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Is genome downsizing associated with diversification in polyploid lineages of *Veronica*?

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The study of genome size evolution in a phylogenetic context in related polyploid and diploid lineages can help us to understand the advantages and disadvantages of genome size changes and their effect on diversification. Here, we contribute 199 new DNA sequences and a nearly threefold increase in genome size estimates in polyploid and diploid *Veronica* (Plantaginaceae) (to 128 species, *c*. 30% of the genus) to provide a comprehensive baseline to explore the effect of genome size changes. We reconstructed internal transcribed spacer (ITS) and *trnL-trnL-trnF* phylogenetic trees and performed phylogenetic generalized least squares (PGLS), ancestral character state reconstruction, molecular dating and diversification analyses. *Veronica* 1C-values range from 0.26 to 3.19 pg. Life history is significantly correlated with 1C-value, whereas ploidy and chromosome number are strongly correlated with both 1C- and 1Cx-values. The estimated ancestral *Veronica* 1Cx-value is 0.65 pg, with significant genome downsizing in the polyploid Southern Hemisphere subgenus *Pseudoveronica* and two Northern Hemisphere subgenera, and significant genome upsizing in two diploid subgenera. These genomic downsizing events are accompanied by increased diversification rates, but a 'core shift' was only detected in the rate of subgenus *Pseudoveronica*. Polyploidy is important in the evolution of the genus, and a link between genome downsizing and polyploid diversification and species radiations is hypothesized. © 2015 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2015, **178**, 243–266.

ADDITIONAL KEYWORDS: annual – alpine – Eurasia – genome size – hebe – New Zealand – Plantaginaceae – PGLS – polyploidy – species radiation.

INTRODUCTION

In recent years, analyses of plant genomes have revealed that polyploidy is ubiquitous in plant evolution (Soltis *et al*., 2009). This has stirred new interest in the question of what causes the success of these polyploid lineages. Although Otto & Whitton (2000) demonstrated a positive correlation between species richness and polyploidy, analyses of the diversification rate demonstrated no difference, or a lower diversification rate, in polyploids relative to diploids (Wood *et al*., 2009; Mayrose *et al*., 2011; Scarpino, Levin & Meyers, 2014). Thus, the essence of these studies is that diploids speciate more rapidly than polyploids and the latter go extinct more frequently, but those polyploids that survive have some inherent advantages over diploid relatives (Soltis *et al*., 2003).

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However, what these inherent advantages are is hotly debated (Soltis & Soltis, 2000; Comai, 2005; Otto, 2007). Here, we explore the effect of genome size changes on related polyploid and diploid lineages in the genus *Veronica* L. (Plantaginaceae).

Veronica is an excellent system in which to study comparative genome size evolution, as polyploidy has evolved several times in parallel in the genus (Albach *et al.*, 2008) in many different ecological and geographical circumstances, in both hemispheres and with different effects on diversification. *Veronica* is a large genus with *c.* 450 species distributed worldwide, but especially in two centres of diversity, Eurasia and New Zealand. Several phylogenetic studies have shown that the Southern Hemisphere *V.* subgenus *Pseudoveronica* J.B.Armstr., including *c.* 150 species from New Zealand [122 species, section *Hebe* (Juss.) Benth.], Australia (23 species, section *Labiatoides* Wettst.) and Papua New Guinea (13 species, section *Hebe*), is a recently evolved polyploid radiation nested in *Veronica* (Wagstaff *et al.*, 2002; Albach *et al.*, 2004b; Albach, Utteridge & Wagstaff, 2005b; Albach & Meudt, 2010). Similarly, polyploid lineages variously comprising few to many species exist in the Northern Hemisphere. Initially, polyploid lineages are expected to have larger genomes than diploids, equivalent to the combined sizes of the diploid parental genomes that come together in the polyploid. However, many studies have demonstrated that loss of DNA, or genome downsizing, is common in polyploids. Polyploids frequently exhibit substantially lower monoploid genome sizes (i.e. DNA amount per basic genome) relative to their diploid progenitors or relatives (Leitch & Bennett, 2004), although genome upsizing in polyploids is also known to occur (e.g. Leitch *et al.*, 2008; Pellicer *et al.*, 2013).

Changes in genome size, i.e. the amount of DNA per cell (C-value), are key biodiversity characters of fundamental importance (Bennett & Leitch, 2005, 2011) and may affect various aspects in the evolution of a species. In addition to affecting growth and reproduction (Bennett, 1972; Francis, Davies & Barlow, 2008; Hessen *et al.*, 2010), genome size has been shown in several studies to be correlated with diverse characters (e.g. ecology, life history; e.g. Knight & Ackerly, 2002; Albach & Greilhuber, 2004; Kang *et al.*, 2014) and with species diversification (Kraaijeveld, 2010). The past decade has seen a sharp rise in the number of species with known genome size estimates through the extended use of flow cytometry (Doležel & Bartoŝ, 2005; Leitch & Bennett, 2007). Genome size data are currently available in the Plant DNA C-value database (release 6.0, December 2012, http://data.kew.org/cvalues) for *c.* 5% of all known plant species. Such sampling allows for the elucidation of some general patterns of plant genome size evolution (Lomax *et al.*, 2014), but, because sampling in many families and genera is still poor, finer scale studies, required for the detection of selective forces acting on genome size, are not yet feasible for most groups.

In a previous study, Albach & Greilhuber (2004) analysed genome sizes for the first time in *Veronica*. They found that the 1C genome size values of 40 *Veronica* spp. ranged from 0.32 to 2.26 pg, i.e. the genomes are very small (1C-value ≤ 1.4 pg) to small (1.4 pg < 1C-value ≤ 3.5 pg) following the categories of Leitch, Chase & Bennett (1998). Albach & Greilhuber (2004) also uncovered some statistically significant trends in their data, i.e. alpines have larger genomes than non-alpines, annuals have smaller genomes than perennials (although this was not significant in all tests), and selfers have smaller genomes than outcrossers. Their study was an important first step towards the understanding of genome size evolution in *Veronica*, but only 40 of 450 species, or < 10% of the genus, was sampled. In particular, sampling of the Southern Hemisphere subgenus *Pseudoveronica* was restricted to only one of the *c.* 150 species, 'because it is felt that genome size evolution in such a large subgroup would be a project of its own' (Albach & Greilhuber, 2004). Furthermore, sampling in that study was not sufficiently dense to provide the monoploid genome size, the size of a single chromosome set (1Cx-value; Greilhuber *et al.*, 2005), for ploidy level analyses in all polyploid complexes of the genus, and this has resulted in at least one case in which ploidy in the genus based on flow cytometry (Castro, Castro & Loureiro, 2012) has been misinterpreted.

In the present study, we aim to fill these knowledge gaps by addressing the following questions using a comparative approach in a phylogenetic context. (1) What is the range of genome sizes found in Southern Hemisphere *Veronica* in comparison with those of the Northern Hemisphere? (2) Are there similar patterns in the Northern vs. Southern Hemisphere polyploid species regarding genomic downsizing and upsizing, and are such changes correlated with changes in diversification rate? (3) Are life history and alpine habitat correlated with genome size given the larger sample size and inclusion of the Southern Hemisphere species? To answer these questions, we generated the most comprehensive phylogenetic analysis of the genus to date, including 199 new sequences, and we estimated new genome size data using flow cytometry for 102 *Veronica* spp., including 81 for which no genome size estimate was previously available. To support our strategy to combine estimates using different methods of analysis of genome size, we also use a subset of our data to test whether flow cytometry measurements are comparable with previous Feulgen densitometry measurements of genome size for a given species, and to what extent measurements

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made on silica gel-dried material are similar to those made using fresh material from the same individual. By achieving the above aims, we endeavour to provide the basis for establishing *Veronica* as a model of polyploid and genome size research. More specifically, we hypothesize that genome downsizing is a prerequisite for the success of polyploid lineages to diversify and may also explain the success of the angiosperms as a whole, which generally have considerably smaller genomes than gymnosperms (Soltis *et al.*, 2003).

