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Phylogeny of *Veronica* in the Southern and Northern Hemispheres based on plastid, nuclear ribosomal and nuclear low-copy DNA

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ABSTRACT

The cosmopolitan and ecologically diverse genus *Veronica* with approximately 450 species is the largest genus of the newly circumscribed Plantaginaceae. Previous analyses of *Veronica* DNA sequences were in stark contrast to traditional systematics. However, analyses did not allow many inferences regarding the relationship between major groups identified, hindering further analysis of diversification and evolutionary trends in the genus. To resolve the backbone relationships of *Veronica*, we added sequences from additional plastid DNA regions to existing data and analyzed matching data sets for 78 taxa and more than 5000 aligned characters from nuclear ribosomal DNA and plastid DNA regions. The results provide the best resolved and supported estimate of relationships among major groups in the Northern (*Veronica* s. str.) and Southern Hemisphere (hebes). We present new informal names for the five main species groups within the Southern Hemisphere sect. *Hebe*. Furthermore, in two instances we provide morphological and karyological characters supporting these relationships. Finally, we present the first evidence from nuclear low-copy *CYCLOIDEA*2-region to compare results from the plastid genome with the nuclear genome.

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1. Introduction

In its most recent circumscription, the genus *Veronica* is the largest genus of Plantaginaceae with about 450 species (Albach et al., 2005a). It is distributed worldwide with centers of diversity in western Asia and New Zealand. The species exemplify a large range of life forms (minute annuals to small trees) and occur in diverse habitats (semideserts to aquatics, tropical to polar, sea level to alpine). Correspondingly, they exhibit a remarkable diversity of different traits in their vegetative morphology. In order to understand the evolution of these characters and to distinguish between parallel evolution from common ancestry, it is necessary to base the interpretation on a robust phylogenetic hypothesis.

The phylogeny of *Veronica* has been the subject of several recent studies using nuclear ribosomal internal transcribed spacer region (ITS) and plastid DNA (cpDNA) *rbc*L, *trn*L-*trn*L-*trn*F and *rps*16 intron sequences (Wagstaff and Garnock-Jones, 1998, 2000; Wagstaff et al., 2002; Albach and Chase, 2001, 2004; Albach et al., 2004a,b, 2005b,c). These studies provided a general framework which allowed the establishment of a new infrageneric classification of the genus (Albach et al., 2004c). In addition, they provided evidence that characters such as life history and position of the inflorescence (i.e., stem ending in an inflorescence or in a vegetative shoot), formerly used for infrageneric classification, evolved several times in parallel. These studies have also led to the inference that the woody Southern Hemisphere species were derived from herbaceous Northern Hemisphere ancestors rather than the other way around (Albach and Chase, 2001; Wagstaff et al., 2002).

Three major issues remain unresolved with respect to *Veronica* phylogeny and classification. First, the relationships among the 12 subgenera in *Veronica* are generally not well supported (e.g., Albach et al., 2004a,b). Such poor resolution may indicate that speciation in *Veronica* has occurred via a rapid species radiation, but it unfortunately also hinders the interpretation of evolutionary transitions on a genus-wide level. Phylogenetic analysis of more DNA sequence markers is warranted to further our understanding of morphological evolution and subgeneric relationships in *Veronica*.

Second, several examples of incongruence were indicated in previous phylogenetic studies. For example, Albach and Chase (2004) demonstrated that the closest relative of the Southern Hemisphere species differed depending on which DNA region was used, but the phylogenetic signal in these regions was not strong enough to reject alternative positions. They suggested that additional, more informative chloroplast markers and other nuclear (low-copy) markers are necessary to shed light on such cases of incongruence.

