653381	HM130889
G. aquifolium Lindl.	ARAPILES1	MELU D110029	TD	Vic. On unmarked track ~20 m from the lookout road at Bluff Picnic Area, Mount Arapiles–Tooan SP.	–	KJ653382	KJ653360	HM130890
G. aquifolium	ARAPILES2	MELU D110031	NM	Vic. At base of Mount Arapiles, on the northern face 1.5 km west of toilet block, Mount Arapiles–Tooan SP.	–	KJ653383	KJ653361	KJ653403
G. aquifolium	ASSES-EARS	MELU D110028	TD	Vic. On Asses Ears Road, 2.6 km from intersection with Mount Victory Road, Grampians NP.	–	KJ653384	KJ653362	HM130883
G. aquifolium	BAGDAD	MELU D110014	TD	SA. On northern border access track, 1.4 km north along eastern border track from intersection with Dairy Range Road, Bagdad NFR.	–	KJ653385	KJ653363	KJ653404
G. aquifolium	BEEHIVE-FALLS	MEL 2376584	PO	Vic. Beehive Falls, along track from car park, Roses Gap Road, Grampians NP.	–	KJ653386	KJ653364	KJ653405
G. aquifolium	BRAY	MELU D110016	TD	SA. On forestry access road, 2 km from the intersection with Robe–Penola road, Bray Junction NFR.	–	KJ653387	KJ653365	HM130888
G. aquifolium	CARPENTER-ROCKS	MELU D110015	TD	SA. On access track, 700 m along Pelican Point Road from intersection with Carpenter Rocks Road, Carpenter Rocks Conservation Park.	–	KJ653388	KJ653367	HM130887
G. aquifolium	CASSIDY	MELU D104197	EAJ	Vic. Cult. RBGM Nursery. Source Cassidys Gap, Grampians NP.	FJ468590	–	–	–
G. aquifolium	COOACK-ROAD1	MEL 2280817	JJ/NW	Vic. Cooack Road, Little Desert NP. Cooack form *sensu* Olde and Marriott (1995*a*).	FJ468591	FJ468669	FJ468598	HM130896

G. aquifolium	DEEP-LEAD	MELU D110022	TD	Vic. At intersection of Stawell–Deep Lead Road and East–West Road, Deep Lead Flora and Fauna Reserve.	–	KJ653389	KJ653368	HM130891
G. aquifolium	FYANS-CREEK	MELU D110030	TD	Vic. Fyans Creek at weir, 1.9 km south of intersection of Halls Gap–Dunkeld road and Mount William Road, Grampians NP.	–	KJ653390	KJ653369	KJ653414
G. aquifolium	GLENISLA-CROSSING	MELU D110021	TD	Vic. On Glenisla Crossing Road, 4 km east of intersection with Henty Highway, Grampians NP.	–	KJ653391	KJ653370	KJ653415
G. aquifolium	KENTBRUCK	MELU D110018	TD	Vic. On Kentbruck Settlement Road between Bark Carters and Cut Out Dam tracks, Kentbruck Heath area, Lower Glenelg NP.	–	KJ653392	KJ653371	KJ653420
G. aquifolium	KN-CARPENTER	MELU D104183	GDH	SA. Cult. RBGM nursery ex Kuranga Nursery, Ringwood. Source Carpenter Rocks area, south-eastern SA. SA form *sensu* Olde and Marriott (1995*a*).	FJ468595	FJ468653	FJ468602	HM130895
G. aquifolium	LAKE-WARTOOK1	MELU D104192	EAJ	Vic. Lake Wartook area, Grampians NP. Lake Wartook prostrate form *sensu* Olde and Marriott (1995*a*).	FJ468589	FJ468666	FJ468603	HM130897
G. aquifolium	MANJA-SHELTER	MELU D110027	TD	Vic. On walking track to Manja Shelter, 14.6 km on Harrop Track north of intersection with Glenelg River Road, Victoria Range, Grampians NP.	–	KJ653393	KJ653372	KJ653424
G. aquifolium	MCDONALD-HWY	MELU D110020	TD	Vic. At intersection of McDonald Highway and Old Nhill Road, central Block, Little Desert NP.	–	KJ653394	KJ653373	HM130885
G. aquifolium	MOYSTON	MELU D104181	EAJ	Vic. Moyston–Pomonal area. Halls Gap form *sensu* Olde and Marriott (1995*a*).	FJ468579[A]	FJ468663	FJ468599	HM130898
G. aquifolium	MTZERO-ROAD	MELU D110023	TD	Vic. On western side of Mount Zero Road; 4.1 km north of intersection with Red Gum Lease Track, Grampians NP.	–	KJ653395	KJ653374	KJ653426

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Table 1. (*continued*)

Taxon	Identifier	Herbarium voucher number	Voucher collector	Locality and reference	GenBank accession numbers			
					ITS	*trn*L–*trn*F	*rpo*B–*trn*C	*trn*Q–5′*rps*16
G. aquifolium	POHLNER-ROAD	MELU D110026	TD	Vic. Corner Pohlner Road and Stapylton Campground Track, 1.7 km from intersection of Pohlner and Halls Gap-Flat Rock roads, Grampians NP.	–	KJ653396	KJ653375	KJ653431
G. aquifolium	POMONAL	MELU D110024	TD	Vic. On Halls Gap–Ararat road, north of Pomonal, 350 m from the intersection of Halls Gap–Ararat road and Church Road, Grampians NP.	–	KJ653397	KJ653376	KJ653432
G. aquifolium	ROSES-GAP	MELU D104182	EAJ	Vic. Roses Gap, Grampians NP.	–	FJ468652	FJ468604	HM130899
G. aquifolium	SERRARANGE-TRACK	MELU D110025	TD	Vic. On Serra Range Track, 7 km from intersection of Fields and Griffin tracks, western side of Serra Range, Grampians NP.	–	KJ653399	KJ653379	HM130884
G. aquifolium	SOUTHERN-BREAK	MELU D110019	TD	Vic. On unmarked track, 2 km east of intersection of Southern Break with Cooack Road and Cooack Settlement Road, Eastern Block, Little Desert NP.	–	KJ653400	KJ653380	KJ653436
G. bedggoodiana J.H.Willis ex McGill.	ENFIELD	MELU D103812	GDH	Vic. Italian Gully Road, Enfield SF, central Vic.	FJ468585	FJ468661	FJ468605	HM130900
G. dilatata (R.Br.) Downing	KANGAROO-ISLAND	AD 99511320	TJC	SA. Cult. WBG. Source near American River, Kangaroo Island.	FJ468572	FJ468646	FJ468606	KJ653419
G. dryophylla N.A.Wakefield	KANGAROO-FLAT	MELU D103816	GDH	Vic. Rail Reserve, Kangaroo Flat, central Vic. Bendigo form *sensu* Olde and Marriott (1995*a*).	FJ468577	FJ468672	FJ468607	KJ653418
G. dryophylla	ST-ARNAUD	MELU D103813	GDH	Vic. St Arnaud (South) SF, central Vic. St Arnaud form *sensu* Olde and Marriott (1995*a*).	FJ468578	FJ468675	FJ468608	KJ653437
G. floripendula R.V.Sm.	MUSICAL-GULLY1	MELU D103797	GDH	Vic. Camp Hill SF, central Vic. Musical Gully form *sensu* Olde and Marriott (1995*a*).	FJ468576	FJ468670	FJ468610	KJ653427
G. floripendula	MUSICAL-GULLY2	MELU D104187	EAJ	Vic. Camp Hill SF, central Vic. Musical Gully form *sensu* Olde and Marriott (1995*a*).	FJ468575	FJ468680	FJ468638	KJ653428