MATERIAL AND METHODS

Genome size estimation

Voucher information for the individuals used in this study is listed in Supporting Information Table S1. For each individual measured, *c*. 1 cm^2 of leaf material was co-chopped with a standard using a razor blade in a Petri dish containing 1.1 mL of cold buffer. We tried several different buffers for samples of *Veronica* and found that 'Otto's Buffer' (Otto, 1992; Doležel & Göhde, 1995) generally gave the best results for fresh material, whereas 'Woody Plant Buffer' [WPB; Loureiro *et al.*, 2007; containing 1–2% polyvinylpyrrolidone (PVP) and using ascorbic acid instead of sodium metabisulphite] gave the best results for silica gel-dried material (data not shown). We primarily used *Solanum pseudocapsicum* L. (1C = 1.2946 pg; Temsch *et al.*, 2010) or *Hedychium gardnerianum* Shepard ex Ker Gawl. (1C = 2.01 pg; see Supporting Information Table S2) as the internal standard, although, depending on the C-value and standard availability, we also used the following: *Zea mays* L. 'CE-777' (1C = 2.7165 pg; Lysák & Doležel, 1998), *Raphanus sativus* L. 'Saxa' (1C = 0.555 pg; Doležel *et al.*, 1998), *Pisum sativum* L. 'Citrad' (1C = 4.545 pg; Doležel *et al.*, 1998), *Pisum sativum* 'Kleine Rheinländerin' (1C = 4.42 pg; Greilhuber & Ebert, 1994) and *Solanum lycopersicum* L. 'Stupicke' (1C = 0.98 pg; Doležel *et al.*, 1998) (see Table S1). The previously unpublished genome size of *Hedychium gardnerianum* was calibrated over 3 days by comparison with the five standards *Pisum sativum*, *Raphanus sativum*, *Solanum lycopersicum*, *Solanum pseudocapsicum* and *Zea mays*, and calculated by demonstrated linearity according to Yokoya *et al*. (2000). We chose the new standard *Hedychium gardnerianum* for a number of reasons: it is widely available as a common perennial ornamental in many botanical gardens; it is distributed as clones; it is a perennial and thus available year-round; it has a genome size between maize and soy bean; and it was established in our laboratory when the standard varieties of maize and soy bean (see Doležel & Greilhuber, 2010) were not available (season-related). The chopped material and buffer were then filtered through a Cell-Tric 30-μm filter into a plastic tube, and 50 μL RNase were added. After incubation in a water bath for 30 min at 37 °C, 450 μL of the solution were transferred to another tube, to which 2 mL propidium iodide (for WPB) or Otto II (propidium iodide + Na_2HPO_4, for Otto's buffer) were added. This solution was placed at 4 °C for 15 min (WPB) or 1 h (Otto's buffer). The samples were analysed using a CyFlow flow cytometer (Partec GmbH, Münster, Germany). For most species, three replicates from one individual, comprising 5000 counts, were measured and averaged; however, in some cases, only one or two replicates were performed (Table S1). In many cases, we used glasshouse or botanical garden material, and thus only one individual per species was available for inclusion in the study. We aimed to use only those analyses in which sample and standard coefficients of variation (CVs) averaged < 5% over all runs, but we also included several samples (particularly silica gel samples from New Zealand) with CVs of 5–8% (Table S1).

In total, we measured the genome sizes of 174 individuals representing 102 *Veronica* spp. and one individual of *Lagotis integrifolia* (Willd.) Schischk. as an outgroup to Veroniceae. Adding our data to the already published genome size estimates brings the total number of *Veronica* individuals with known genome sizes to 237 (including 13 with multiple measurements), representing 128 *Veronica* spp. [including 43 with multiple (two to 15) individuals sampled]. All new and previously published data are shown in Table S1. We performed a literature search to code each individual for ploidy, base chromosome number, chromosome number, annual vs. perennial and alpine vs. non-alpine following and updating Albach & Greilhuber (2004), including two corrected 1C-values (*V. gentianoides* Vahl, 1C = 2.234 pg; *V. baumgartenii* Roem. & Schult., 1C = 0.639 pg), four corrected ploidies (*V. perfoliata* R.Br., 6*x*; *V. ciliata* Fisch., 4*x*; *V. hederifolia* L., 4*x*; *V. urticifolia* Jacq., 4*x*) from Albach & Greilhuber (2004) and three corrected ploidies (*V. peregrina* L., 6*x*; *V. micrantha* Hoffmans. & Link, 2*x*; *V. chamaedrys* L., 2*x* or 4*x*) from Castro *et al*. (2012). In eight cases in which ploidy and chromosome number were previously unknown for the species, ploidy was inferred from the flow cytometry measurements by comparing the genome size with that of phylogenetically related species. Inferring ploidy was usually straightforward and our inferences are robust because of the greatly increased sampling of genome size measurements in *Veronica* in the present study. Although hidden intraspecific cytotype diversity (Husband, Baldwin & Suda 2013) could be a problem as chromosome counts for the particular individuals sampled were not available, in most cases

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(with the exception of subgenus *Pentasepalae*), different ploidies were unambiguously indicated by clearly differentiated 1C-values. Because of difficulties in coding for breeding system, we did not code this trait, and thus could not test the hypothesis that selfing species have smaller genome sizes than outcrossing species (Albach & Greilhuber, 2004). The analysis of breeding system will require a more in-depth analysis of quantitative characters associated with the reproductive mode, as initiated by Scalone, Kolf & Albach (2013).

Approximately one-half of our genome size measurements were made using fresh material growing in the field, botanical garden or glasshouse, whereas the other half were made using silica gel-dried material that was measured as soon as possible following drying (46% within 6 weeks, 66% within 3 months, 78% within 6 months, 87% within 1 year; Table S1). Ten samples (13%) were stored for more than 1 year, including one (*V. orientalis* Mill., *Albach 701*, WU) for more than 8 years (Table S1). Silica gel-dried material has been employed successfully for the measurement of genome size using flow cytometry in *Veronica* (Bardy *et al*., 2011), as well as in other genera with differing degrees of success (Suda & Trávníček, 2006; Cires *et al*., 2009; Bainard *et al*., 2011; Sánchez-Jiménez *et al*., 2012; Dyer *et al*., 2013). The use of fresh material is not always feasible, especially for many of the New Zealand *Veronica* spp. sampled here. To test experimentally whether our genome size estimates based on silica gel-dried material were equivalent to those using fresh material, we performed flow cytometric measurements on leaves from six individuals sent to us from the Royal Botanic Gardens, Kew (UK), first using fresh leaves and again 6 weeks and 5 months later using silica gel-dried leaves from the same plant. Similarly, some of the genome size estimates in our dataset come from previously published studies that used Feulgen densitometry instead of flow cytometry. Although some studies have already shown that the two methods give similar results (Baranyi & Greilhuber, 1996; Doležel *et al*., 1998; Moscone *et al*., 2003), differences have been noted (Greilhuber, 1998). To obtain a general idea about the extent to which Feulgen and flow cytometry measurements may differ in our dataset, we compared measurements from samples ($N = 19$) made using both techniques for the 17 species for which we had at least one Feulgen and at least one flow cytometry measurement (albeit from different individuals of the same species). Diploid and tetraploid *V. chamaedrys* and tetraploid and hexaploid *V. cymbalaria* Bodard were treated as separate samples. Twelve of the comparisons used averages of flow cytometry measurements from two to five different individuals, and two of these also used averages of Feulgen measurements from two different individuals. The non-parametric paired Mann–Whitney *U*-test was performed in R (R Core Team, 2014) using wilcox.test (paired=TRUE) to compare genome sizes from the different types of material (fresh vs. silica) and the different methods (flow cytometry vs. Feulgen).

DNA SEQUENCING AND PHYLOGENETIC ANALYSES

We chose the nuclear ribosomal internal transcribed spacer (ITS) region and plastid DNA *trnL* intron plus *trnL-trnF* intergenic spacer regions (referred to as *trnL-trnL-trnF* hereafter) for phylogenetic analysis. Both of these markers have been shown to be useful for the phylogenetics of *Veronica* (Wagstaff & Garnock-Jones, 1998, 2000; Albach & Chase, 2001, 2004; Wagstaff *et al*., 2002; Albach, Martínez-Ortega & Chase, 2004a; Albach *et al*., 2004b, 2005b; Albach, Meudt & Oxelman, 2005a; Albach & Briggs, 2012) and many sequences were already available from our previous studies. The use of *trnL-trnL-trnF* further allows us to update and compare our results with those of the previous study of genome size (Albach & Greilhuber, 2004), whereas ITS allows us to expand upon that study, use a more informative marker and test whether our results are robust to marker choice. Although these markers have been used previously, 199 sequences were newly generated (see below, Table S1).

Genomic DNA was isolated using a modified cetyltrimethylammonium bromide (CTAB) method (Doyle & Doyle, 1987) or the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). PCR amplification and sequencing of the ITS region were accomplished using primers ITSA, ITSC, ITSD (Blattner, 1999), LEU1 (Vargas, Baldwin & Constance, 1998) and ITS4 (White *et al*., 1990) or ITS2, ITS3, ITS5 (White *et al*., 1990) and ITS28cc (Wagstaff & Garnock-Jones, 1998). PCR amplification and sequencing of the *trnL-trnL-trnF* region were performed with primers c, d, e and f (Taberlet *et al*., 1991). PCRs were set up in 25 μL as in Low (2005) or Albach & Meudt (2010) with the following PCR profile: 94 °C for 1–5 min, followed by 30–35 cycles of 94 °C for 18–30 s, 50–55 °C for 30 s and 72 °C for 1–2 min, and, finally, 72 °C for 7–10 min. For some sequences (i.e. those from Low, 2005), PCR fragments were separated on low-melting-point agarose gels (Nusieve FMC Bio-Products, Rockland, ME, USA) stained with ethidium bromide, cut from the gel and purified using the MinElute Gel Extraction Kit (Qiagen, Valencia, CA, USA); following cycle sequencing, ethanol precipitation, re-elution of the PCR fragments and sequencing (Perkin-Elmer ABI PRISM 377 Automated DNA Sequencer, Victoria University, Wellington, New Zealand), forward and reverse sequences were edited and aligned using

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SeqMan (DNAStar). For the remaining sequences, we followed Albach & Meudt (2010) or sent PCR products directly to GATC Biotech (Konstanz, Germany) or Macrogen Inc. (Seoul, South Korea) for forward and reverse sequencing. All generated sequences were aligned to the GenBank sequences using PhyDE v. 0.9971 (Müller *et al*., 2006).

