

Phylogenetic analysis of Australian species of *Veronica* (*V.* section *Labiatooides*; Plantaginaceae)

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Abstract. Phylogenetic analyses of DNA-sequence data have revealed that the southern hemisphere species of *Veronica* are derived from within the northern hemisphere *Veronica* clade. Previous analyses focussed on the species in New Zealand and included at maximum 7 of 23 species of section *Labiatooides* from Australia. In the present study, we used nuclear ribosomal-ITS and plastid *ndhF-rpl32*-spacer sequence data of all species currently recognised in Australia to analyse phylogenetic patterns. Most importantly, herbaceous species from coastal calcareous sands or limestone habitats do not form a clade with those from shady, moist forest habitats, as formerly believed, but seem to be independently derived from woody species. Incongruence between results from nuclear- and plastid-DNA markers suggest hybridisation to be an important factor in the evolution of the group. Our sample of *V. parnkalliana* included alleles similar to *V. decorosa* and *V. novae-hollandiae* at both loci, which suggests a hybrid origin.

Received 4 May 2012, accepted 12 July 2012, published online 10 October 2012

Introduction

Veronica, the largest genus of Plantaginaceae in its new circumscription (Angiosperm-Phylogeny-Group 2003; Albach *et al.* 2005a) is most species rich in Europe, south-western Asia and New Zealand (*V.* section *Hebe*). Therefore, recent phylogenetic analyses in the genus have focussed on species from these three areas (e.g. Wagstaff and Garnock-Jones 1998; Albach and Chase 2001; Wagstaff *et al.* 2002; Albach *et al.* 2004a). These analyses have demonstrated the inadequacy of previous classifications to delimit coherent evolutionary units. Consequently, the evolution of various characters had to be reinterpreted (e.g. Albach *et al.* 2004a, 2004b, 2005b, 2008) and the intrageneric classification of the genus was revised (Albach *et al.* 2004c; Garnock-Jones *et al.* 2007; Albach 2008). Most notably, the large genus *Hebe* and its segregate genera, such as *Parahebe* W.R.B.Oliv., *Chionohebe* B.G.Briggs & Ehrend. and *Derwentia* Raf., were (re-)classified under the genus *Veronica*. Despite these efforts, the ~23 native Australian species of the genus, some of which have also been referred in the past to the genera *Parahebe* (Briggs and Ehrendorfer 1968; Heads 1994) and *Derwentia* (Briggs and Ehrendorfer 1992), have mostly been neglected in these analyses. Most previous DNA-based phylogenetic analyses exclusively relied on nuclear ribosomal ITS sequences. For example, Wagstaff and Garnock-Jones (1998) included four Australian species and demonstrated a well supported sister-group relationship of three of these species with the

New Zealand species, whereas *Veronica* (*Chionohebe*) *densifolia* was embedded among New Zealand species. Wagstaff *et al.* (2002) increased the sampling to nine Australian species, seven of which (i.e. all except *V. densifolia* and *V. ciliolata*) were again moderately supported as a clade that is sister to the New Zealand *Hebe* complex; however, resolution of relationships within the group was limited. In contrast, their analysis of three plastid-*rbcL* sequences revealed the Australian species as a polyphyletic assemblage. A similar result was retrieved by Albach *et al.* (2005c) using plastid *trnL*-intron and *trnL-trnF*-spacer sequences (subsequently *trnL-F*), although the resolution was low because of the low sequence variability. Furthermore, the combined analysis of ITS and *trnL-F* sequences by Albach *et al.* (2005c) gave strong support for the monophyly of the Australian clade and even stronger support than in the analysis of ITS alone. A similar pattern was retrieved by Albach and Meudt (2010).

Given the great progress in understanding phylogenetic relationships by molecular systematic analyses and especially the controversy around generic delimitation in *Veronica sensu lato* (Brummitt 2006; Garnock-Jones *et al.* 2007; Albach 2008), it is important to investigate the phylogenetic relationships among the Australian species of *Veronica* in more detail. These species exemplify well the problems of differentiating *Veronica* from *Hebe* and *Parahebe*, especially because they include herbaceous species within the broad and mostly