G. ilicifolia (R.Br.) R.Br. subsp. *ilicifolia*	NERICON	MELU D103821	GDH	NSW. Greening Australia Reserve, Nericon, central-southern NSW.	FJ468568	FJ468649	FJ468611	HM130902
G. ilicifolia subsp. *lobata* (F.Muell.) Downing	BLACKRANGE	MELU D103801	EAJ	Vic. Cultivated RBGM nursery. Source Black Range SP, western Vic.	FJ468570	–	–	–
G. ilicifolia subsp. *lobata*	DUNOLLY	MELU D103815	GDH	Vic. Erskines Flora Reserve number 2, Dunolly, central Vic. Eastern-most population in Victoria.	FJ468569	FJ468651	FJ468613	HM130901
G. infecunda McGill.	BREAKFAST-CREEK	MEL 2346941	EAJ	Vic. Breakfast Creek Road, ~1 km S of Hammonds Road intersection, Great Otway NP *sensu* Kimpton *et al.* (2002).	–	KF724187	KJ653366	KJ653406
G. infecunda	GREVILLEA-TRACK	MELU D103985	GDH	Vic. Grevillea Track, Great Otway NP, southern Vic. Love population *sensu* Kimpton *et al.* (2002).	–	FJ468676	FJ468614	KJ653416
G. infecunda	GUMFLAT	MELU D103984	GDH	Vic. Gumflat Tk, Great Otway NP, southern Vic. Gumflat population *sensu* Kimpton *et al.* (2002).	FJ468677	–	–	–
G. infecunda	PROVING-GROUND	MEL 2346940	EAJ	Vic. Australian Automotive Research Centre, northern side of Gumflat Road, Anglesea.	–	KF724188	KJ653377	KJ653433
G. microstegia Molyneux	LIBRARIAN-TRACK	MELU D103800	EAJ	Vic. Librarian Track, Pomonal, western Vic.	FJ468582	FJ468664	FJ468616	HM130904
G. microstegia	MN-GRAMPIANS	MELU D104185	GDH	Vic. Cult. Maranoa Gardens, Balwyn Vic. Source Grampians NP (site unknown).	–	FJ468659	FJ468617	HM130903
G. montis-cole R.V.Sm. subsp. *montis-cole*	CAMP-ROAD	MELU D104177	GDH	Vic. Camp Road, Mount Buangor SF, central Vic.	–	FJ468674	FJ468621	KJ653408
G. montis-cole subsp. *montis-cole*	THE-GLUT	MELU D103798	GDH	Vic. Near The Glut picnic ground, Mount Buangor SF, central Vic.	FJ468584	FJ468673	FJ468622	KJ653439
G. montis-cole R.V.Sm. subsp. *brevistyla* R.V.Sm.	LANGI-GHIRAN1	MELU D103799	GDH	Vic. Langi Ghiran SP, central Vic.	FJ468580	FJ468668	FJ468618	KJ653421
G. montis-cole subsp. *brevistyla*	LANGI-GHIRAN2	MELU D104011	GDH	Vic. Langi Ghiran SP, central Vic.	FJ468581	FJ468658	FJ468619	KJ653422
G. montis-cole subsp. *brevistyla*	LANGI-GHIRAN3	MELU D104176	GDH	Vic. Langi Ghiran SP, central Vic.	–	FJ468657	FJ468620	KJ653423

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Table 1. (*continued*)

Taxon	Identifier	Herbarium voucher number	Voucher collector	Locality and reference	GenBank accession numbers			
					ITS	*trn*L–*trn*F	*rpo*B–*trn*C	*trn*Q–5′*rps*16
G. obtecta Molyneux	CASTLEMAINE1	MELU D103887	GDH	Vic. Porcupine Ridge Road, Castlemaine Diggings NHP, central Vic. Intermediate leaf size form *sensu* Molyneux (1985).	FJ468573	FJ468654	FJ468624	KJ653409
G. obtecta	CASTLEMAINE2	MELU D103810	GDH	Vic. Freyers Ridge Road, Castlemaine Diggings NHP, central Vic. Freyers Range form *sensu* Olde and Marriott (1995*a*); small leaf size form *sensu* Molyneux (1985).	FJ468574[A]	FJ468656	FJ468623	KJ653410
G. renwickiana F.Muell.	MORTON	MELU D104184	KM	NSW. Morton NP, south-eastern NSW.	–	FJ468679	FJ468636	HM130830
G. renwickiana	NERRIGA	MELU D104189	EAJ	NSW. Nerriga, south-eastern NSW. Nerriga form *sensu* Olde and Marriott (1995*a*).	FJ468593	FJ468643	FJ468627	KJ653429
G. repens F.Muell. ex Meisn.	AMBLERS-LANE	MELU D104010	GDH	Vic. Amblers Lane, north-west Lerderderg SP, central Vic. AL population of Holmes (2008).	FJ468565	FJ468644	FJ468628	KJ653402
G. repens	MASONS-FALLS	MELU D103817	GDH	Vic. Masons Falls, Kinglake NP, central Vic. Mount Slide form *sensu* Olde and Marriott (1995*a*); MF population of Holmes *et al.* (2009).	–	FJ468655	FJ468625	KJ653425
G. repens	PAULSRANGE-TRACK	MELU D104190	GDH	Vic. Pauls Range Track, Toolangi SF, central Vic. Mount Slide form *sensu* Olde and Marriott (1995*a*); PR suckering form of Holmes *et al.* (2009).	FJ468567	FJ468678	FJ468637	KJ653430
G. repens	STAR-TRACK	MELU D103802	GDH	Vic. Star Track, Muskvale, Wombat SF, central Vic. Daylesford form *sensu* Olde and Marriott (1995*a*); ST population of Holmes *et al.* (2009).	FJ468566	FJ468665	FJ468629	KJ653438

G. scortechinii (F.Muell. ex Scort.) F.Muell. subsp. *scortechinii*	STANTHORPE	MELU D104180	GDH	Qld. Cult. Vaughan's Nursery, Curlewis. Source Stanthorpe area, southern Qld.	FJ468586	–	–	–
G. scortechinii subsp. *sarmentosa* (Blakely & McKie) F.Muell.	SARA-RIVER	NE 96725	AKG	NSW. Private property, north of Sara River, Mount Mitchell, northern NSW.	–	KJ653398	KJ653378	KJ653434
G. steiglitziana N.A.Wakefield	BRISBANE-RANGES	MELU D103811	GDH	Vic. McCleans Highway, Brisbane Ranges NP, central Vic.	FJ468583	FJ468671	FJ468609	KJ653407
G. steiglitziana	INGLISTON	MELU D103983	GDH	Vic. Ingliston Gorge, Werribee Gorge SP, central Vic. Isolated from Brisbane Ranges populations.	FJ468592	FJ468681	FJ468630	KJ653417

[A]Partial 18S, complete ITS-1, 5.8S and ITS-2, partial 26S.

a GeneAmp 9700 (Perkin–Elmer, Waltham, MA, USA) or a Mastercycler gradient thermal cycler (Eppendorf, North Ryde, NSW).

After an initial screening of nucleotide variation across multiple samples within several chloroplast DNA regions (Holmes 2008; Downing 2012), three intergenic spacers from the large single-copy genome region (*trn*Q$^{(UUG)}$–5′*rps*16, *trn*L$^{(UAA)}$–*trn*F$^{(GAA)}$ and *rpo*B–*trn*C$^{(GCA)}$) were amplified for all samples. The primers used for the *trn*Q–5′*rps*16 spacer were *trn*Q$^{(UUG)}$ and *rps*16x1 (Shaw *et al*. 2007). For the *trn*L–*trn*F spacer, the primers 'e' and 'f' from (Taberlet *et al*. 1991) were used. The *trn*C end of the *rpo*B–*trn*C spacer was amplified using the primer *trn*C^{GCA}R (Shaw *et al*. 2005) and the new grevillea-specific internal primer BCif (TCGCAGGACAAAGAACA AAG); PCR amplification using the latter primer was successful for all sampled taxa, except *G. dryandroides* subsp. *hirsuta*, which was found to have a deletion overlapping the primer site.

For cpDNA amplification, several protocols were used initially, dependent on the DNA region of interest and availability of reagents (Holmes 2008). Most PCR reactions (25 μL) contained 0.2 μM of each forward and reverse primer, 2.5 mM of each dNTP, 0.1 μL of HotStar*Taq* DNA polymerase with 2.5 μL of 10 × PCR buffer (QIAGEN), 5–50 ng of genomic DNA, and dH_20 to volume. For the *rpo*B–*trn*C and *trn*Q–5′*rps*16 intergenic spacers, reactions contained 2.0 mM $MgCl_2$ and 0.5 μL of non-acetylated BSA (Fermentas, Vilnius, Lithuania), 0.1 μL of HotStar*Taq* DNA polymerase, 2.5 μL of 10 × PCR buffer (QIAGEN), 2.0 μL of DNA isolate and dH_20 to volume.

CpDNA PCR amplifications were performed on an Eppendorf Mastercycler with the lid heated to 105°C. A 'touch-down' PCR protocol was employed for the *trn*L–*trn*F spacer. The PCR conditions were as follows: one cycle at 95°C for 15 min; five cycles of 95°C for 30 s, 64°C for 30 s (decreasing by 2.0°C each cycle), 72°C for 1 min; 30 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 1 min; a final extension of 72°C for 10 min. For the *trn*Q–5′*rps*16 and *rpo*B–*trn*C spacers, the PCR protocol was based on the 'slow and cold' '*rpl*16' program of Shaw *et al*. (2007). PCR conditions consisted of one cycle at 95°C for 15 min; 30 cycles of 95°C for 1 min, 50°C for 1 min (increasing by 0.5°C each cycle), 65°C for 4 min; followed by a final extension of 65°C for 5 min.