Whenever possible, we used sequences from the same individual or population as the individual that was used for the genome size estimates, but, when this was not possible (i.e. DNA was not available, PCR amplification did not work or sequences were not clean after multiple attempts), we chose another individual of the same species from roughly the same geographical area (Table S1). In one case (*V. bozakmanii* M.A.Fisch.), we used a related species for the ITS sequence (*V. reuteriana Boiss*.) and, in another case (*V. orientalis*), we used a related species for the *trnL-trnL-trnF* sequence (*V. oltensis* Woronow ex Elenevsky) based on more in-depth phylogenetic analyses of the respective group (Sonibare *et al*., 2014; D. C. Albach & E. Mayland-Quellhorst, unpubl. data). In two cases (*V. orchidea* Crantz and *V. macrocarpa* Vahl), we obtained genome size measurements from two different individuals of differing ploidy, but DNA sequences were available from only one individual, such that we used the same DNA sequences to represent these species so that we could include multiple ploidies in the analyses. This was justified because multiple individuals of the same species with differing ploidy had similar sequences in the present study, as did those in another study in subgenus *Pseudolysimachium* (Opiz) Buchenau, of which *V. orchidea* is a member (P. Kosachev, L. Behcet & D. C. Albach, unpubl. data.). To examine patterns of the evolution of genome size in *Veronica*, we aimed to sample species from throughout the main lineages (subgenera), with a special emphasis on the Southern Hemisphere species. Therefore, from the genome size dataset of 237 individuals/128 species of *Veronica*, we chose a subset of 146 individuals/110 species plus the five other species of Veroniceae for which the genome size is known [i.e. *Lagotis integrifolia*, *Picrorhiza kurrooa* Royle, *Veronicastrum axillare* Siebold & Zucc., *Veronicastrum virginicum* (L) Farw. and *Wulfenia carinthiaca* Jacq.], generating matching ITS and *trnL-trnL-trnF* datasets for downstream phylogenetic analyses and statistical tests. Of the 151 ITS sequences and 151 *trnL-trnL-trnF* sequences analysed here, just over two-thirds (96 ITS and 103 *trnL-trnL-trnF* sequences, respectively) were newly sequenced, including 45 ITS and 45 *trnL-trnL-trnF* sequences from unpublished theses (Low, 2005; Prebble, 2008; see Table S1 for GenBank accession numbers). For several samples, only ITS1 (*V. gentianoides*, *V. barrelieri* H.Schott ex Roem. & Schult., *V. incana* L., *V. longifolia* L. from Turkey, *V. nakaiana* Ohwi, *V. orchidea*, *V. spicata* L., *V. thymifolia* Sibth. & Sm.) or ITS2 (*V. copelandii* Eastw., *V. anagallis-aquatica* var. *nilotica* R.Uechtr., *V. anagalloides* ssp. *taeckholmiorum* Chrtek & Osb.-Kos., *V. catenata* ssp. *pseudocatenata* Chrtek & Osb.-Kos., *V. hederifolia*, *V. ciliata*) were available. For all species for which only ITS1 was available (except *V. thymifolia*), the ITS1 sequence was obtained using the consensus of the contig with the most reads from a 454 sequencing run (D. C. Albach *et al*., unpubl. data). For 46 New Zealand samples, including all 38 from Low (2005), the first *c*. 410 bp of *trnL-trnL-trnF* was missing because of sequencing difficulties caused by a polyA/T region, and the *V. baumgartenii trnL-trnL-trnF* sequence was also missing *c*. 340 bp in the middle.

We estimated the optimal model of evolution to be GTR + Γ + I for ITS and combined (ITS + *trnL-trnL-trnF*) datasets, and GTR + Γ for *trnL-trnL-trnF*, based on the Akaike information criterion (AIC) (Akaike, 1973) as executed in jModelTest 2.1.3 (Guindon & Gascuel, 2003; Darriba *et al*., 2012). We used RAxML v 7.0.4 (Stamatakis, 2006) to perform phylogenetic and bootstrap analysis of *Veronica* simultaneously (1000 replicates), with *Lagotis integrifolia* chosen as the outgroup for each dataset. As the RAxML manual does not recommend the use of invariant sites in the model, we implemented the GTR + Γ model for all three datasets. We also combined our ITS dataset with that of Surina *et al*. (2014) in an expanded ITS dataset, which enabled the molecular dating of *Veronica* and lineages within it using BEAST version 1.8.0 (Drummond *et al*., 2012). The optimal model of evolution for this expanded ITS dataset was GTR + Γ + I, which we used in the BEAST analysis. The BEAST .XML input file was generated using BEAUTi version 1.8.0 (Drummond *et al*., 2012). To allow for rate variation among lineages, rate evolution was modelled in an uncorrelated lognormal relaxed clock framework (Drummond *et al*., 2006). The branch rate prior (ucld-.mean) was set to follow a normal distribution with a mean of $4.125 \times 10^{-3} \pm 1.808 \times 10^{-3}$, which is the mean ITS substitution rate of several herbaceous species from Kay, Whittall & Hodges (2006), except the outlier *Gentiana* section *Ciminalis* (Adans.) Dumort., following Surina *et al*. (2014). We used a Yule tree prior as recommended for species-level phylogenies in the BEAST manual. We ran three runs of 40 million iterations each, sampling every 2000th iteration. Chain convergence and estimated sample sizes (ESSs) were confirmed to be sufficiently high (> 200) in Tracer v 1.6.0 (Rambaut *et al*., 2014). The resulting tree files were combined with LogCombiner v 1.8.0, discarding the first 10% of the trees as burn-in. TreeAnnotator v 1.8.0 was used to compute the maximum clade credibility tree with node heights being the median of the

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age estimates. Both programs are part of the BEAST package (Drummond *et al*., 2012). Nodes were calibrated following Surina *et al*. (2014) in that we: (1) constrained the *Plantago* L. + *Aragoa* Kunth stem to be monophyletic using an exponential distribution with a mean of 1, and an offset of 19.4 million years ago (Mya), and (2) set a uniform age prior for the crown age of *Aragoa* that spans 3.3–0.0 Mya, likewise constrained to monophyly. These calibrations are based on palaeobotanical, geomorphological and fossil data (for details, see Surina *et al*., 2014).

All statistical tests were performed in R using R Studio v 0.98.501 (R Studio Inc., Boston, MA, USA). Although all five continuous and categorical variables deviated from a normal distribution (qqnorm and qqline plots in 'stats' package) and the significant tests of normality ('nortest' package; Gross, 2012), below we report only the results based on untransformed (raw) data for the following reasons: the characters do not have large ranges, log transformation did not greatly improve the QQ plots, and tests using log-transformed data gave similar results relative to those using raw data (data not shown). As phylogenetic relatedness may confound standard statistical analysis (Felsenstein, 1985), we calculated the relationship between genome size and each of the characters of interest using a phylogenetic generalized least-squares (PGLS) approach (Grafen, 1989; Symonds & Blomberg, 2014) employing the best RAxML tree for each dataset. First, we used GLS to determine which model received the best AIC and log-likelihood scores employing different types of correlation structures (corBrownian, corMartins, corPagel, corBlomberg) in the 'nlme' (R Core Team, 2014) and 'ape' packages (v. 3.0–11; Paradis, Claude & Strimmer, 2004; Paradis, 2010). For all pairs of characters, corPagel (which is derived from the Brownian motion model by estimating the λ statistic of Pagel, 1999 and multiplying the covariances by it; see R documentation for an explanation of the other correlation structures) was found to be the best model. Then, we employed PGLS with lambda="ML" in the 'caper' package (Orme, 2013) to estimate the phylogenetic signal using maximum likelihood simultaneously with the regression (Revell, 2010) using Pagel's λ, which ranges from $0 < \lambda < 1$, where $\lambda = 0$ is the equivalent to a random walk and no phylogenetic signal present, and $\lambda = 1$ equates to a Brownian motion model and the presence of phylogenetic signal.