woody *Hebe* clade (Fig. 1). Understanding character evolution in the group may therefore help understand the differentiation of *Hebe* within *Veronica*. Bentham (1846, 1869) classified the 13 Australian species known at that time almost equally in the following two sections: section *Hebe*, including also the species from New Zealand, and section *Chamaedrys*, including also many species from Eurasia. Section *Hebe* was distinguished from section *Chamaedrys* by being (1) ‘evergreen shrubs or densely tufted or tall and erect perennial herbs’ in contrast to ‘herbs, from a perennial usually creeping rootstock, diffuse ascending or erect’ and (2) ‘capsule more or less turgid and septicidally dividing when ripe’ not ‘capsule compressed, opening loculicidally on the margin’ (Bentham 1869). This distinction was kept up by following authors, although Wettstein (1891) elevated the Australian members of section *Hebe* to a separate section, namely, section *Labiatoides*. This group was recognised at generic rank under the name *Derwentia* Raf. by Briggs and Ehrendorfer (1992). Garnock-Jones (1993), in a cladistic analysis of morphological characters, found this genus to be intermediate between *Veronica* and *Hebe sensu lato*, although he could not give an unambiguous synapomorphy for the genus. Other Australian species here recognised within the Australian *Veronica* section *Labiatoides* (*V. formosa*, *V. lithophila*) clade are nested within *Hebe* in the cladistic analysis of Garnock-Jones (1993). Finally, Heads (1994) did not recognise *Derwentia* at generic rank, but considered the species of *Derwentia* to belong to *Parahebe*, along with species distributed also in New Zealand and New Guinea.

We here provide a phylogenetic analysis of nuclear ITS sequences and plastid *ndhF-rpl32*-spacer sequences to test these taxonomic concepts. We consider the main clade of native Australian species to belong to *Veronica* section *Labiatoides* (Garnock-Jones *et al.* 2007) equating to the Australian clade identified by Wagstaff *et al.* (2002), and are here seeking to identify intra-sectional groupings. Furthermore, we use these phylogenetic hypotheses to search for patterns of hybridisation and infer trends in habitat, chromosomal, habit and fruit morphological evolution.

Material and methods

Plant materials

There are 23 species of *Veronica* native to Australia recognised in *V.* section *Labiatoides* (Garnock-Jones *et al.* 2007). On the basis of previous analyses, the section is most likely monophyletic (see above). We have sampled all 23 species and three of the five subspecies recognised in *V. derwentiana* (Table 1). As outgroups, we have chosen four accessions of three species from the same subgenus, *V.* subgenus *Pseudoveronica* J.B.Armstr., from New Guinea and New Zealand, for which sequences were already available from previous studies. Including more divergent outgroups has been difficult in the *ndhF-rpl32* dataset because of alignment ambiguities and they have, therefore, been left out in both datasets.

DNA isolation and sequencing

DNA was isolated from ~20 mg of tissue from silica gel-dried leaves by using the DNeasy plant minikit (Qiagen GmbH, Hilden, Germany), following the manufacturer’s instructions. The quality of the extracted DNA was checked on 0.8% TBE-agarose gels and the concentration was measured spectrophotometrically with a GeneQuant RNA/DNA calculator (Pharmacia, Cambridge, UK).

For the phylogenetic analyses, we used two DNA regions demonstrated to have suitable variation in *Veronica*, namely the nuclear ribosomal internal-spacer region (ITS) and the plastid-DNA *ndhF-rpl32* intergenic-spacer region (subsequently *ndhF-rpl32*). Whereas the former has been used extensively across the genus (Wagstaff and Garnock-Jones 1998; Albach and Chase 2001; Albach and Meudt 2010), *ndhF-rpl32* has not been used in analyses of *Veronica* before; however, it has been demonstrated to have high variation in the study group in preliminary investigations. For amplification and sequencing of ITS, we used primers ITS A (Blattner 1999) and ITS 4 (White *et al.* 1990), whereas for *ndhF-rpl32* we used primers *ndhF* and *rpl32F* (both Shaw *et al.* 2007). PCR reactions

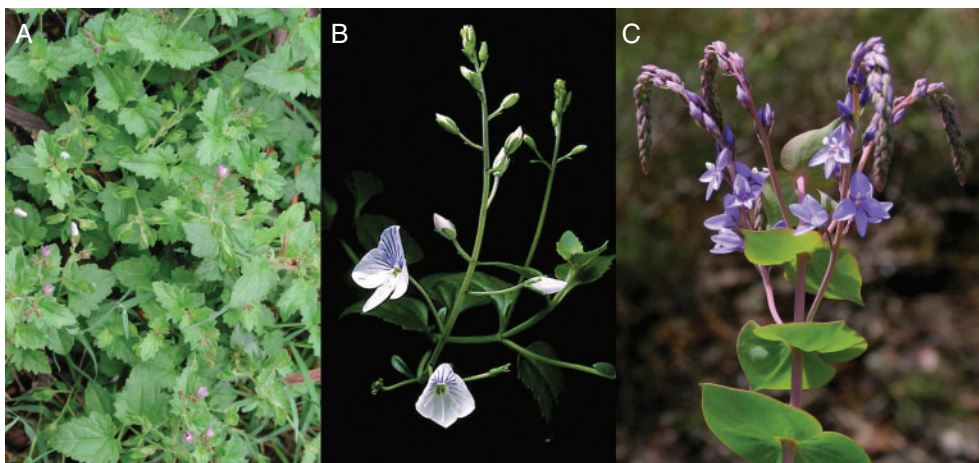


Fig. 1. A. *Veronica plebeia*; B. *V. lithophila*; C. *V. perfoliata*. A and B are herbaceous and stoloniferous; C has soft-woody stems that are mostly replaced annually. Photos: L. von Richter (A), J. Plaza (B) and A. N. Rodd (C).