After checking PCR amplification success, products were purified using a QIAquick purification kit (QIAGEN), Illustra GFX PCR DNA purification kit (GE Healthcare, Little Chalfont, UK) or enzymatically by Macrogen Inc. (Seoul, Korea). Sequencing reactions were performed in-house with an ABI Prism BIG Dye Terminator v.3.1 Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) or by either AGRF or Macrogen, followed by capillary separation by AGRF, Macrogen or the Applied Genetics Diagnostic Group (University of Melbourne, Australia).

Sequence editing and alignment

Contiguous sequences for each region were assembled and edited using Sequencher v.4.8 (Gene Codes Corporation, Ann Arbor, MI, USA). Edited sequences were aligned manually using Se-Al

Sequence Alignment Editor v. 2.0a11 (Rambaut 2002). Gaps were inserted to ensure positional homology and to minimise nucleotide mismatches (Kelchner and Clark 1997). All variable nucleotide positions were checked against the original chromatogram files to ensure the accuracy of base calls.

Insertion–deletion events (INDELs) were coded using a method equivalent to the 'simple coding' of Simmons and Ochoterena (2000), with a single character representing each INDEL whether single- or multi-base. There was a deletion of 69 bp in the *trn*Q–5′*rps*16 intergenic spacer of two accessions of *G. aquifolium* from Roses Gap in the Grampians Ranges, Victoria. Because the sequence data corresponding to this deletion in other accessions contained several base substitutions and single-base insertions, all variable base positions were included, but with the two Roses Gap accessions scored as 'missing' for those positions. Also, in the *trn*Q–5′*rps*16 spacer, there is a short inverted (and complimented) region (Bases 346–350 in the alignment). Although initially included as a binary character, on closer examination, this region was found to be the loop of a putative stem–loop structure and homoplasious, and so was excluded from the final analyses. Alignments included four variable poly-N regions, two each in the *trn*L–*trn*F and *rpo*B–*trn*C spacers. Two were highly variable and homoplasious and were excluded from analysis. The other two were coded as multistate characters for phylogenetic analysis. Alignments used for analysis are available on request.

In an initial study, one accession of *G. bedggoodiana* (MELU D104178; not listed in Table 1) was found to contain a small number of polymorphic nucleotide sites distributed across the *trn*L–*trn*F and *rpo*B–*trn*C spacers. Heteroplasmy was confirmed by the re-isolation of DNA and subsequent sequencing of cloned copies of an *rpo*B–*trn*C segment (Holmes 2008). Data from this sample were not used in subsequent analyses.

Comparison of ITS paralogues

For several taxa in our dataset, we were able to amplify and sequence paralogous copies of ITS. Paralogues within and among individuals were compared, with a view to distinguishing potentially functional copies from pseudogenes. ITS-1 was highly divergent among paralogues and difficult to align; therefore, our comparisons focussed on the 5.8S gene and ITS-2. Comparisons included G/C content, the number of CpG and CpNpG methylation sites and the presence of conserved motifs, all of which have been shown to vary between functional and pseudogenic rDNA (Buckler *et al*. 1997; Bayly and Ladiges 2007). Sequences were examined for the presence of four motifs in the 5.8S gene previously identified as highly conserved in flowering-plant rDNA. These were as follows: 5′-GAATTGCAGAATC (Jobes and Thien 1997); an EcoRV restriction site (GATATC) near the 5′ end (Liston *et al*. 1996); the motifs M1 and M3 identified by Harpke and Peterson (2008). We also compared, among paralogues, the distribution of variable sites across the 5.8S gene and ITS-2. Boundaries between these regions were identified by comparisons with previously published sequences, including those for *Macadamia* (Proteaceae; Mast *et al*. 2008).

Phylogenetic analyses

Parsimony analyses were performed using PAUP* 4.0b10 (Swofford 2001). For all analyses, individual nucleotide sites (base positions) were coded as equally weighted, unordered multistate characters; missing data were coded as '?', and gaps (coded in the matrix as '–') were treated as a new state. Accessions exhibiting multiple states for a character (i.e. ambiguities) were interpreted as polymorphic at that character or position. Starting trees were obtained by stepwise addition, using a closest addition sequence (holding one tree at each step), and then subjected to tree-bisection–reconnection (TBR) branch-swapping, with the MULTREES option on, with no limit on the maximum number of trees to be saved (MaxTrees). All other options were left on their default settings. A strict consensus tree for each dataset was derived from the set of equally most parsimonious trees. Branch lengths were calculated for one of the equally most parsimonious trees using DELTRAN character state optimisation. The consistency index (CI) (Kluge and Farris 1969) and the retention index (RI) (Farris 1989) were calculated to determine the amount of homoplasy and synapomorphies within the cladogram (Kitching *et al*. 1998). Support for nodes was tested by bootstrap analysis, using a full heuristic search, with 1000 bootstrap replicates, starting by random stepwise addition, with TBR branch-swapping and saving no more than 5000 trees per addition replicate.

For the ITS regions, two separate datasets were analysed; the first compared divergent copies from a limited number of samples (putative functional copies and pseudogenes) and the second included only putatively functional copies across a broader sample of taxa. Outgroups were *Grevillea papuana* and *G. robusta* for the first analysis, and *Hakea nodosa* and *H. ulicina* for the second analysis.

Datasets from each cpDNA region were analysed separately and the results compared. Outgroups for all cpDNA analyses were *G. acanthifolia*, *G. laurifolia* and *G. willisii*. The incongruence length difference test (ILD; Farris *et al*. 1994, 1995) was used to test for congruence between phylogenetic signals across the different cpDNA regions. The ILD tests were implemented using the 'partition homogeneity' (HomPart) option in PAUP*, using only informative characters of each dataset. Tests were run using 1000 randomisations (homogeneity replicates) and a heuristic search to obtain the sum of tree lengths for data from each DNA region (Swofford 2001). The heuristic search options employed were start by stepwise addition, closest addition sequence, one tree held at each step and TBR branch-swapping. All other settings were left on default. All ILD tests were conducted on the University of Oslo Bioportal (Kumar *et al*. 2009), previously available at www.bioportal.uio.no (accessed 2012).

Results

ITS variation and phylogeny

For many samples in our dataset, PCR amplification of the combined ITS region (including ITS-1, 5.8S, ITS-2) identified multiple fragment-length variants within individuals. The number and size of amplicons for any individual varied with PCR conditions, particularly with differing concentrations of

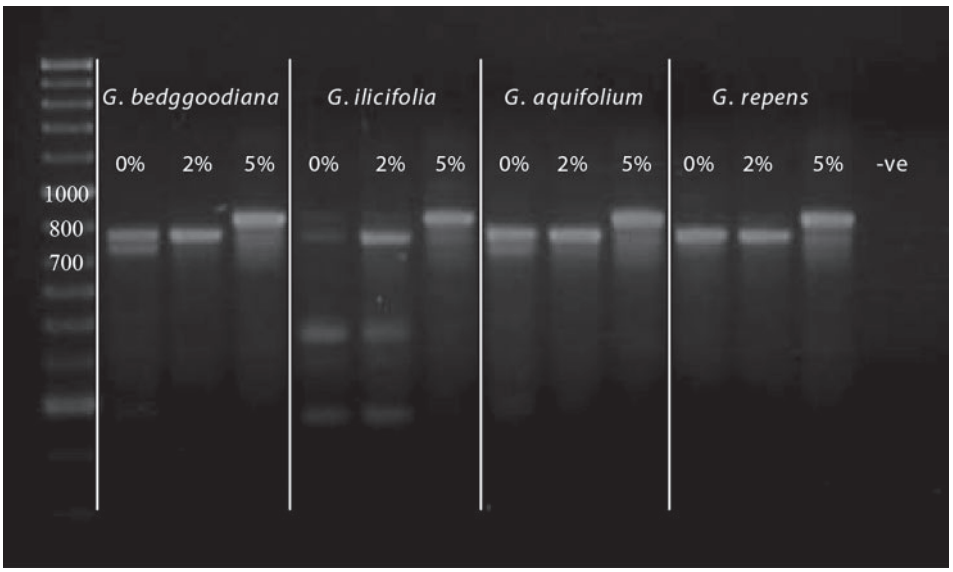

Fig. 2. Agarose gel showing amplicons from four species of *Grevillea*, using primers for the nrDNA internal transcribed spacer (ITS) region and varying percentages of dimethyl sulfoxide (DMSO) (0%, 2%, 5% v/v) in polymerase chain reactions (PCRs). Numbers on the left of the figure indicate DNA-ladder fragment sizes (bp). The largest bands amplified with 5 and 2% DMSO are respectively referred to in the text as the 'a' and 'b' ITS copies. Accessions: *G. aquifolium* MOYSTON, *G. bedggoodiana* ENFIELD, *G. ilicifolia* subsp. *lobata* DUNOLLY, *G. repens* STAR-TRACK as per Table 1.