Bar plots of all characters of interest were visually assessed by mapping them on the side of each phylogenetic tree using the package 'phytools' (Revell, 2012). Ancestral character states for the continuous characters 1C- and 1Cx-values were calculated and mapped on the best RAxML tree for each dataset using contMap in 'phytools', which employs fastAnc to estimate the maximum likelihood ancestral states for continuous traits. Significant genome downsizing or upsizing events were detected by comparing ancestral vs. random node values which were calculated using ace (method="GLS", corStruct=corBrownian) with randomly reshuffled tip values ($N = 999$ replicates) following Šmarda *et al*. (2014). We also plotted observed and expected ploidy vs. 1C-value and ploidy vs. 1Cx-value. Expected values were extrapolated for polyploids based on mean diploid observed values for each base number separately (i.e. $x = 7$, $x = 8$ and $x = 9$) and all diploids combined ($x = 7$, 8 and 9).

To determine the net diversification rates in *Veronica* lineages (subgenera) and the number and location of monoploid genome size evolution rate shifts on the tree, we used Bayesian Analysis of Macroevolutionary Mixtures (BAMM) (Rabosky, 2014) and the package 'BAMMtools' (Rabosky *et al*., 2014; bamm-project.org). The BEAST tree (with extra outgroups from Surina *et al*., 2014 removed) was used as input for running both the trait (1Cx-value) and speciation/extinction analyses in BAMM. Priors were set in 'BAMMtools'. We set globalSamplingFraction to 0.33 in the diversification analysis to account for incomplete sampling (~150/450 *Veronica* species). Three replicates of each analysis were run in BAMM for 30 000 000 generations each, sampling every 10 000 for a total of 3000 samples per run. 'BAMMtools' was used to process the BAMM output files (using 10% burnin), including the assessment of the stationarity and convergence of replicate runs, ensuring that ESS of number of shifts and the log-likelihood were > 200, determining posterior probabilities for different rate shifts, calculating Bayes factors (BFs), plotting the best shift rate configurations and estimating rates of evolution of monoploid genome size (trait analysis) and speciation/extinction (diversification analysis). Finally, to test whether uncertainties regarding ploidy estimation affected our results, the PGLS analysis and BAMM were re-run without the 18 sampled individuals of subgenus *Pentasepalae*.

RESULTS

There is a strong correlation ($R^2 = 0.998$; $y = 0.963x + 0.028$) between flow cytometric measurements based on fresh vs. silica gel-dried leaf material for the respective subset of our *Veronica* dataset ($N = 6$) (results not shown; see Table S1 for 1C-values used in comparison). The silica gel-dried measurements were 0.0–0.15 pg (0.1–6.7%) higher than their fresh counterparts at 6 weeks ($N = 6$) and −0.01 to 0.07 pg (0.7–2.4%) different from their fresh counterparts at 5 months ($N = 4$). In addition, intra-individual variation (i.e. between runs of the same individual) was lower for fresh material (0.0–0.02; 0.1–0.5%) compared with

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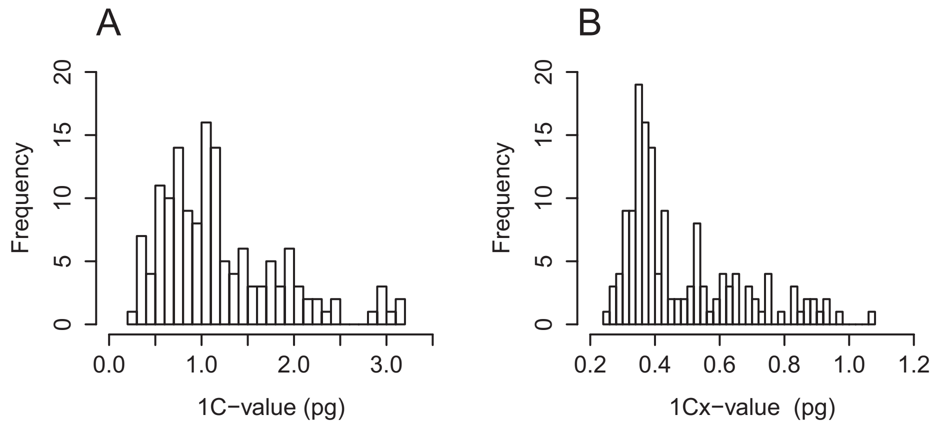

Figure 1. A, Distribution of 1C-values in *Veronica*. B, Distribution of 1Cx-values in *Veronica*.

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silica gel-dried material (0.0–0.05, 0.1–3.7%). CV values from fresh material (2.8–5.2%, $N = 6$) were lower than those from silica gel-dried material, but CV values at 6 weeks (5.3–8.1%, $N = 6$) were similar to those at 5 months (4.8–7.5%, $N = 4$). Overall, *c*. 70% of the CV values obtained from silica gel-dried material were between 5 and 8%, whereas just 6% of the CV values obtained from fresh material were > 5%. Although the genome sizes of the two samples were statistically significantly different at $P < 0.05$ ($U = 0$, $P = 0.03125$), because of the high correlation and small differences between samples using fresh vs. silica gel-dried material, we felt confident using silica gel-dried material from other species (when CV values were < 8%) for which fresh material was not available for genome size measurement.

Similarly, there is a strong correlation ($R^2 = 0.958$; $y = 1.114x - 0.008$) and no statistically significant difference ($U = 73$, $P = 0.3955$) between flow cytometry vs. Feulgen densitometry for the respective subset of our *Veronica* ($N = 19$ comparisons for 17 species) (results not shown; see Table S1 for 1C-values used in comparison). The Feulgen measurements differed by 0–0.3 pg relative to their flow cytometry counterparts (0–30.5%; median, 5.5%), with nine Feulgen measurements lower vs. ten measurements higher than the corresponding flow cytometry measurements. Although the difference between both types of measurements for *V. beccabunga*, *V. arvensis*, *V. triphyllos* and *V. chamaedrys* (4*x*) was 17.5–30.5%, for the vast majority (15 of 19) of the comparisons there was ≤ 9.1% difference. Because of this, we felt confident including Feulgen measurements in our comparisons when flow cytometry measurements were not available.

The genome sizes for all 237 individuals of 128 *Veronica* spp., plus five individuals of Veroniceae outgroups, from the current study plus previous publications are listed in Table S1. For 81 of the 101 species of *Veronica* sampled in this study, the genome sizes are published here for the first time, including 54 from New Zealand (50) and Australia (4). The 1C genome size values for *Veronica* found here range from 0.26 pg (*V. javanica* Blume, $2n = 2x = 16$) to 3.19 pg (*V. baylyi* Garn.-Jones, preliminary count of $2n = 18x = c$. 116 by B. Murray, University of Auckland, New Zealand, unpubl. data; several similar and probably related species have $2n = 120$; Fig. 1A). Southern Hemisphere species ($N = 79$ individuals, 54 species) range from 0.73 pg (*V. raoulii*, $2n = 6x = 42$) to 3.19 pg (*V. baylyi*). The 1Cx-value in the genus ranges from 0.24 pg (*V. raoulii*) to 1.08 pg (*V. micrantha*, $2n = 2x = 16$) (Fig. 1B) and, in the Southern Hemisphere, only from 0.24 to 0.52 pg. Previously unreported DNA ploidies are inferred from our genome size estimates for *V. catenata* Pennell (2*x*), *V. pectinata* L. (4*x*), *V. ciliata* (4*x*), *V. densiflora* Ledeb. (4*x*), *V. rotunda* Nakai (8*x*), *V. scutellata* L. (4*x*) and *V. bozakmanii* (6*x*), and DNA ploidies are inferred for the first time for *V. donii* Römpp. (2*x*), *V. kaiseri* Tächk. (2*x*), *V. anagalloides* ssp. *taeckholmiorum* (2*x*), *V. vendetta-deae* Albach (4*x*), *V. macrostemon* Bunge ex Ledeb. (4*x*), *V. polifolia* Benth. (4*x*), *V. leiocarpa* Boiss. (8*x*) and *V. baylyi* (18*x*).

Phylogenetic trees based on ITS (Fig. 2, Supporting Information Fig. S1), *trnL-trnL-trnF* (Supporting Information Fig. S2) and combined datasets (Supporting Information Fig. S3) were generally similar to previously published trees using these markers. However, there were some differences with regard to topology and sampling. In general, relationships and support values in the combined tree were more similar to the better resolved and more strongly supported ITS tree (rather than the *trnL-trnL-trnF* tree). In the ITS analysis (Fig. S1), subgenus *Cochlidiosperma* (Rchb.) M.M.Mart.Ort. & Albach is polyphyletic, whereas the subgenus is monophyletic in the