Table 1. Voucher and sequence information for accessions used in the analyses
Chromosome numbers based on Briggs and Ehrendorfer (2006)

Species	Chromosome number (2n)	Locality data	Voucher information	ITS	Accession number <i>ndhF-pp132</i>
<i>V. sect. Labiatioides</i> Wettst.					
<i>V. arcuata</i> (B.G.Briggs & Ehrend.) B.G.Briggs	42	20 km from Glen Innes to Emmaville, NSW, 29°34'S, 151°40'E	Bean 17286, NSW 521397	JX193660	JX193691
<i>V. arenaria</i> A.Cunn. ex Benth.	38	Cult. RBG Sydney, original from Dingo Creek, NSW, 31°46'S, 152°21'E	Abell s.n., NSW 256680	JX193658	JX193687
<i>V. blakeyi</i> (B.G.Briggs & Ehrend.) B.G.Briggs	42	Clarence Colliery Road, NSW, 33°27'S, 150°10'E	D. Aubert 598, NSW 222284	JX193661	JX193692
<i>V. brownii</i> Roem. & Schult.	72	Victoria Falls, NSW, 33°34'S, 150°18'E	Briggs 8210, NSW 285360	JX193647	JX193675
<i>V. calycina</i> R.Br.	36	Ben Bullen State Forest, NSW, 33°19'S, 150°06'E	Hind 5498, NSW 210378	JX193643	JX193670
<i>V. continua</i> B.G.Briggs	?	Harding Falls, Tas., 41°52'S, 148°08'E	Briggs 9148, NSW 264960	JX193642	JX193669
<i>V. decorosa</i> F.Muell.	38	Waukarie Falls, SA, 32°28'S, 138°00'E	Haegi 3464 NSW 281740	JX193659	JX193688
<i>V. derwentiana</i> Andrews subsp. <i>derwentiana</i>	40	NW of Ben Lomond, NSW, 29°57'S, 15°33'E	White s.n., NSW 219825	JX193655	JX193685
<i>V. derwentiana</i> subsp. <i>maideniana</i> (Gand.) B.G.Briggs	?	Mount Gingera, NSW, 35°34'S, 148°46'E	Coveny 11537, NSW 258913		JX193684
<i>V. derwentiana</i> subsp. <i>subglauca</i> (B.G.Briggs and Ehrend.) B.G.Briggs	40	Mount Werong, NSW, 34°05'S, 148°57'E	Bishop 652, NSW 201648	JX193656	JX193683
<i>V. distans</i>	72	Alkimos Block, Carabooda Lake, WA, 31°37'S, 115°43'E	Keighery 823 B, NSW 420541	JX193644	JX193671
<i>V. formosa</i> R.Br.	42	Mount Wellington, Tas., 42°54'S, 147°13'E	Briggs 9121, NSW 264726	JX193641	JX193668
<i>V. gracilis</i> R.Br.	72	Coxs River, NSW, 33°19'S, 15°6'E	Coveny 15078, NSW 504160	JX193648	JX193676
<i>V. grosseserrata</i> B.G.Briggs & Ehrend.	72	Barrington Tops, NSW, 32°09'S, 151°41'E	Perkins s.n., 16.7.2006, NSW 734687	JX193646	JX193674
<i>V. hillebrandii</i> F.Muell.	36	Kangaroo Island, SA, 35°50'S, 137°52'E	Overton, 40672, NSW 726803	JX193649	JX193677
<i>V. lithophila</i> (B.G.Briggs & Ehrend.) B.G.Briggs	42	Cult. RBG Sydney, original from south of Wentworth Falls, NSW, 33°53'S, 150°23'E	Briggs 8938, NSW 254406	JX193662	JX193693
<i>V. nivea</i> Lindl.	40	Perisher Valley, NSW, 36°24'S, 152°24'E	Wright 110, NSW 517879	JX193663	JX193689
<i>V. notabilis</i> F.Muell. ex Benth.	36	Cochrane Lake, NSW, 36°35'S, 149°26'E	Whalen 366, NSW 500617	JX193652	JX193680
<i>V. novae-hollandiae</i> Poir.	?	Arthur Pieman Protected Area, Tas., 41°30'S, 144°48'E	Schahinger 2. Apr. 2006, NSW 726478	JX193654	JX193682
<i>V. parmaliiana</i> J.M. Black	?	Telowie Gorge CP, SA, 33°02'S, 138°07'E	Bates 7319, NSW 420609	JX193650, JX193651	JX193678, JX193679
<i>V. perfoliata</i> R.Br.	40	Baldy Range, NSW, 35°14'S, 148°49'E	Lyne 2526, NSW 502261	JX196844	JX193690
<i>V. plebeia</i> R.Br.	34	Mebbin State Forest, NSW, 28°28'S, 153°09'E	Bean 14554, NSW 508659	JX193653	JX193681
<i>V. sobolifera</i> B.G.Briggs & Ehrend.	72	Junction Pools Camping area, NSW, 32°02'S, 151°27'E	Hosking 2918, NSW 744760	Available from first author	JX193672
<i>V. subtilis</i> B.G.Briggs & Ehrend.	36	Little Peppercorn Creek, NSW, 3°33'S, 148°37'E	Jobson 5427, NSW 429111	on request JX193645	JX193673
<i>V. velutina</i> (B.G.Briggs & Ehrend.) B.G.Briggs	40	Mount Kaputar NP, NSW, 30°17'S, 150°10'E	Wieck 13005, NSW 427435	JX193657	JX193686
<i>V. sect. Hebe</i> (Juss.) G.Don (outgroup)					
<i>V. salicifolia</i> G.Forst. – Chile	40	Buxton s.n., 1992, WELTU 16898		AF037386	JX193664
<i>V. salicifolia</i> G.Forst. – New Zealand	40	Nelson, CHR 512466		AF037385	JX193665
<i>V. albiflora</i> (Pennell) A.Bach – PNG	42	Triono 149, K		AY540869	JX193666
<i>V. vandewateri</i> Wernham – Indonesia	?	Banker 59, K		EU224209	JX193667