dimethyl sulfoxide (DMSO) in the reactions (e.g. Fig. 2). Preferential amplification of the largest 'a' copy of this region was generally most efficient with the addition of 5–6% (v/v) DMSO, and the shorter 'b' copy with 1–2% (v/v) DMSO. In some cases, this approach allowed sequencing of the predominant ITS paralogue size classes without the need for cloning or gel extraction and was used to compare the 5.8S/ITS-2 regions for two of the largest distinct band sizes ('a' and 'b') for five ingroup accessions. The size classes fell into two divergent clades in the first phylogenetic analysis (Fig. 3). Characters separating the two clades were distributed across the 5.8S gene and ITS-2 (Fig. 4). Variation among 'a' copy sequences was low and restricted to sites in ITS-2, whereas variation among the shorter 'b' copies was greater and variable sites were distributed throughout the 5.8S and ITS-2 (Fig. 4). The 5.8S–ITS-2 portion of the sequence for the larger 'a' copy in each sample had a higher G/C content and higher number of methylation sites (Fig. 5). The 'b' copies also lacked the conserved angiosperm motif M1 in the 5.8S identified by Harpke and Peterson (2008). These features of the 'b' copies are characteristic of rDNA pseudogenes, as observed in many other plant groups (e.g. Buckler *et al*. 1997; Mayol and Roselló 2001; Bayly *et al*. 2008; Burke *et al*. 2008). Given this, our subsequent analyses focused purely on the 'a' copy amplified from a broader range of samples.

Parsimony analysis of the larger dataset of 'a' copy sequences yielded 5763 equally most parsimonious trees (length 42 steps, CI = 0.91, RI = 0.93). The holly grevilleas (Fig. 6) are supported as monophyletic and distinct from the outgroup and *Grevillea alpina* (in the *Floribunda* group of Makinson 2000). Branch lengths within the holly grevilleas were short and the strict consensus tree showed little resolution of relationships (Fig. 6), with all holly grevilleas being united by a large polytomous node. Within this polytomy, only three further nodes were identified with bootstrap support (BS) of >50%. One of these united the three samples of *G. repens*, one united the two samples of *G. obtecta*, and the third united the single accessions of *G. bipinnatifida* and *G. dryandroide*s subsp. *hirsuta*.

cpDNA phylogeny

A complete dataset of sequences for the three cpDNA intergenic spacers studied (*trn*L–*trn*F, *trn*Q–5′*rps*16 and partial *rpo*B–*trn*C) was obtained for 57 samples (Table 1). The observed sequence characteristics for each of these regions are listed in Table 2. Results from the three DNA regions were highly congruent and the ILD tests were non-significant ($P > 0.05$) for all comparisons. Given this, and the fact that all regions are part of the same non-recombining chloroplast genome, only the results of a combined analysis of chloroplast regions are presented here.

Parsimony analyses of the combined dataset recovered 60 equally most-parsimonious trees, with a length of 114, CI of 0.92 (CI excluding uninformative characters = 0.90) and RI of 0.97. One of the shortest trees, showing branch lengths and support values, is shown in Fig. 7. Only two of the nodes on this tree (indicated) were not present on the strict consensus; all others have BS of >50%.

The ingroup taxa, together with one outgroup taxon, *G. willisii*, form a monophyletic group with 100% BS (Fig. 7, Node 1). *G. willisii* and the northern holly grevillea taxa, *G. renwickiana* (two accessions, Node 3) and *G. scortechinii* subsp. *sarmentosa*, are separate lineages that are unresolved in the polytomy at Node 1, whereas the other members of the ingroup form a monophyletic group with 100% BS (Fig. 7, Node 4). These

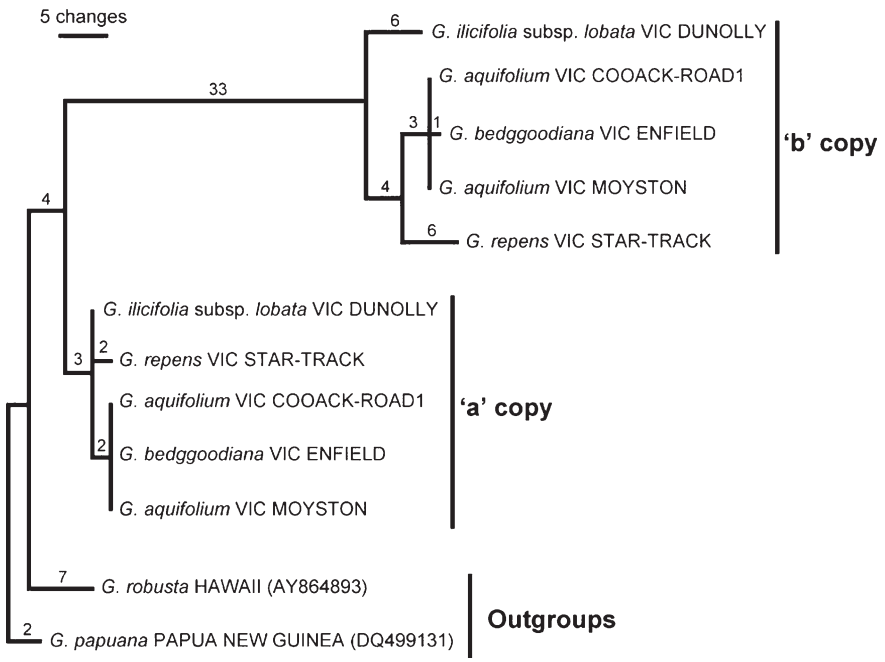

Fig. 3. Relationships between 'a' and 'b' paralogues of internal transcribed spacer (ITS) from five holly grevillea samples. This is one of three trees produced by parsimony analysis (length = 73 steps, consistency index (CI) = 0.97, retention index (RI) = 0.99), and it has the same topology as the strict consensus tree. Numbers represent the number of sequence changes.

Fig. 4. Diagram comparing 'a' and 'b' paralogues of internal transcribed spacer (ITS) from five holly grevillea samples (as included on Fig. 3). Variable sites among 'a' copies (denoted by black vertical bars) are found only in ITS-2. Variable sites among 'b' copies also occur in the 5.8S gene. Consistent nucleotide differences between 'a' and 'b' copies (denoted by grey vertical bars) are distributed across ITS-2 and 5.8S.

are the southern holly grevilleas, which include the following four main clades: the '*G. ilicifolia*', '*G. dryophylla*', '*G. repens*' and '*G. aquifolium*'. The geographic distributions of these clades are shown in Fig. 8. The '*G. ilicifolia* group' includes *G. ilicifolia*, *G. angustiloba* and *G. dilatata*, and the clade has a strong BS (100%, Node 5). This clade, which has the broadest distribution and extends into South Australia, is the sister group to the remaining three clades of southern holly grevilleas (Node 7, with 94% BS). Of the three other clades, the '*G. repens*' and '*G. dryophylla*' clades of central Victoria have moderate (63%, Node 8) and high (92%, Node 13) BS respectively. The '*G. aquifolium*' clade occurs west of these two groups and has moderate BS (63%, Node 18); however, the species *G. aquifolium* is not resolved as monophyletic (see Nodes 18 and 13).

The '*G. repens*' clade (Node 8, Fig. 7) is composed of three taxa, namely, *G. repens*, *G. obtecta* and *G. montis-cole* subsp. *brevistyla*. Each taxon was confirmed as monophyletic, as follows: *G. obtecta* (Node 9, 78% BS), *G. montis-cole* subsp. *brevistyla* (Node 10, 91% BS) and *G. repens* (Node 11, 86% BS). *G. repens* (Nodes 11 and 12) exhibits some within-species differentiation, with two western Victoria accessions (locality codes AMBLERS-LANE and STAR-TRACK) being grouped at Node 12 with 86% BS; this is consistent with a genetic split between eastern and western populations in this species reported by Holmes *et al.* (2009) on the basis of nuclear microsatellite DNA data. *G. montis-cole* is not shown as monophyletic, with its two subspecies being placed in different clades (Nodes 10 and 15).