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Finally, relationships among the Southern Hemisphere species have remained poorly resolved. Previous studies were based largely on ITS markers (Wagstaff and Garnock-Jones, 1998, 2000; Wagstaff et al., 2002; Albach et al., 2005b), because phylogenetic analyses of cpDNA markers lacked sufficient taxon sampling and/or were unresolved due to the low number of informative sites (*rbc*L, Wagstaff et al., 2002; *trn*L-*trn*L-*trn*F, Albach et al., 2005b; Low, 2005). The ITS phylogeny with the best taxonomic sampling to date of the Southern Hemisphere species showed that although some of the segregate genera were monophyletic, few of the relationships among these groups received high bootstrap support (Wagstaff et al., 2002). In particular, the limits and evolutionary relationships of *Chionohebe* and *Parahebe*, which formed a grade along the backbone of the Southern Hemisphere clade, were not well resolved (Wagstaff et al., 2002). (Throughout this paper we refer to the main species groups within the Southern Hemisphere sect. *Hebe* by new informal names: semi-whipcord hebes, snow hebes, speedwell hebes, sun hebes, and hebes; see Table 4 and Section 4.) More DNA markers are needed to test the relationships found in the ITS phylogeny, and to understand more fully the phylogenetic history of *Veronica* in the Southern Hemisphere, especially in New Zealand.

The main aim of this study is to reconstruct the phylogeny of *Veronica* using six DNA markers to address the subgeneric relationships, topological incongruence found in previous studies, and relationships among the main species groups in the Southern Hemisphere. To reconstruct the phylogeny of *Veronica*, we chose four cpDNA regions (*trn*L-*trn*L-*trn*F, *rps*16, *rpo*B-*trn*C, and *psb*A-*trn*H), the nuclear ribosomal ITS region, and the nuclear low-copy gene, *CYCLOIDEA* (more precisely *CYCLOIDEA*2; see below, in the following called *CYC*2). Of these six markers, four have been used previously in phylogenetic analyses of *Veronica.* For example, the ITS and *trn*L-*trn*L-*trn*F regions have been used extensively in many studies of *Veronica* (Wagstaff and Garnock-Jones, 1998, 2000; Wagstaff et al., 2002; Albach and Chase, 2001, 2004; Albach et al., 2004a,b, 2005b,c), whereas the *rps*16 intron (in the following called *rps*16) has only been used in the Northern Hemisphere species (Albach and Chase, 2004). In addition, the *rpo*B-*trn*C spacer region, ranked as one of the most variable plant markers of the plastid genome by Shaw et al. (2005), was found to be informative in an analysis of *V.* subg. *Stenocarpon* (von Sternburg, 2007; Albach et al., 2009) and was also used in a phylogeographic study of the Southern Hemisphere snow hebes (sensu Table 4; Meudt and Bayly, 2008). The *psb*A-*trn*H spacer is another highly variable cpDNA region (Sang et al., 1997; Shaw et al., 2005, 2007) that has been suggested as a potential DNA barcode marker in plants due to its high variability, short length, and consistent amplification across land plants (Kress et al., 2005; Chase et al., 2007; Edwards et al., 2008), but it has not yet been used for *Veronica*. We therefore chose *psb*A-*trn*H as a fourth cpDNA region to assess its variability and usefulness as a phylogenetic marker in *Veronica*. Finally, we wanted to compare the results of these ITS and cpDNA markers with a low-copy nuclear marker. A test of several nuclear markers (Albach, unpublished) revealed that the *CYC*2 gene is sufficiently variable to resolve relationships in the genus and at the same time can be confidently aligned across Veroniceae.