(25 μ L) included 2–2.5 mM MgCl₂, 8 mM bovine serum albumin, 0.4- μ M primer, 0.2 mM dNTP, 1 U μ L⁻¹ *Taq* polymerase (New England Biolabs, Ipswich, MA, USA), 1 \times polymerase buffer and 1 μ L of DNA. ITS sequences were amplified using the following program: 2 min at 95°C; 36 cycles of 1 min at 95°C, 1 min at 50–55°C, and 1.5–2 min at 72°C; and 5 min at 72°C.

PCR products were cleaned using QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's protocols. PCR fragments of cpDNA of the highly polymorphic *V. parnkalliana* and the ITS sequence of *V. derwentiana* subsp. *maideniana* were cloned into the pGEM T-easy vector (Promega GmbH, Mannheim, Germany) and processed following the manufacturer's instructions. Three plasmids were picked and sequenced.

Sequencing reactions (10 μ L) were carried out using 1 μ L of the *Taq* DyeDeoxy Terminator Cycle Sequencing mix (Applied Biosystems Inc., Foster City, CA, USA) and the same primers as for PCR, and run out on automated sequencers. Both strands were sequenced. Sequences were assembled and edited using Sequencher 4.7.2 (Gene Codes Corporation, Ann Arbor, MI, USA). Heterogeneous sites were coded as polymorphic. Assembled sequences were manually aligned before analysis (available from the first author on request).

Data analysis – DNA sequences

DNA sequences were analysed in parsimony, likelihood and Bayesian frameworks. Parsimony analyses were conducted in PAUP v.4a120 (Swofford 2002), with random starting tree, random taxon addition (10 replicates), tree bisection reconnection (TBR) branch swapping and 'MulTrees' in effect. To test the influence of indels, analyses were run with either gaps treated as missing data or gap positions coded according to the modified complex indel coding, as defined by Simmons *et al.* (2007), using SeqState v.1.4.1 (Müller 2005). Parsimony analyses were repeated three times, finding the same most parsimonious trees. We analysed 1000 bootstrap replicates with the same settings; however, the maximum number of trees kept for each replicate was restricted to 50. Likelihood analyses were likewise analysed in PAUP v.4a120 (Swofford 2002), after evaluating different models in jModeltest (Posada 2008), and choosing a model based on the Akaike's information criterion, which found the F81+ Γ -model to be the best for the *ndhF-rpl32* dataset and the TPM3uf+ Γ - model for the ITS dataset. A likelihood analysis of the *ndhF-rpl32* dataset constrained so that *V. formosa* and *V. continua* were sister taxa was conducted and the optimal constrained trees were compared using the Shimodaira–Hasegawa tests (SH) as implemented in PAUP4.0b10 (Swofford 2002), with the model estimated as above, using RELL optimisation and 10 000 bootstrap (BS) replicates. Bayesian analyses were conducted using MrBayes v.3.1.2 (Ronquist and Huelsenbeck 2003), after evaluating the best model in MrModeltest v.2.3 (see <http://www.abc.se/~nylander/mrmodeltest2/mrmodeltest2.htm>, accessed 27 July 2012), which was the GTR+ Γ +I in both cases. Analyses were run for three million generations, sampling every 100th generation and discarding the first 25% as burnin. Stationarity was checked in Tracer v. 1.5 (see <http://tree.bio.ed.ac.uk/>

software/tracer/, accessed 27 July 2012). Because including gap characters in parsimony analyses had no influence on the resulting topology, gap characters were ignored in likelihood and Bayesian analyses. To affirm monophyly of *V.* section *Labiatooides*, the new ITS sequences generated for the present study were combined with the dataset of Albach and Meudt (2010), which sampled all subgenera and major clades of *Veronica*, and analysed using MrBayes v. 3.1 (Ronquist and Huelsenbeck 2003), as described by Albach and Meudt (2010).