The '*G. dryophylla*' clade (Node 13, Fig. 7) includes *G. dryophylla*, *G. floripendula*, *G. steiglitziana*, *G. infecunda* and *G. montis-cole* subsp. *montis-cole*, together with three accessions of *G. aquifolium*, each from a different location in the Grampians Ranges. Three characters, one from each of the intergenic spacers (*trn*L–*trn*F, *trn*Q–5′*rps*16, *rpo*B–*trn*C),

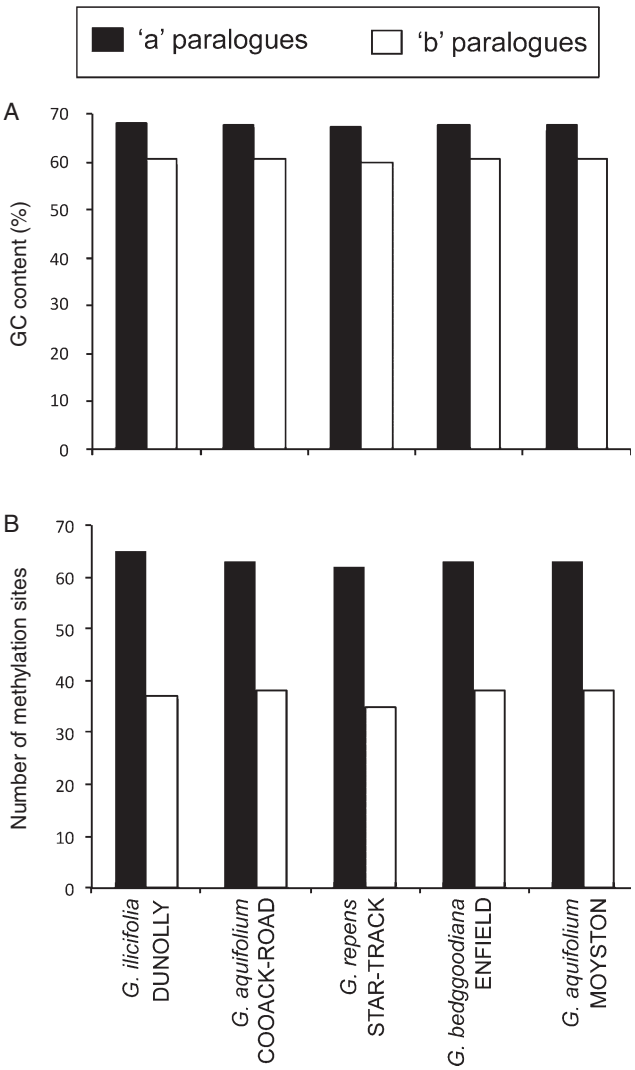

Fig. 5. Graphs comparing 'a' and 'b' paralogues of internal transcribed spacer (ITS) from five holly grevillea samples (as included in Fig. 3). A, G/C content; B, number of CpG and CpNpG methylation sites. Comparisons are based on 394 aligned base pairs including parts of ITS-2 and the 5.8S gene (the same regions as compared in Fig. 4).

support the placement of these three *G. aquifolium* accessions within the '*G. dryophylla*' clade. All three characters are single-base substitutions and all have a CI = 1.0. Within the '*G. dryophylla*' clade, the two accessions of *G. montis-cole* subsp. *montis-cole* cluster together (63% BS, Node 15). *G. steiglitziana* is resolved as non-monophyletic, with one accession clustering with *G. dryophylla* and *G. floripendula* at Node 17 (65% BS). The two accessions of *G. steiglitziana* are separated by two single-base substitutions (CI = 1.0), one from each of the *rpo*B–*trn*C and *trn*Q–5′*rps*16 spacers.

The '*G. aquifolium*' clade (Node 18, 63% BS) includes the remaining *G. aquifolium* accessions in two main lineages on the strict consensus (Nodes 19 and 24), with *G. microstegia* being nested within one of them. Four accessions of *G. aquifolium* are unresolved in the polytomy at Node 18, where *G. bedggoodiana* is also positioned. These four *G. aquifolium* accessions are from different localities across the Grampians Ranges and areas immediately to the east. One lineage with strong support (Node 19, 93% BS) relates all *G. aquifolium* accessions from South Australia, the Little Desert and Mount Arapiles. There is evidence also of geographic differentiation in this clade, with the South Australian (Node 20) and Victorian (Node 21) accessions as sister clades. The second lineage (Node 24, 63% BS) includes seven accessions of *G. aquifolium* from central and western Grampians and south-western Victoria, with *G. microstegia* from Mount Cassell, eastern Grampians, nested within it (Node 27, 64% BS).

Discussion

This study has presented the first molecular phylogeny for any group of species in the genus *Grevillea*, one of Australia's largest plant genera, and provided insight into the evolutionary relationships of the holly grevilleas. Circumscription of this group and delimitation of taxa has so far been based wholly on morphology, which shows complex patterns of variation. Molecular data here have identified major clades and their geographic distributions, allowing a comparison with current taxonomy and the distinctiveness of narrowly endemic species of conservation interest.

Monophyly of holly grevilleas

Without a phylogenetic framework for *Grevillea*, either morphological or molecular, identification of monophyletic groups is difficult. Our *a priori* circumscription of the holly grevilleas was based on the morphological groups of McGillivray and Makinson (1993) and Makinson (2000), and we chose three potentially related species, also from the Aspleniifolia–Hookeriana subgroup (Makinson 2000), as outgroups for the cpDNA analysis. Our cpDNA trees could not be rooted in such a way that the ingroup was monophyletic. Of the three outgroup species, two (*G. acanthifolia* and *G. laurifolia*) are more distinctive from the ingroup on the basis of both cpDNA sequences (longer branch lengths) and on morphology than is the third species, *G. willisii*. On this basis, we chose to root cpDNA trees on the branch connecting *G. acanthifolia* and *G. laurifolia*. Very low ITS sequence variation between these taxa and the ingroup species precluded their use as outgroups for the nrDNA analysis, for which two *Hakea* accessions were utilised.

Grevillea willisii, a species from eastern Victoria which is similar in cpDNA sequences to *G. renwickiana* and *G. scortechinii* (Fig. 7), exhibits overall morphology similar to that of the holly grevilleas, sharing characters such as pinnatipartite leaves (e.g. *G. dryophylla*, *G. floripendula*, *G. obtecta*, *G. steiglitziana*) and secund (toothbrush) conflorescences; the latter character is shared by all species included in the Pteridifolia group of Makinson (2000). However, *G. willisii* has a sessile ovary, differentiating it from the holly grevilleas, which all have a clearly stipitate, or stalked, ovary (McGillivray and Makinson 1993; Makinson 2000).

Exact relationships among the basal nodes of the cpDNA tree are unclear and *G. willisii* (outgroup) forms a polytomy with holly grevilleas on the strict consensus tree (Fig. 7). Resolving these