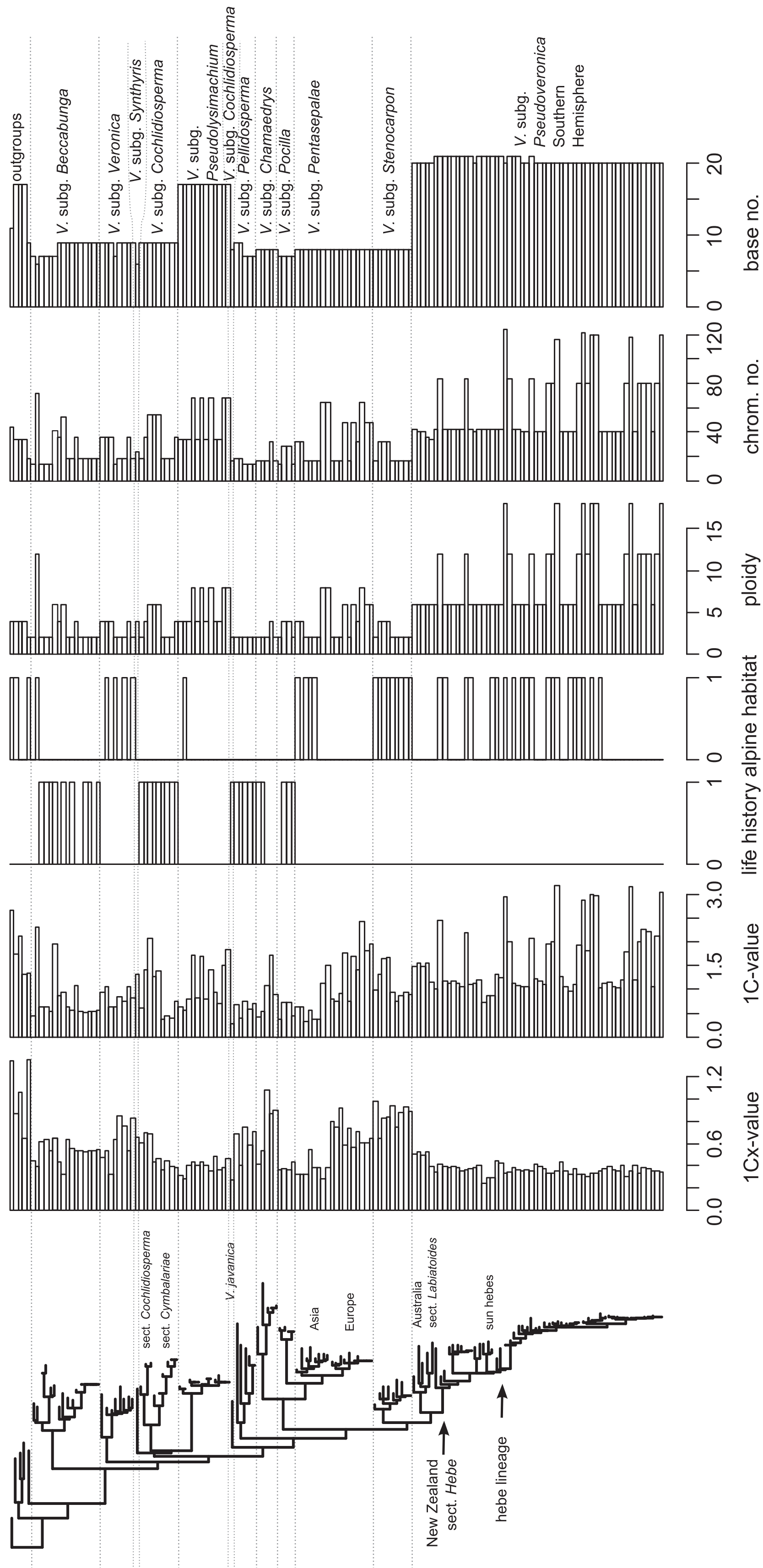

Figure 2. RAxML maximum likelihood tree of *Veronica* and outgroups based on internal transcribed spacer (ITS) with categorical variables of interest mapped to the right, including 1Cx-value (0.24–1.35 pg), 1C-value (0.27–3.19 pg), life history (annual, bar; perennial, no bar), habitat (non-alpine, no bar; alpine, bar), ploidy (2*x* to 18*x*), chromosome number (14–126) and base chromosome number (6–21).

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trnL-trnL-trnF and combined trees (Figs S2, S3). *Veronica* subgenus *Beccabunga* (Hill) M.M.Mart.Ort., Albach & M.A.Fisch. is sister to the remaining subgenera in the ITS tree, whereas, in the *trnL-trnL-trnF* analysis, subgenus *Pseudolysimachium* is sister to the rest followed by subgenus *Beccabunga* (Fig. S2). In the combined analysis, subgenus *Veronica* is in this position (Fig. S3). The combined dataset topology (Fig. S3) contained a mixture of results from the separate analyses (but see above) and increased support where both datasets agree and decreased support for incongruent relationships. In all cases, these incongruences have little or no support in at least one of the datasets.

The Southern Hemisphere *V.* subgenus *Pseudoveronica* is supported as monophyletic in all analyses (ITS, 77 BS (bootstrap support); *trnL-trnL-trnF*, 82 BS; combined, 96 BS; Figs S1–S3). The five Australian species form a clade (section *Labiatoides*) sister to the New Zealand clade (section *Hebe*) in the ITS tree and combined trees. In the *trnL-trnL-trnF* tree, only four of the five Australian species form a clade, and with low support; the fifth species *V. formosa* R.Br. is unresolved in subgenus *Pseudoveronica*. In section *Hebe*, the following species form highly supported clades in the ITS and combined trees: *V. catarractae* G.Forst., *V. hookeriana* Walp., *V. lanceolata* Benth., *V. lyallii* Hook.f and *V. melanocaulon* Garn.-Jones (speedwell hebes, 100 BS); *V. raoulii* Hook.f., *V. hulkeana* F.Muell. and *V. lavaudiana* Raoul (sun hebes, 100 BS); and 36 species comprising the hebe lineage (*sensu* Albach & Meudt, 2010; 100 BS); whereas the positions of *V. planopetiolata* G.Simpson & J.S.Thomson, *V. macrantha* Hook.f., *V. densifolia* (F.Muell.) F.Muell. and *V. colostylis* Garn.-Jones are unresolved and not supported as belonging in any particular clade. Two additional speedwell hebes, *V. senex* (Garn.-Jones) Garn.-Jones and *V. decora* (Ashwin) Garn.-Jones, are sister species (100 BS), but their relationship to the larger speedwell hebe clade is unresolved in this tree. In the hebe lineage, there is a moderately supported lineage involving 19 species [*V. topiaria* (L.B.Moore) Garn.-Jones to *V. macrocarpa,* 80 BS], plus several other lineages with little support. In this lineage, a smaller clade of species characterized by large leaves and mostly with a northern and lowland distribution [*V. flavida* (Bayly, Kellow & de Lange) Garn.-Jones to *V. macrocarpa*] is weakly supported (63 BS) and sister to a weakly supported clade of species characterized by smaller leaves (*V. topiaria* to *V. albicans* Petrie). At the base of the hebe clade is a grade of several species [*V. ochracea* (Ashwin) Garn.-Jones to *V. lycopodioides* Hook.f.] characterized by whipcord habit (small imbricating appressed leaves), but this grade also includes *V. vernicosa* Hook.f. with larger leaves. In general, although similar relationships in Southern Hemisphere subgenus *Pseudoveronica* were also found in the *trnL-trnL-trnF* tree, they are not well resolved or supported. In that tree, a clade of hebes is sister to a clade comprising speedwell hebes, sun hebes and semi-whipcord hebes, but the support values for this topology are low.

BEAST analyses on the expanded ITS dataset (Fig. 3) allowed molecular dating of several crown and stem lineages in *Veronica* (Supporting Information Table S3). The topology of the BEAST tree was largely congruent with the topologies from the two datasets from which it is made, i.e. the RAxML ITS tree (Fig. S1) and the Surina *et al*. (2014) tree. *Paederota lutea* L.f. was sister to *Veronica* subgenus *Veronica*, as has been seen in some previous ITS studies (e.g. Albach & Chase, 2001, 2004), even though plastid, morphological and other ITS analyses place it as sister to *Veronica* (e.g. Albach & Meudt, 2010). The crown and stem ages for *Veronica* (including *Paederota lutea*) are 16.13 Mya [95% highest posterior density (HPD), 20.59–12.46 Mya] and 19.91 Mya (95% HPD, 25.09–15.47 Mya), respectively. Northern Hemisphere subgenera range in mean age from 8.65 to 2.30 Mya (crown; 95% HPD, 12.12–0.84 Mya) and from 14.37 to 6.92 Mya (stem; 95% HPD, 18.87–9.96 Mya), whereas Southern Hemisphere subgenus *Pseudoveronica* is estimated to have originated at 10.21 Mya (crown; 95% HPD, 13.33–7.21 Mya) and 11.43 Mya (stem; 95% HPD, 14.85–8.39 Mya) (Table S3).

Mapping the categorical and binary characters of interest on the side of the tree shows visually that several of these characters indeed show phylogenetic patterns (Fig. 2). The PGLS analyses for all datasets were similar (compare Table 1 with Supporting Information Table S4). In all analyses, λ was near 1 for all five trait pairs involving the 1Cx-value and for two trait pairs involving the 1C-value (ploidy and chromosome number), indicating strong phylogenetic signal for these characters, whereas λ was near 0.6 for alpine habitat, life history and base number vs. 1C-value, suggesting moderate phylogenetic signal (Tables 1, S4). 1C- and 1Cx-values were weakly to strongly correlated with certain traits, including a weak but significant correlation of 1C-value with life history, but no significant correlations were found between 1C- or 1Cx-values and alpine habitat in any of the datasets (Table S4). The results of PGLS analyses excluding the subgenus *Pentasepalae* (Benth.)M .M.Mart.Ort., Albach & M.A.Fisch. were similar (data not shown).