Results

The *ndhF-rpl32* dataset included 30 accessions and 785 aligned nucleotide sites, plus 13 gap characters. Of these, 106 were variable and 43 were parsimony-informative (including seven gap characters). Plasmid sequences of *V. parnkalliana* revealed two different haplotypes among the three sequences (termed Clones 1 and 2). The analysis with gaps treated as missing data resulted in 875 most parsimonious trees (111 steps, consistency index (CI; excluding uninformative characters) 0.82, retention index (RI) 0.91), whereas the analysis with gaps coded under modified complex indel coding was stopped when 5000 most parsimonious trees (147 steps, CI 0.75, RI 0.83) were reached. The optimal topology retrieved by the latter analysis was congruent with that of the former analysis. The likelihood analysis (GC content 26%, gamma parameter 0.596) found five optimal trees that are a subset of the most parsimonious trees without gap characters (Fig. 2). All nodes supported by more than 70% posterior probability in the Bayesian analysis were also found in the likelihood analysis (Fig. 2).

All analyses of *ndhF-rpl32* data found four main clades, one consisting of *V. parnkalliana* (Clone 1) and *V. decorosa*, the second of *V. blakeyi*, *V. arcuata*, *V. perfoliata* and *V. derwentiana* subsp. *maideniana*, the third of *V. distans*, *V. hillebrandii*, *V. parnkalliana* (Clone 2) and *V. novae-hollandiae*, and the fourth consisting of all other species. A topology with *V. formosa* and *V. continua* forming sister species, as suggested by morphological features, was rejected by the Shimodaira–Hasegawa test ($P < 0.02$).

The ITS analyses included 750 aligned characters, with 569 of them constant and 145 parsimony-informative, plus 13 gap characters, four of them parsimony-informative. Plasmid sequences of *V. parnkalliana* revealed two different haplotypes among the three sequences (termed Clones 1 and 2). Direct sequencing of the ITS region of *V. derwentiana* subsp. *maideniana* gave highly heterogeneous sequences and plasmid sequencing remained unsuccessful despite several attempts. Only a small piece of sequence could be retrieved for *V. sobolifera*. Analyses were repeated without the sequence and did not differ from those including the sequence. Therefore, we show the topology including the sequence and support values without it. The analysis with gaps treated as missing data resulted in 18 most parsimonious trees (419 steps, CI 0.57, RI 0.70). The analyses with gaps coded under the modified complex indel coding found the same 18 trees as optimal result (434 steps). The likelihood analysis retrieved an optimal phylogeny with the same topology as one of the most parsimonious trees (Fig. 3). Support for internal clades in

unlikely to solve them. Results from the two datasets will be discussed in the light of these difficulties. Both markers suggest multiple shifts between the soft-wooded habit of the former *Derwentia* species and a fully herbaceous habit and disagree with previous hypotheses on relationships to a considerable extent.

The Tasmanian endemic species *V. continua* and *V. formosa* are supported as sister taxa in the ITS analysis (Fig. 3) but not the *ndhF-rpl32* analysis. Indeed *V. continua* has strikingly incongruent placements in the two trees, whereas the position of *V. formosa* is more consistent. This raises the possibility that *V. continua* may be of hybrid origin or, through introgression, shares a cpDNA lineage with *V. derwentiana* subsp. *derwentiana*, which has a widespread distribution, including Tasmania. The Tasmanian endemics show marked similarity in their oblong to lanceolate thick-textured leaves that are stem-clasping, often recurved, with entire margins and glabrous, except for rigid white cilia on the margins near the base. In this combination of characters, they differ from all other Australian species. *V. continua* differs from *V. formosa* mainly in the taller growth, less stem branching and longer racemes, all being features in which it resembles *V. derwentiana*. If these differences do not indicate hybridogeneous influence from *V. derwentiana* in *V. continua*, they could alternatively represent adaptations to the sheltered (although rocky) habitats of *V. continua*, whereas *V. formosa* mostly occurs at more exposed sites.