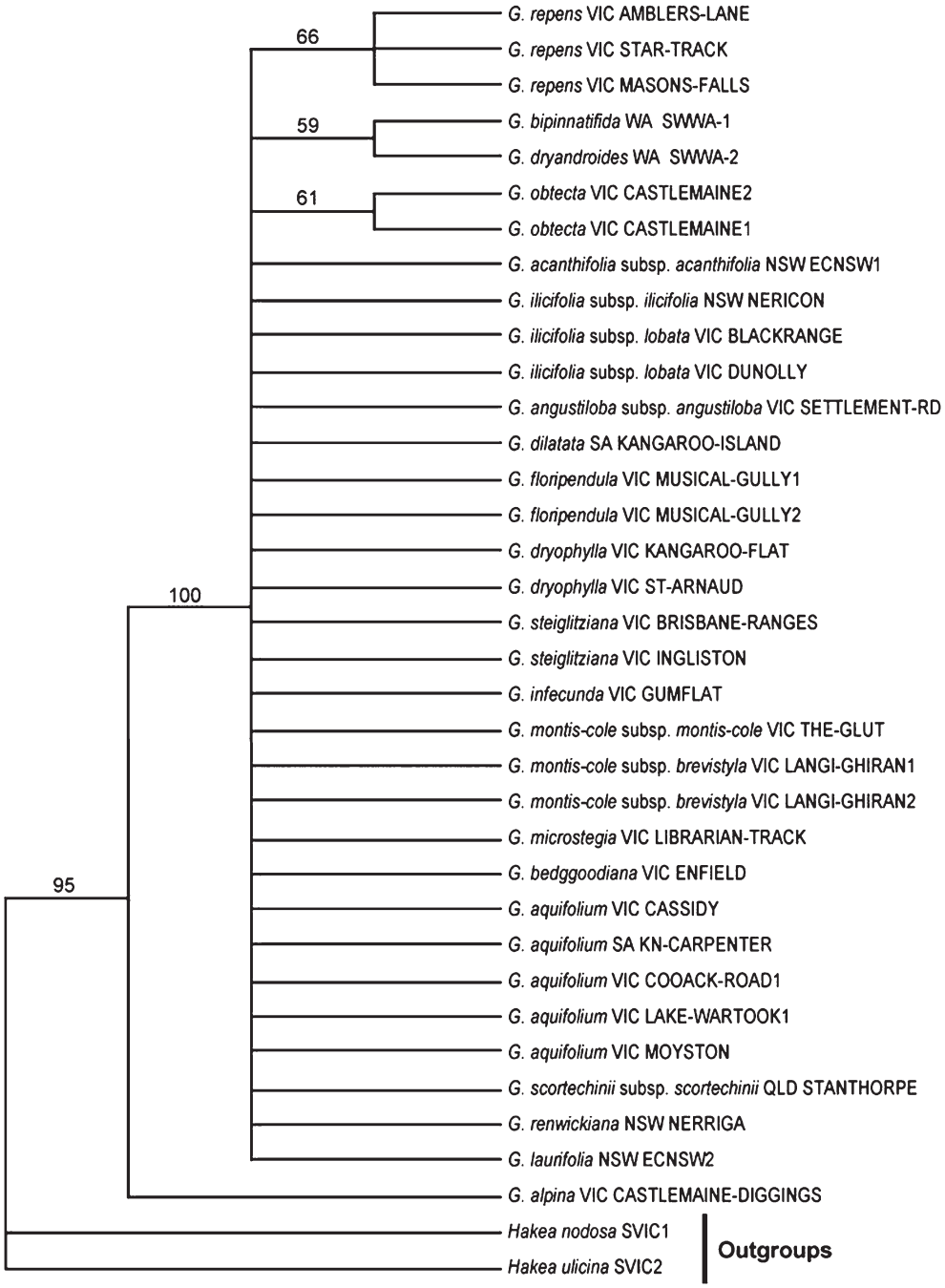

Fig. 6. Strict consensus of 5763 trees (length 42 steps, consistency index (CI) = 0.91, retention index (RI) = 0.93) produced by parsimony analysis of the 'a' paralogues of rDNA. The analysis included 425 aligned base pairs of internal transcribed spacer (ITS)-2 and the 5.8S gene (19 parsimony-informative characters). Bootstrap support (BS) is shown above nodes.

relationships and testing the monophyly of the holly grevillea group would be best undertaken in the context of a broader phylogenetic analysis, using additional and more variable molecular markers. Including a wider sample of taxa from the Aspleniifolia–Hookeriana subgroup (Makinson 2000) in such a study would be worthwhile. In particular, *G. pachylostyla*, a segregate of, and presumed sister to, *G. willisii* (Olde and Marriott 1994; Makinson 2000) would be a valuable inclusion.

Major clades of holly grevilleas

The combined analysis of sequence data from three cpDNA regions provides insight into relationships of the holly

Table 2. Characteristics of cpDNA datasets, including summary statistics for each region utilised in the combined maximum parsimony (MP) analysis

Parameter	*trn*L–*trn*F	*rpo*B–*trn*C	*trn*Q–5′*rps*16	Combined analysis
Sequence characteristics				
Aligned length (bp)[A]	448	656	1259	2363
Unaligned length range (bp)	411–478	656–765	1162–1243	
G+C content (%)	32.20–34.47	26.60–27.34	31.32–31.98	
Characters included				
Total[B]	410	607	1166	2183
Constant	395 (96.34%)	586 (96.54%)	1104 (94.68%)	2085 (95.51%)
Variable but uninformative	1 (0.24%)	9 (1.48%)	21 (1.8%)	31 (1.42%)
Parsimony-informative				
Overall (across all taxa)	14 (3.41%)	12 (1.98%)	41 (3.52%)	67 (3.07%)
Within *G. aquifolium* only	4 (0.98%)	5 (0.82%)	8 (0.69%)	
Within *G. aquifolium* as % of overall	28.57%	41.67%	19.51%	
Single-base indels (binary coding)	0	0	5	5
Multi-base indels (binary coding)	0	3	2	5
Variable-length indels (multistate coding)	1	1	0	2
Parsimony analyses				
Number of equally most parsimonious trees	–	–	–	60
Tree length	20	24	70	114
Consistency index (CI)	0.95	0.96	0.90	0.92
Homoplasy index (HI)	0.05	0.04	0.10	0.08
Retention index (RI)	0.99	0.99	0.96	0.97
Rescaled consistency index (RC)	0.94	0.95	0.86	0.90

[A]Excludes separately coded characters (i.e. only the aligned DNA base positions are included in this total).
[B]Excludes regions of ambiguous alignment. Numbers in parentheses are categories expressed as a percentage of the total number of included characters.

grevilleas and, in particular, identifies several major clades. Of note is the strong support (the longest branch on the tree; 100% BS) for the southern holly grevilleas (Node 4, Fig. 7) forming a clade differentiated from *G. willisii*, *G. renwickiana* and *G. scortechinii*. This suggests an early geographic divergence of this clade (in central and western Victoria, South Australia and western New South Wales) from taxa occurring further east and north, mostly on and east of the Great Dividing Range (Fig. 1).

Among the southern holly grevilleas, the basal split, with strong support (Fig. 7), differentiates the *G. ilicifolia* clade from all other taxa. There has been some confusion between taxa of this group and *G. aquifolium*, including misidentification of specimens (Makinson 2000; Downing *et al*. 2004). Despite this, and despite some geographic overlap of the predominantly western *G. ilicifolia* clade with *G. aquifolium* and related species at the south-eastern portion of it range (Fig. 8), the cpDNA data support this clade being a distinct lineage and show no evidence of introgression or gene flow with *G. aquifolium* and close relatives. This is consistent with the morphological assessment of Downing *et al*. (2004).

In addition to the *G. ilicifolia* clade, the southern holly grevilleas include three closely related cpDNA clades, namely, the '*G. repens*', '*G. dryophylla*' and '*G. aquifolium*' clades that range from central to western Victoria and south-eastern South Australia. These groups show some geographic differentiation (Fig. 8), which suggests the possibility of a vicariant history. What historical events this might relate to is a matter of speculation, but there is considerable climatic, topographic and edaphic variation across the region and a history of geological and climatic upheaval. For example, the Grampians Ranges (Fig. 8) were isolated by mid- and late-Miocene marine incursions into the lower Murray Basin, and more recent changes include Pleistocene climate fluctuations and substantial volcanic activity from the Pliocene to Holocene (e.g. Dodson 1974; Abele *et al*. 1976; Bowler *et al*. 1976; Costermans 1981; Nelson 1981; Marginson and Ladiges 1988; D'Costa *et al*. 1989; Crisp *et al*. 2001; Byrne 2008; Byrne *et al*. 2008; Pollock *et al*. 2013). Volcanic activity has potentially disrupted the distributions of holly grevilleas, which are effectively absent from volcanic soils. Likewise, aridity during Pleistocene glacials would have made some areas, especially north and west of the Grampians Ranges (Byrne 2008), less hospitable for holly grevilleas. Patterns of cpDNA variation in these plants might show evidence of vicariance events because the seeds have a limited capacity for dispersal, i.e. seeds are assumed to fall by gravity over short distances from parent plants, possibly with some secondary dispersal by ants for taxa such as *G. repens* that have elaiosomes.

cpDNA variation and species-level taxonomy

The combined analysis of three cpDNA regions did not resolve all species as monophyletic. This could be partly explained by a lack of divergence in the cpDNA regions sequenced (e.g. in the case of *G. floripendula*, *G. dryophylla*, *G. infecunda*, *G. angustiloba* and *G. ilicifolia*). However, there is clear incongruence between accepted species limits and the combined cpDNA tree (Fig. 7). Such patterns of incongruence are common in plants (e.g. McKinnon *et al*. 1999, 2001; Tsitrone *et al*. 2003; Meudt and Bayly 2008; Nevill *et al*. 2014), making the use of cpDNA sequences problematic for identification using a 'DNA barcoding' approach (e.g. Hollingsworth *et al*. 2011). Possible explanations for this incongruence include relatively low cpDNA mutation rates, homoplasy of DNA base changes, effects of incomplete chloroplast lineage sorting (e.g. the variable