Plots of ploidy vs. 1C- and 1Cx-values suggest that both genomic downsizing and upsizing have occurred in *Veronica* (Fig. 4). The means of the Northern Hemisphere diploid, tetraploid and hexaploid species of *Veronica* (excluding subgenus *Pseudolysimachium*) are close to expected values, with observed values

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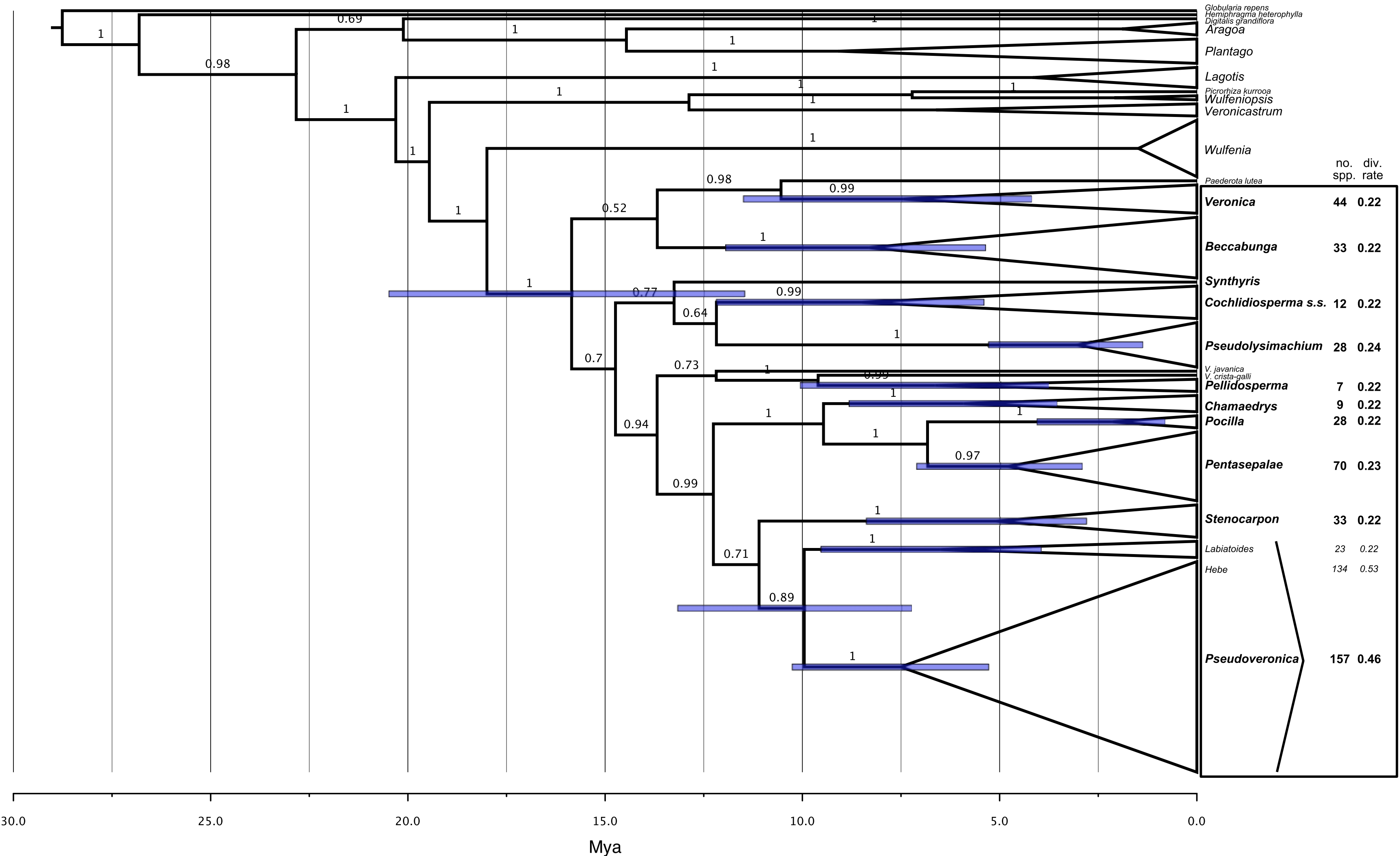

Figure 3. BEAST time tree for *Veronica* and outgroups of the expanded internal transcribed spacer (ITS) dataset showing the major lineages, their bootstrap support (numbers above branches) and their estimated ages (millions of years ago, Mya). *Veronica* subgenera are listed in bold italics within the box, sections are listed in italics. no. spp., total number of species in each subgenus or section; div. rate, net diversification rate per million years. See also Supporting Information Table S3.

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Table 1. Results of phylogenetic generalized least-squares (PGLS) analysis for *Veronica* and outgroup genome size and character data on the internal transcribed spacer (ITS) phylogenetic tree. Significant *P* values are indicated in bold type

	Character	λ	Slope	Multiple R^2	Adjusted R^2	F-statistic	d.f.	P
1C-value	Alpine habitat	0.63	0.19	0.02	0.01	3	149	0.09
1Cx-value	Alpine habitat	0.99	−0.01	0.00	0.00	0	149	0.53
1C-value	Life history	0.57	−0.36	0.03	0.02	5	149	**0.03**
1Cx-value	Life history	0.99	−0.01	0.00	−0.01	0	149	0.67
1C-value	Base number	0.60	0.03	0.01	0.00	1	149	0.24
1Cx-value	Base number	0.99	−0.02	0.02	0.02	4	149	0.05
1C-value	Chromosome number	0.97	0.02	0.91	0.91	1517	149	**0.00**
1Cx-value	Chromosome number	0.99	0.00	0.07	0.07	12	149	**0.00**
1C-value	Ploidy	0.97	0.17	0.90	0.90	1358	149	**0.00**
1Cx-value	Ploidy	1.00	0.00	0.07	0.07	11	149	**0.00**

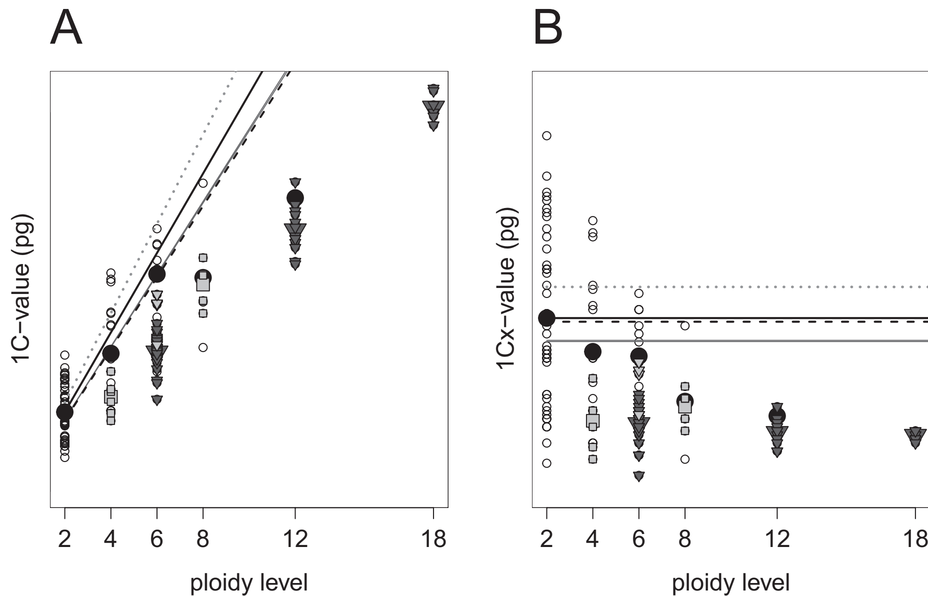

Figure 4. Expected and observed 1C-values (A) and 1Cx-values (B) in *Veronica*. Lines represent expected values based on mean observed diploid values from species with $x = 7$ or $x = 9$ (overlapping, nearly identical grey full lines), $x = 8$ (grey dotted line) and $x = 7$, 8 and 9 combined (black full line), as well as expected values based on the estimated ancestral values from all species (black broken line, based on 1C-value = 1.13 pg and 1Cx-value = 0.62 pg). Symbol shape signifies location or subgenus: Northern Hemisphere excluding *V.* subgenus *Pseudolysimachium* (black and white circles), New Zealand (black triangles), Australia (grey triangles), *V.* subgenus *Pseudolysimachium* (grey squares). Symbol size signifies observed measurements (small) vs. means (large).