The differentiation of the *Distans* clade from the *Calycina* clade in analyses from both markers is plausible considering the ecology (*Distans* clade on calcareous sand or limestone, *Calycina* clade in shady, moist forests or grasslands) and morphology. The members of the *Distans* clade are widely separated, with *V. distans* occurring in southern Western Australia, *V. novae-hollandiae* in Tasmania and *V. hillebrandii* in South Australia, although all are coastal. They share a habit of erect or sprawling stems linked by stolons or rhizomes, serrate leaves and large flowers, mostly few in the raceme. Unless these are symplesiomorphies, *V. notabilis* should, on the basis of this differentiation, be considered part of the *Calycina* clade. Our sample of *V. parnkalliana* would be an inter-clade hybrid on the basis of the presence of divergent alleles in the investigated individual (see below).

The next branches in the ITS tree lead to *V. notabilis* and to the *Formosa* clade of Briggs and Ehrendorfer (2006), together with *V. lithophila* and *V. nivea*. The coherence of the *Formosa* clade has been questioned above; however, *V. formosa* and *V. nivea* also associate in the plastid tree. *V. lithophila* and *V. nivea* both appear to be distinctive and somewhat isolated species, with different relationships in the two trees. Eight taxa formerly referred to *Derwentia* form the next branches of the ITS tree, although they are not grouped as a clade and form the greater part of two distinct clades in the plastid tree. The *Derwentia* assemblage is paraphyletic with respect to the *Calycina* clade of herbaceous species in the ITS tree, with the subspecies of *V. derwentiana* being widely separated in the plastid tree and less so in the ITS tree. However, the monophyly of *V. derwentiana* cannot be tested rigorously because two subspecies have not been sampled and no clear ITS sequence of *V. derwentiana* subsp. *maideniana* could be retrieved. The

Calycina clade appears in both trees in the same general relationship to species formerly placed in *Derwentia*.

Hybridisation in Australian Veronica

Hybridisation is a common theme in *Veronica* and polyploid hybridisation was estimated to be responsible for 25% of speciation events in the genus (Albach *et al.* 2008). Hybridisation is likely also in the Australian *Veronica* because 5 of the 20 species, for which chromosome numbers are available (Table 1), have polyploid chromosome numbers. However, even homoploid hybrid speciation has been inferred in the genus (Albach *et al.* 2009). Furthermore, because all species are native to the mesic zone of Australia (with the exception of *V. distans* in Western Australia), there is some continuity of suitable habitats between southern Queensland and Tasmania, so that hybridisation is not excluded by geographic isolation.

The distributions of *V. derwentiana* subsp. *derwentiana* and *V. derwentiana* subsp. *maideniana* differ altitudinally and Briggs and Ehrendorfer (1992) reported that in southern New South Wales and Victoria 'above ~750 m alt. [subsp. *derwentiana*] is largely replaced by subsp. *maideniana* and plants morphologically intermediate between these two subspecies.' They also observed that 'plants with intermediate morphological features are common where the ranges of the subspecies adjoin'. The subspecies differ mainly in the broader leaves, often with a cordate base, and more compact inflorescences of subsp. *maideniana*. However, we find the subspecies to be widely separated in the phylogenetic analyses and what looked like a transitional zone between two subspecies could also be a hybrid zone, possibly also reflected in the highly heterogeneous ITS sequences of subsp. *maideniana*. However, our sampling in *V. derwentiana* was not dense enough to exclude geographic structure of cpDNA haplotypes in *V. derwentiana* subsp. *derwentiana*; analysis of *V. derwentiana* subsp. *derwentiana* sympatric with subsp. *maideniana* will be necessary in addition to our allopatric sample. A similar case may be made for *V. derwentiana* subsp. *subglauca*, which grows geographically close to *V. velutina* and with which it shares a similar cpDNA haplotype (Fig. 2); however, it is morphologically and in the ITS tree closer to *V. derwentiana* subsp. *derwentiana* (Fig. 3). Again, broader sampling *V. derwentiana*, in a phylogeographic framework, would be necessary to distinguish between possible scenarios.

The most surprising case of hybridisation and, yet, that with the strongest signal is the case of *V. parnkalliana*. The following three kinds of evidence revealed by DNA sequencing and phylogenetic analysis suggest some gene exchange between *V. decorosa* and *V. novae-hollandiae* or of the stem lineages of these species: heterogeneous sequences after direct sequencing, multiple alleles after cloning and consistent placement of sequences from different DNA regions after cloning in topological positions close to the putative parents. Alternative explanations, such as retention of ancestral polymorphism, differential lineage sorting or paralogy, could explain the presence of multiple alleles, although they are unlikely to explain the consistent placement with the same putative parents, given that the DNA regions represent the

nuclear and the plastid genome. Other samples of *V. parnkalliana* would need to be analysed to differentiate among a hybrid origin, some recent introgression influencing the genotype of our analysed sample and other causes.