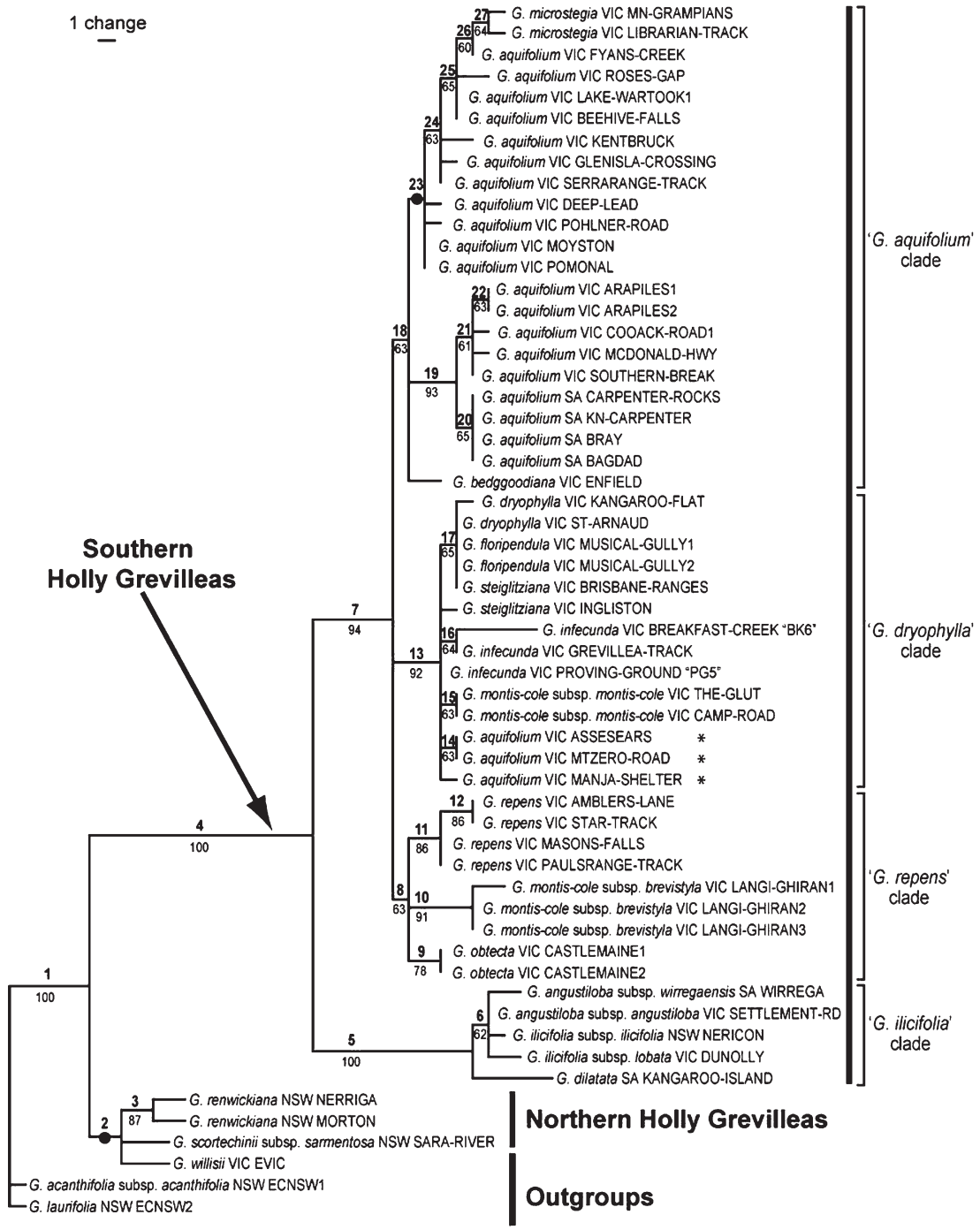

Fig. 7. One of sixty equally shortest trees produced by parsimony analysis of the combined cpDNA dataset. (Length = 114 steps, consistency index (CI) excluding uninformative characters = 0.90, retention index (RI) = 0.97). Only two nodes, indicated by black circles on branches, were not present on the strict consensus tree. Nodes are numbered (above branches) and bootstrap-support values are shown below branches. Asterisks indicate the three accessions of *Grevillea aquifolium* that fall outside the '*G. aquifolium*' clade.

retention of ancestral cpDNA polymorphisms among descendant taxa), gene flow resulting from hybridisation–introgression (chloroplast capture) and poor taxonomy, the latter including excessive splitting, or reliance on homoplasious morphological traits.

For the holly grevilleas, it is unlikely that the mismatch between taxonomy and cpDNA variation is a result of genetic homoplasy (convergence of sequences). The potential for convergence in sequences among taxa arising by chance decreases as the number of character differences among

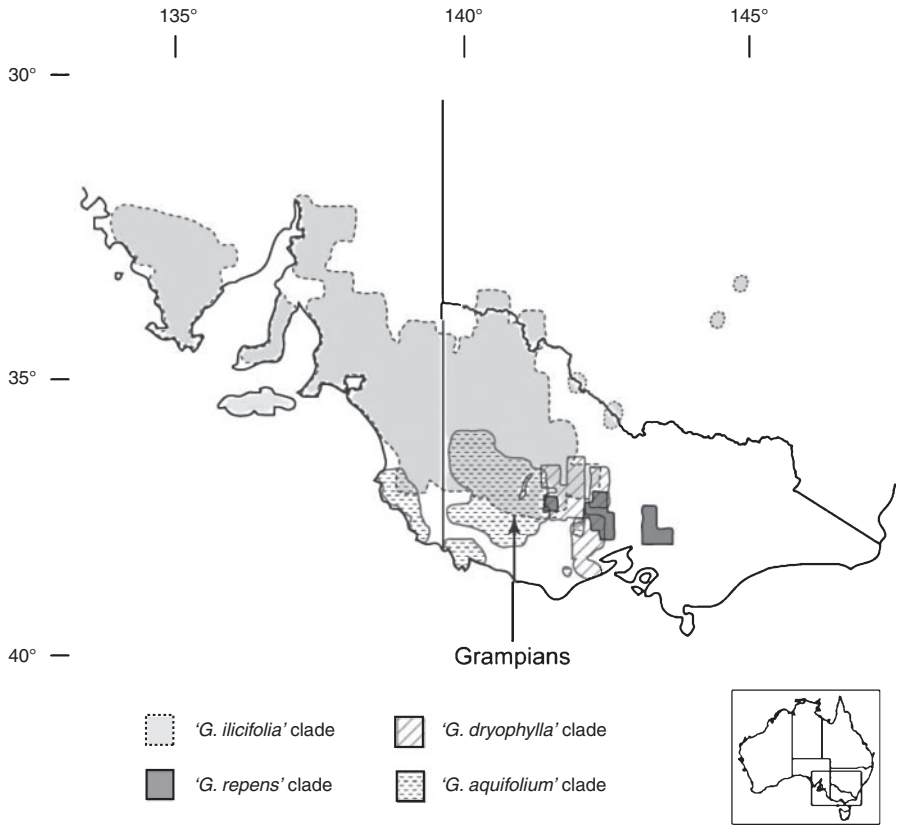

Fig. 8. Distribution of major clades among the southern holly grevilleas on the basis of combined analysis of cpDNA regions. Clade names follow those shown in Fig. 7. The location of the Grampians Ranges in western Victoria is indicated.

lineages and number of DNA regions used increases (McKinnon *et al*. 1999, 2001). In our dataset, similar phylogenetic signal was observed in each of the three cpDNA regions, making genetic homoplasy an unlikely explanation for the observed patterns.

Incomplete chloroplast lineage sorting and/or hybridisation and introgression could affect some patterns of cpDNA variation observed in the holly grevilleas; distinguishing the influence of each of these processes is not straightforward, but evidence from the geographic distribution and relationships of cpDNA lineages can be used. If sharing of cpDNA haplotypes or lineages among species occurs in individuals from overlapping or proximal geographic areas, this is commonly taken as evidence of local introgression, as seen in eucalypts and other plant groups (e.g. Jackson *et al*. 1999; McKinnon *et al*. 1999, 2004; Pollock *et al*. 2013; Nevill *et al*. 2014); lineage sorting is not expected to produce such a geographic pattern (Schaal *et al*. 1998; Avise 2004). Gene flow associated with introgression can also result in the sharing of highly derived cpDNA lineages among species (e.g. among those located towards the tips of phylogenetic trees or haplotype networks), whereas incomplete lineage sorting is more likely to result in sharing of older, ancestral lineages (Schaal *et al*. 1998; Schaal and Leverich 2001).

Of particular interest here are the relationships and classification of four species, namely, *G. microstegia*, *G. montis-cole*, *G. bedggoodiana* and *G. aquifolium.* We will consider, in turn, what inferences can be made regarding these taxa.

Grevillea microstegia

Grevillea microstegia is confined to the top and slopes of Mount Cassell in the Mount William Range in the eastern Grampians (Molyneux 1975), where it grows over a few square kilometres. When first describing the species, Molyneux (1975) suggested that its closest morphological affinities were with *G. floripendula* (then undescribed) from the Ben Major area, near Beaufort, Victoria. However, in the cpDNA analysis *G. microstegia* is nested within the '*G. aquifolium*' clade (Nodes 24–26; Fig. 7), clustered with *G. aquifolium* accessions from the Grampians and Lower Glenelg region in south-west Victoria. The closest *G. aquifolium* accession (Fyans Creek) to *G. microstegia* (Mount Cassell) differs from the latter by only two nucleotide substitutions. Specimens of *G. microstegia* can be distinguished from *G. aquifolium* by having more deeply lobed leaves, sometimes with tertiary lobing, and a sparser indumentum on the lower leaf surface (Makinson 2000; Downing 2012). However, Olde and Marriott (1995*a*) noted some minor variation in the size of leaf lobes in *G. microstegia* and reported the presence of putative hybrids of variable morphology, presumed to be between

G. microstegia and *G. aquifolium*, on the lower slopes of Mount Cassell and nearby streams where the two species grow in close proximity.