falling both above and below the expected values (Fig. 4). By contrast, the mean of the Northern Hemisphere octoploid species is lower than the expected value, as are the means (and in fact all observed data) of the Southern Hemisphere species (Fig. 4). Ancestral state reconstruction of the continuous genome size variables shows that *Veronica* had an ancestrally small genome (1C-value = 1.13 pg; Fig. 5A). There have been several increases (dark green to blue in Fig. 5A) and decreases (dark orange to red in Fig. 5A) of 1C-value along the tree, some of which are statistically significant ($P < 0.05$; triangles in Fig. 5A). Ancestral state reconstruction of 1Cx-values shows significant genomic downsizing from the ancestral *Veronica* 1Cx-value = 0.62 pg within New Zealand section *Hebe* of subgenus *Pseudoveronica* (1Cx-value = 0.42 pg), Northern Hemisphere subgenus *Pseudolysimachium* (1Cx-value = 0.34 pg) and

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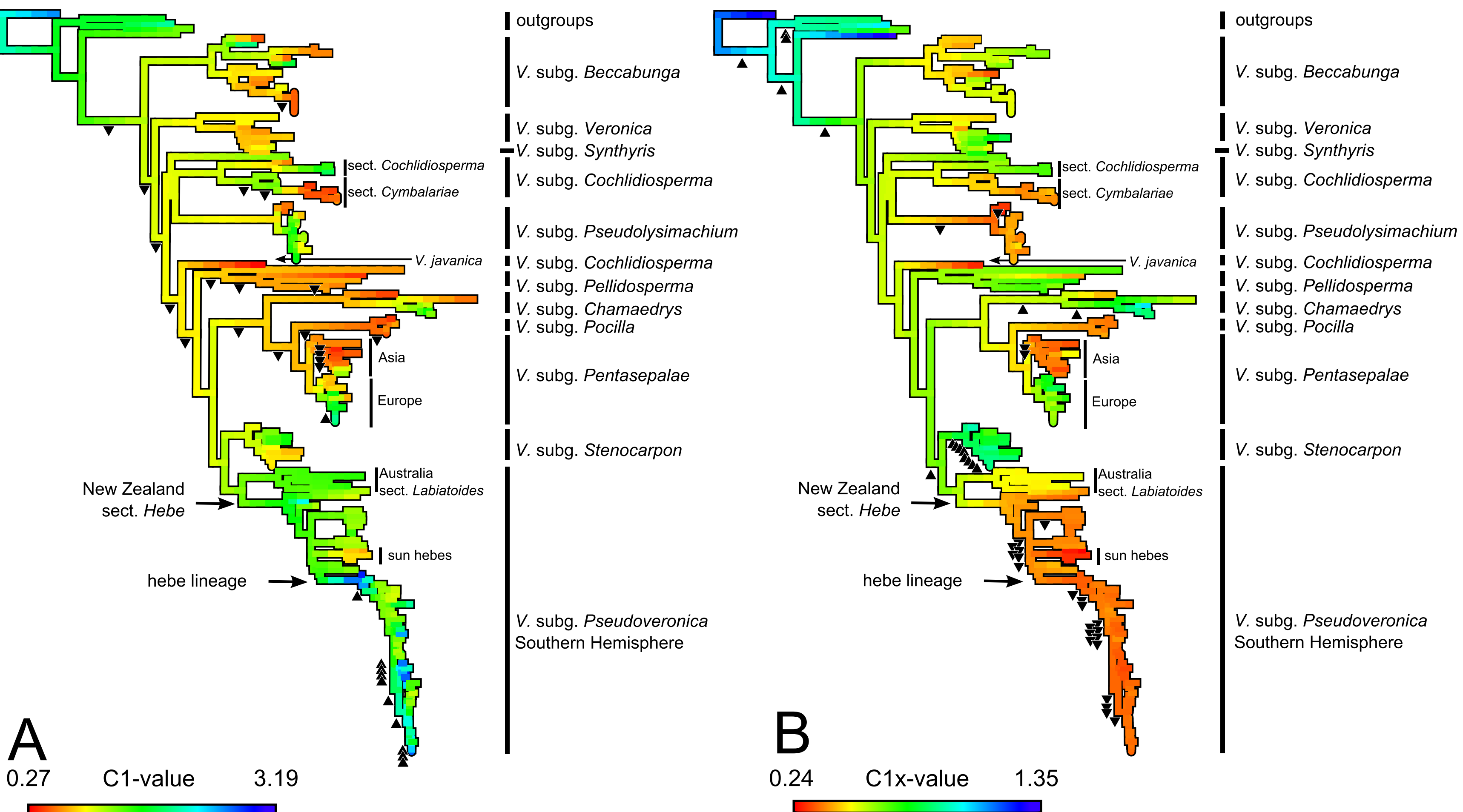

Figure 5. RAxML maximum likelihood tree of *Veronica* and outgroups based on internal transcribed spacer (ITS), with ancestral genome size values calculated by fastAnc in the R package 'phytools' shown as colours. A, 1C-values; B, 1Cx-values. Branches with significant genome downsizing or upsizing ($P < 0.05$), calculated using ace in the R package 'ape', are shown by down or up black triangles, respectively.

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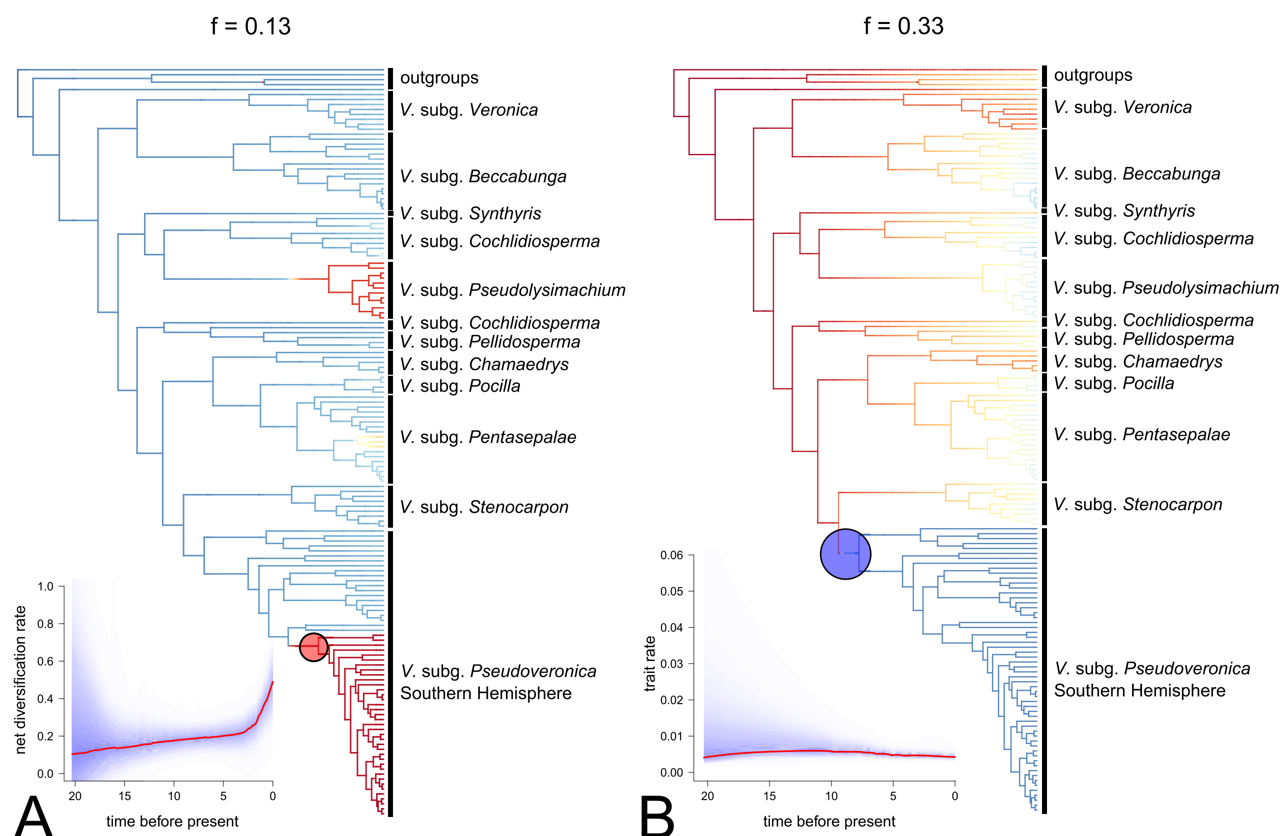

Figure 6. Results from BAMM (Bayesian Analysis of Macroevolutionary Mixtures) diversification and trait analyses of *Veronica*. A, The most frequent shift configuration (i.e. that with the highest posterior probability, $f = 0.13$) in the 95% credibility shift set for the diversification analysis. Colours refer to decreases (blue) or increases (red) in diversification rates. The red circle shows the large 'core shift' to a higher rate in *V.* subgenus *Pseudoveronica* section *Hebe*. Inset shows the rate vs. time plot from the diversification analysis, where the 'net diversification rate' is given as diversification (speciation events – extinction events) per million years, and 'time before present' is in millions of years. B, The most frequent shift configuration (i.e. that with the highest posterior probability, $f = 0.33$) in the 95% credibility shift set for the trait analysis of monoploid genome size. Colours refer to decreases (blue) or increases (red) in rates of monoploid genome size evolution. The blue circle shows the large 'core shift' to a lower rate in *V.* subgenus *Pseudoveronica*. Inset shows the rate vs. time plot from the trait analysis, where 'trait rate' is given as change per million years, and 'time before present' is in millions of years.

the Asian lineage of subgenus *Pentasepalae* (1Cx-value = 0.37 pg) ($P < 0.05$; dark orange to red and down triangles in Fig. 5B). Two significant increases are also noted, namely in subgenus *Stenocarpon* (Boriss.) M.M.Mart.Ort., Albach & M.A.Fisch. and subgenus *Chamaedrys* (W.D.J.Koch) Buchaenau ($P < 0.05$: blue and up triangles in Fig. 5B).