The relationship of *V. parnkalliana* with *V. decorosa* is not unexpected on the basis of morphological characteristics and occurrence in similar rocky habitats in South Australia, whereas *V. novae-hollandiae* is not sympatric with either species and is morphologically quite different. *V. parnkalliana* has been collected on the South Australian mainland at rocky sites, along a sandstone ridge in the Flinders Range and near Mount Remarkable, and also previously occurred closer to the coast near Port Lincoln (Fig. 5). Although this species is very rarely collected and is extinct in some of these locations, a collection was made from the Northern Lofty District, along the northern boundary of Telowie Gorge Conservation Park, in 1986 by R. J. Bates, who noted in the collection data that ~500 plants were counted at the collection site. *V. decorosa* occurs in close vicinity to *V. parnkalliana* on the southern Australian mainland, mostly on cliffs and in gorges of the Flinders ranges, with a single disjunct location further to the south-east. Its main occurrences are now separated from *V. novae-hollandiae* in Tasmania by over 1000 km and, for the disjunct site, over 500 km, across Bass Strait. However, during Pleistocene glacial periods, when the sea level

was ~80 m lower than it is now, Tasmania was connected to the Australian mainland until ~12 000 years before present (Rawlinson 1974). There were then extensive dunefields on the Bassian Plain. *V. novae-hollandiae* appears well suited to range expansion under these circumstances because its occurrences are coastal, with specimen records from ‘near-coastal vegetated sand dunes’ or ‘sands just above high water’.

Veronica decorosa has erect (although soft-wooded and presumably short-lived) stems from a perennial base, upwardly curved hairs on stems and leaves, narrow-linear leaves that are mostly entire, elongated racemes of up to 40 flowers and short, broad, but not emarginate, capsules. *V. novae-hollandiae* is a soft herb with short erect stems connected by rhizomes, stems and leaves pilose with recurved hairs, short broad-toothed leaves, large flowers solitary or in few-flowered racemes and obcordate capsules. The stems of *V. parnkalliana* appear to be softly woody, hairs sparse, very short and upwardly curved, the leaves pinnately lobed, with short linear segments, the racemes elongated, with up to 15 flowers widely spaced, and the capsules flattened and emarginate. The leaf shape, inflorescences and capsules of *V. parnkalliana* may show some degree of intermediate morphology between *V. decorosa* and *V. novae-hollandiae*; however, there is little sign of such intermediacy in indumentum.

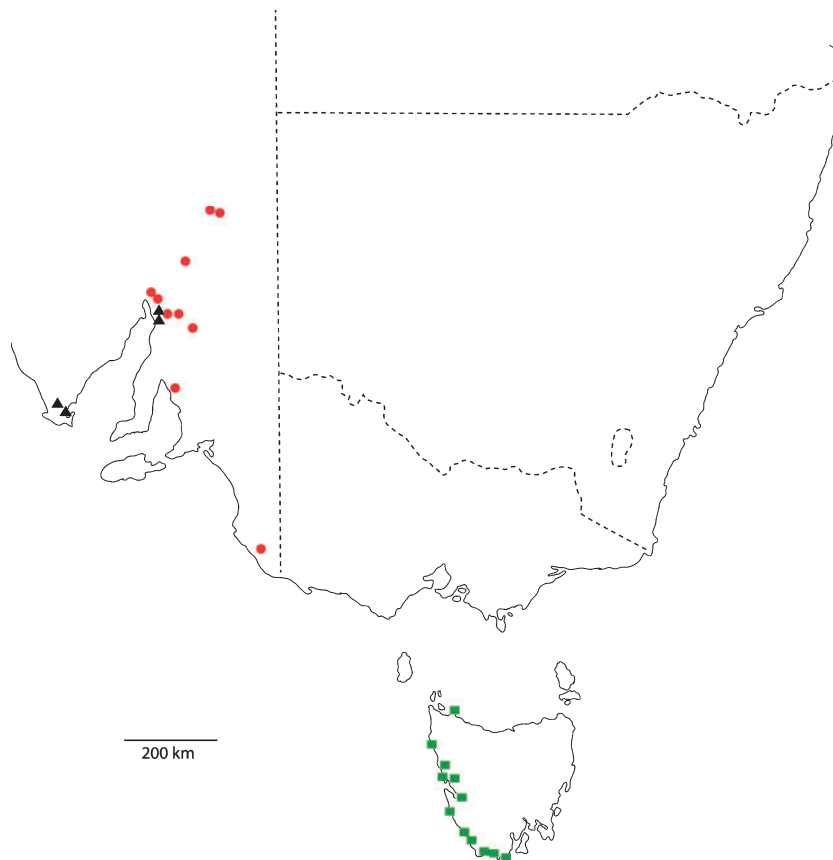


Fig. 5. South-eastern Australia, showing the distribution of *Veronica decorosa* (circles), *V. parnkalliana* (triangles) and *V. novae-hollandiae* (squares). (Adapted from Australia's Virtual Herbarium 2012, see www.avh.ala.org.au, accessed 18 June 2012).