2. Materials and methods

2.1. Sampling and plant material

A total of 78 individuals were included in this study representing 68 species (71 individuals) of *Veronica*, all 12 subgenera (sensu Albach et al., 2004c; Garnock-Jones et al., 2007) and all major clades within these subgenera. Seven individuals of six other genera of Veroniceae (*Lagotis*, *Paederota*, *Picrorhiza*, *Wulfeniopsis*, *Wulfenia*, and *Veronicastrum*) were designated as outgroups. For all but eight terminals (*Veronica abyssinica*, *V. campylopoda*, *V. catarractae*, *V. filiformis*, *V. javanica*, *V. planopetiolata*, *V. spicata*, and *V. triphyllos*) the same DNA was used for all DNA regions (Table 1). Two individuals each of four species were sampled from different geographic localities (e.g., *V. densifolia*, New Zealand and Australia; *V. salicifolia*, New Zealand and Chile; and *V. macrantha* and *V. thomsonii* (incl. *V. myosotoides*) from different New Zealand locations). Due to the poor quality of herbarium extracted DNA, sequences of *Veronica tubata* could not be generated for the *rps*16, *psb*A-*trn*H spacer and *rpo*B-*trn*C spacer. ITS sequences of *Veronica lanceolata* and *trn*L-*trn*L-*trn*F-sequences of *V. acuta*, *V. hulkeana*, *V. lavaudiana*, and *V. pentasepalae* could not be sequenced cleanly in both directions and were not included in those data sets. Finally, the *rpo*B-*trn*C could not be reliably amplified in all species of *Veronica* and is lacking for 12 terminals. Sequences newly generated for this study include all 70 *CYC*2 sequences (from 60 individuals), all 77 *psb*A-*trn*H sequences, 21 ITS sequences, 25 *trn*L-*trn*L-*trn*F sequences, 50 *rpo*B-*trn*C sequences, and 54 *rps*16 sequences. The classification follows Albach et al. (2004c) and Garnock-Jones et al. (2007). Voucher specimens for all plants used in this study are listed in Table 1.

2.2. DNA extraction, amplification and DNA sequencing

For 12 of the sampled individuals, DNA was extracted using DNeasy Plant Mini Kits (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. The remaining individuals sampled in this study had been extracted for previous studies. The *trn*L-*trn*L-*trn*F region was amplified with primers c and f of Taberlet et al. (1991), ITS with primers ITSA (Blattner, 1999) or ITS1a (Downie and Katz-Downie, 1996) and ITS4 (White et al., 1990), the *rpo*B-*trn*C spacer with primers *rpoB* and *trnC*-R (Shaw et al., 2005), the *psb*A-*trn*H spacer using primers *psb*A (Sang et al., 1997) and *trn*H (Tate and Simpson, 2003), the *rps*16 with primers *rps*F and *rps*R2 (Oxelman et al., 1997), and *CYC*2 with primers (DYTVTCCATCGGCATTGC) and (GATGAAYTTRTGCTGATCCAAAATG) (C.N. Wang, pers. comm.). The PCR program of 95 °C 2 min, 95 °C 1 min, 50–55 °C 1 min, 72 °C 1.5–2 min, 36× to 2, 72 °C 5 min, 10 °C hold was used for all markers except *CYC*2 (94 °C 2 min, five times (94 °C 30 s, 55 °C 1 min, 72 °C 2 min), 35 times (94 °C 30 s, 60 °C 1 min, 72 °C 2 min), 72 °C 5 min, 10 °C hold). PCR products were cleaned using QIAquick PCR purification and gel extraction kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocols, or by adding 5 U exonuclease I (EXO) and 1 U shrimp alkaline phosphatase (SAP), and returning the tubes to the PCR machine for 37 °C 30 min, 80 °C 15 min, followed by 10 °C hold. PCR fragments of *CYC*2 that proved to be heterogeneous after direct sequencing were cloned into the pGEM T-easy vector and processed following the manufacturer's instructions (Promega GmbH, Mannheim, Germany). Up to six plasmids containing cloned products were sequenced. Cloning of *CYC*2 was not attempted for the Southern Hemisphere species. Sequencing reactions (10 μL) were carried out using 1 μL 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 Corp., Ann Arbor, MI, USA) or Geneious (Biomatters Ltd., New Zealand, www.geneious.com). *CYC*2 sequences retrieved after cloning, which were markedly shorter (>100 bp shorter) and considered as pseudogenes, were excluded from the analysis. Heterogeneous sites were coded as polymorphic. Assembled sequences were manually aligned prior to analysis.

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