The geographic range of G. *microstegia* is wholly enclosed within that of *G. aquifolium* and it is possible that *G. microstegia* is simply part of the spectrum of morphological variation within *G. aquifolium*, and that this is reflected in the observed pattern of cpDNA relationship. However, it is also possible that *G. microstegia* represents a separate lineage that has obtained a '*G. aquifolium* chloroplast' by introgression; an effect of incomplete chloroplast lineage sorting seems less likely because of the geographic proximity of related haplotypes and their derived position in the phylogenetic tree (Fig. 7). Further sampling within *G. microstegia*, and of *G. aquifolium* populations on, and close to, Mount Cassell, and analysis using variable nuclear DNA markers would help confirm or refute the paraphyly of *G. aquifolium* inferred by our cpDNA data.

Grevillea montis-cole

Grevillea montis-cole was first described by Smith (1981), including two allopatric subspecies separated by ~10–15 km in western Victoria, namely, subsp. *montis-cole* from the Mount Buangor–Mount Cole area and subsp. *brevistyla* from neighbouring Mount Langi Ghiran. The two subspecies were not recognised by McGillivray and Makinson (1993), but were accepted by Makinson (1996, 2000), Olde and Marriott (1995*b*) and Walsh and Stasjic (2007). They are distinguished by characters such as style length (shorter in subsp. *brevistyla*) and leaf lobe and outline dimensions (Smith 1983; Olde and Marriott 1995*b*), and possibly occupy different niches; subsp. *montis-cole* is found as an understorey shrub in granitic loam soil, whereas subsp. *brevistyla* grows mainly in cracks and depressions in large granite outcrops at more exposed sites at higher altitude (Makinson 2000, G. D. Holmes, pers. obs.).

CpDNA data show distinction between the subspecies of *G. montis-cole* and other lineages sampled. All three cpDNA regions analysed show clear differences between the two subspecies (consistent with their taxonomic separation), but do not resolve the species as monophyletic (Fig. 7). Subsp. *montis-cole* is placed in the '*G. dryophylla*' clade, which includes its suggested relatives on morphological grounds (*G. steiglitziana* and *G. floripendula*; Smith 1983), whereas subsp. *brevistyla* is placed in the '*G. repens*' clade. This pattern of variation was unexpected for morphologically similar plants separated by just a few kilometres, and its basis is unclear. It could reflect historical hybridisation and introgression (e.g. between subsp. *brevistyla* and members of the '*G. repens*' clade, or between subsp. *montis-cole* and the '*G. dryophylla*' clade) or the stochastic effects of incomplete chloroplast lineage sorting. A population-level study using suitable nuclear markers would help elucidate the degree of differentiation between the two subspecies (e.g. whether they might warrant species-level recognition) and provide additional evidence of their relationships with other species.

Grevillea bedggoodiana

Grevillea bedggoodiana was first described by McGillivray (1986; attributed as J.H.Willis ex McGill.), and had been previously considered part of *G. aquifolium*, or an undescribed species allied to *G. aquifolium* or *G. obtecta* (Willis 1973; McGillivray and Makinson 1993). It is restricted to a small geographic range near Enfield, south of Ballarat, Victoria (Fig. 1), in an area with soil of marine sedimentary origin that has been encircled by newer volcanics. It is difficult to make firm inferences on the status of *G. bedggoodiana* because only one accession was included for analyses in the present study; however, in the combined cpDNA analysis it is related to *G. aquifolium*, being a genetically distinct lineage placed on the basal polytomy of the '*G. aquifolium*' clade (Fig. 7). It is geographically disjunct from the main distribution of the '*G. aquifolium*' clade and it may well be a distinct taxon whose history relates to allopatric peripheral isolation. However, greater population sampling and additional markers are required to confirm its phylogenetic position and degree of genetic differentiation from *G. aquifolium*. Additional study is also warranted to clarify the significance of the heteroplasmy identified in a second sample of *G. bedggoodiana* (Holmes 2008). Divergent cpDNA haplotypes may occur among (and within) individuals or populations of this species, which could affect inference of its phylogenetic placement.

Grevillea aquifolium

Grevillea aquifolium is a widespread (Fig. 1) and morphologically variable species (Olde and Marriott 1995*a*) and was a particular focus of sampling in the present study. Analysis of cpDNA placed the majority of samples in the '*G. aquifolium*' clade, along with *G. microstegia* and *G. bedggoodiana*, as discussed above. However, three accessions of *G. aquifolium* were not resolved within this clade, but instead clustered within the '*G. dryophylla*' clade (Node 13; Fig. 7). Three characters, one from each of three cpDNA regions (*trn*L–*trn*F, *trn*Q–5′*rps*16, *rpo*B–*trn*C), support the inclusion of these accessions in the '*G. dryophylla*' clade. These accessions of *G. aquifolium* are each from different populations in the Grampians in western Victoria, an area where the species is very common. They are not morphologically distinct from surrounding plants or populations of *G. aquifolium* and they are geographically disjunct from other members of the '*G. dryophylla*' clade, which occur in central Victoria (Fig. 8). This geographic pattern is not consistent with recent introgression between taxa in different clades, especially maternal (seed-mediated) gene flow between disjunct areas, but may result from historical introgression events between lineages from distinct clades if their ranges previously overlapped. Another plausible explanation for this pattern is related to incomplete chloroplast lineage sorting, i.e. that the distinct chloroplast clades predate the differentiation of at least some taxa and that representatives of both clades have been variably retained in different populations/individuals of *G. aquifolium*. If this is the case for *G. aquifolium*, it also means that such explanations might apply to other taxa of holly grevilleas that share related chloroplast haplotypes. Again, further data would help better understand the evolutionary history of the group, including nuclear markers and additional individuals/populations for taxa other than *G. aquifolium*.

Utility of ITS sequences for phylogeny reconstruction

Individual plant genomes include many copies of the ITS regions of nrDNA. These are commonly homogenised through concerted evolution (Arnheim 1983), such that there is little within-individual variation, allowing these DNA regions to be widely used in phylogenetic studies of flowering plants (Hershkovitz *et al*. 1999). However, numerous studies have reported the presence of divergent ITS paralogues within genomes, often including putative non-functional 'pseudogenes' (e.g. Buckler *et al*. 1997; Mayol and Roselló 2001; Bayly *et al*. 2008; Burke *et al*. 2008). This also appears to be the case in *Grevillea*. Presence of paralogues, when not recognised, can confound phylogeny reconstruction and lead to misleading phylogenetic trees (Sanderson and Doyle 1992; Bailey *et al*. 2003).

In the present study, we identified multiple ITS paralogues from PCR fragment-size and sequence comparisons. We attempted to compare only orthologues for phylogeny reconstruction, in particular using the presumed functional copies, based on sequence characteristics (G/C content, distribution of variable sites, number of methylation sites), as has been done in studies of other groups (e.g. Buckler *et al*. 1997; Bayly *et al*. 2008). Among the presumed functional orthologues, we found some phylogenetic signal, but there was inadequate resolution to separate closely related *Grevillea* lineages (Fig. 6), even those from different taxonomic subgroups (e.g. *G. bipinnatifida v. G. aquifolium*). Although the percentage of parsimony-informative sites relative to sequence length in the ITS was similar to that of cpDNA regions, much of this variation was found at nucleotide sites that were polymorphic within individuals. This level of within-genome variation makes the ITS regions highly problematic for use in phylogeny reconstruction in *Grevillea*, and most likely in other genera of Proteaceae (Mast and Givnish 2002).

Implications for conservation

All species of *Grevillea* in Victoria are protected under the *Flora and Fauna Guarantee (FFG) Act*; however, active conservation is dependent on priority listings. Our findings raise questions about the evolutionary distinctness of some recognised taxa and suggest a review of their conservation status. The differing level of DNA sequence variation observed among species of the *G. aquifolium* group hints at unrecognised taxa and possible instances of over-splitting. In particular, the large genetic differentiation between the two subspecies of *G. montis-cole* suggests that these lineages may constitute separate species. Pending a detailed population-genetic study, both lineages warrant recognition of conservation status that is currently afforded only to subsp. *brevistyla*. Considerable cpDNA and morphological variation exists within *G. aquifolium*, indicative of a long history of population isolation. Although there is evidence of cpDNA sequence divergence among lineages within *G. aquifolium*, we suggest that the current taxonomic status of this species should be maintained pending further examination. The species *G. microstegia* should also be examined in more detail to ascertain its taxonomic status because its highly restricted distribution defines it as an endangered species under the *FFG Act* (1988) and the *EPBC Act* (1999).

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