Unfortunately, no chromosome number is available for *V. parnkalliana* (and *V. novae-hollandiae*) to distinguish homoploid from polyploid hybridisation. The most surprising finding of the present study is the presence of two different plastid types in the plant of *V. parnkalliana* that we sampled (heteroplasmy). Maternal inheritance of plastids is considered predominant in angiosperms and was found in *V. polita* Fr. and *V. linariifolia* Pall. ex Link (Zhang *et al.* 2003). Despite the wide use of plastid-DNA sequences in phylogenetic and phylogeographic analyses in the genus, such heterogeneous sequences indicative of the presence of two plastid types in one individual have not been found before, although the presence of two types occurs at least sometimes in ~20% of angiosperms (Nagata 2010). Therefore, some closer inspection of the origin of *V. parnkalliana* is warranted and paternal inheritance of plastid DNA in *Veronica* is still considered a rare event. Nevertheless, given the prevalence of hybridisation in the genus, even a rare event with subsequent return to a homoplasmic condition could confound inference from phylogenetic analyses (Wolfe and Randle 2004).

Character evolution

Because the species of this group have at times been classified in four different genera, it is worth discussing some of the characters previously used for classifying these species. Hong (1984), in a cladistic analysis of the tribe Veroniceae, used the woody habit, presence of a pulvinus (a swelling on the stem below the base of the petiole) and a crustaceous fruit texture to delimit a clade comprising *Detzneria* and a 'Hebe group' consisting of *Hebe*, *Parahebe* and *Chionohebe* (now *Veronica* section *Hebe* and *Labiatooides*). However, Hong (1984) did not consider the Australian species explicitly and possibly considered the herbaceous species as part of *Veronica* and the somewhat woody species as part of the 'Hebe group'. The shrubby habit has been used to classify *V. formosa* in the genus *Hebe* when all other species in Australia have been classified in other genera; however, on the basis of the present phylogenetic understanding of the Australian and the New Zealand species, this is a parallelism, as is likely the connate leaves stated by Briggs and Ehrendorfer (1992) as a synapomorphy for *Hebe* including *V. formosa*. Indeed, those authors pointed out that the stems of *V. formosa*, although erect and woody, are short-lived, becoming procumbent after a few flowering seasons. Also, they indicated that there is a gradation of habit types between this and the species with stems that are replaced annually. The difficulties of classifying woodiness in *Veronica* extend to *V.* subgenus *Stenocarpon* (Boriss.) M.M.Mart. Ort., Albach & M.A.Fisch., sister group to the southern hemisphere species on the basis of ITS sequence data (e.g. Albach and Meudt 2010).

The higher chromosome base number $x=20$ or $x=21$ was an essential part of the justification for segregating *Parahebe* from *Veronica* (Oliver 1944) and has been used by, for example, Briggs and Ehrendorfer (1992) to delimit the southern hemisphere species from those in the northern hemisphere. Wagstaff and Garnock-Jones (1998) hypothesised an ancestral chromosome number of $x=21$ for the southern hemisphere species of *Veronica*, with $x=20$ being derived multiple times

from that base. Comparison of known chromosome numbers (Table 1) with our phylogenies revealed a more complex picture, with no easy inference for an ancestral chromosome number, although $x=21$ still appears to be the most plausible number on the basis of outgroup comparison, with $x=18$ apparently being derived in the *Calycina* clade and *V. notabilis* and *V. hillebrandii-V. distans*. The base number $x=19$ appears either homoplasious (Fig. 2) or transitional to $x=18$ (Fig. 3).

Briggs and Ehrendorfer (1992) originally considered that *V. lithophila* shared with *Parahebe* the distinctive corolla-lobe folds shown in a photograph purported to be of newly opened flowers of that species; however, later (Briggs and Ehrendorfer 2006) they reported that the photographed plant had been misidentified and that *V. lithophila* has flat lateral corolla lobes.

The *Derwentia* clade and the *Calycina* clade of Briggs and Ehrendorfer (1992) had also been distinguished by Bentham (1846) on the basis of capsule shape. Indeed, capsules in the *Calycina* clade are broader than long, laterally compressed and shallowly emarginate, whereas those in the *Derwentia* clade are mostly longer than wide, scarcely compressed and range from acute to emarginate. Although, capsule shape in the *Calycina* clade resembles that in most species of *Veronica*, capsule shape in *V. derwentiana* is very similar to that of many species in *V.* subgenus *Stenocarpon*. Thus, we can assume an evolution towards broader capsules in the *Calycina* clade and, possibly, a plesiomorphic type of capsule shape in *V. derwentiana*, although a character optimisation across the genus would be necessary to test this pattern.

Acknowledgements

The authors are grateful to M. Kever for technical support and H. Meudt for sharing unpublished sequences of *V. salicifolia*. We also thank Michael Bayly, Phil Garnock-Jones and Peter Weston for valuable comments on the manuscript.

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