In BAMM, the three replicate runs of diversification and trait analyses gave similar results (including log likelihoods and number of shifts; data not shown), and so only the run with the best likelihood is discussed. For the diversification analyses, the rate shift models with one, two and three rate shifts had the highest posterior probabilities (0.27, 0.40 and 0.23, respectively). Very large BFs > 1000 were seen for all models with one to seven rate shifts, and the model with three rate shifts had the highest BF (4200). The most frequent shift configuration of the 95% credible set of shift configurations showed multiple shifts to higher diversification rates and a 'core shift' in the Southern Hemisphere subgenus *Pseudoveronica* (red circle in Fig. 6A). An additional 'core shift' could be seen in *Veronica* as a whole in some of the other shift configurations (data not shown). The mean net diversification rate in *Veronica* as calculated by BAMM was 0.29 speciation events per million years (Myr), ranging from 0.22–0.24 in the Northern Hemisphere subgenera to 0.46 in the Southern Hemisphere subgenus *Pseudoveronica* (and 0.53 in section *Hebe*; Table S3, Fig. 3). The net diversification rate has

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increased over time in *Veronica*, especially in the last 2 Myr (Fig. 6A, inset). In the BAMM trait analysis of monoploid genome size (1Cx-value), models with one to three rate shifts had the highest posterior probabilities of 0.22, 0.38 and 0.24, respectively. BFs > 20 were seen for all models with one to nine rate shifts, and the model with nine rate shifts had the highest BF (BF = 461). The most frequent shift configuration of the 95% credible set of shift configurations showed one large 'core shift' to a decreased rate in the Southern Hemisphere lineage (blue circle and branches in Fig. 6B). A subsequent smaller 'core shift' to an increased rate in section *Hebe* was seen in some of the other shift configurations (data not shown) The mean rate of monoploid genome size evolution in *Veronica* as calculated by BAMM was 0.005 and has not varied much over time (Fig. 6B, inset). The results of BAMM excluding subgenus *Pentasepalae* did not differ (data not shown).

DISCUSSION

General patterns of genome size variation and evolution in *Veronica*

Our study represents a nearly three-fold increase in the number of *Veronica* spp. for which the genome size is now known, which means that such data are now available for *c*. 30% of all *Veronica* spp. worldwide, including 35% of the species in the Southern Hemisphere subgenus *Pseudoveronica* (Table S3). Previously, most genome size estimates had been generated using Feulgen densitometry (> 90% in Albach & Greilhuber, 2004), whereas now more than 70% of all estimates are from flow cytometry using both fresh and silica gel-preserved material (see below for a discussion of methodological comparisons). In general, the range of genome sizes for *Veronica* fits in the lower half of the range of genome sizes known for Plantaginaceae (0.26–8.98 pg; $N = 204$ species in 18 genera, including *Callitriche* L., *Penstemon* Mitch., *Plantago* and *Veronica*; Broderick *et al*., 2011; Bennett & Leitch, 2012; Wong & Murray, 2012; Prančl *et al*., 2014).

Veronica 1C-values showed a significant correlation with life history (PGLS, $P < 0.05$: Tables 1, S4). As stated by Bennett (1972) and demonstrated previously for *Veronica* by Albach & Greilhuber (2004), this correlation is not caused by a direct relationship, but instead by a restriction of annuals to lower genome sizes (1C-value = 0.26–2.08 pg) compared with perennials (1C-value = 0.32–3.19 pg). For annuals, only *V. bozakmanii* and polyploids of subgenus *Cochlidiosperma* have genomes in the upper end of this range (1C-value > 1.0 pg), whereas annuals with smaller genomes (1C-value < 0.75 pg) are frequent across most Northern Hemisphere subgenera of *Veronica*. By contrast, although alpine *Veronica* spp. tend to have larger 1C-values than non-alpines, this correlation was not significant (PGLS, $P = 0.09$; Tables 1, S4). The occupation of an alpine habitat is more randomly distributed over the phylogenetic tree (and is found in both Northern and Southern Hemispheres, see Fig. 2).

Veronica has smaller ancestral 1C- and 1Cx-values than the outgroups, which is significant for the 1C-value (Fig. 5A). As confidence intervals for ancestral genome size values can be large (data not shown), caution is required in the interpretation of genome size evolution at these deeper branches of the tree. The strong positive correlations between both 1C- and 1Cx-values vs. both ploidy and chromosome number in *Veronica* (PGLS, $P < 0.001$; Tables 1, S4) indicate that polyploidy, which initially brings about an instant doubling, has a strong influence on the evolution of genome size in *Veronica* (Albach *et al*., 2008). Polyploidy is in fact rampant across the genus in both hemispheres, with 76% of all species of known ploidy being polyploid (Table S3; 56% considering Northern Hemisphere species only), and estimates of 20–25% of all speciation events being associated with polyploidy (Albach *et al*., 2008). Numerous studies have investigated some of these polyploid complexes and have highlighted the importance of detailed analyses for the distribution of particular ploidies (Martínez-Ortega *et al*., 2004; Albach, 2007; Bardy *et al*., 2010, 2011; Sonibare *et al*., 2014). Although polyploidy occurs in almost all subgenera of *Veronica*, polyploid lineages have had mixed diversification success (Table S3; see also table 3 from Albach *et al*., 2008). Two species radiations comprised entirely of polyploid species are the Southern Hemisphere subgenus *Pseudoveronica* (6*x*, 12*x*, 18*x*) based on hexaploid ancestors, and the Northern Hemisphere subgenus *Pseudolysimachium* (4*x*, 8*x*) based on tetraploid ancestors. These are two of the three lineages which show significant monoploid (1Cx-value) genome downsizing (Fig. 5). Most of the genome downsizing events in these lineages are estimated to have occurred during the last ~3.5 Myr, although the first downsizing event in New Zealand may have occurred by ~6.4 Mya (Figs 3, 5). Clearly, genome downsizing is not universal in the genus and, indeed, the monoploid genome size (1Cx-value) increased significantly in at least two lineages at ~6.0–5.3 Mya (95% HPD, 8.82–2.77 Mya; i.e. subgenera *Stenocarpon* and *Chamaedrys*, Figs 3, 5), both with the rare occurrence of polyploids that have not diversified (Albach *et al*., 2008; Table S3) and both with low diversification rates (Table S3). Analyses both with and without subgenus *Pentasepalae* gave similar results in PGLS and BAMM (data not shown), and therefore we are confident that our conclusions are not affected by any ploidy level uncertainties.

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Our analyses point to the intriguing hypothesis that genome downsizing may have played an important role in the diversification of species-rich lineages of both the Northern and Southern Hemispheres. They provide strong evidence for multiple rate shifts along the *Veronica* tree (Fig. 6A), with subgenus *Pseudoveronica* (with 157 species, all polyploid) showing a 'core rate shift' (Fig. 6A) and a diversification rate (0.46) which is 1.8–2.2 times higher than those in Northern Hemisphere subgenera (Table S3). The other two Northern Hemisphere lineages for which genome downsizing has occurred [subgenus *Pseudolysimachium*, 28 species, 100% polyploid; and subgenus *Pentasepalae* (Asian lineage), 70 species, 46% polyploid] have the next two highest diversification rates (0.23–0.24), but these are only marginally higher than those of the other Northern Hemisphere subgenera (0.22; Table S3). Thus, genome downsizing may be a prerequisite in polyploid lineages for subsequent radiation. However, this may be difficult to assess as current methods do not allow the testing of correlations among two rates on a phylogenetic tree, and genome size downsizing and diversification shift may have occurred subsequent to polyploidization and, in that case, would not be a trait of the whole polyploid lineage. Nevertheless, a potential mechanism for such a correlation between higher diversification and genome downsizing could be the stochastic loss of genes, which creates Bateson–Dobzhansky–Muller incompatibilities between different polyploid lineages, thus increasing the non-adaptive speciation rate (Werth & Windham, 1991; Lynch & Force, 2000). However, alternative hypotheses, such as organisms with small genomes have more stably inherited mutations, or a nucleotypic effect, in which organisms with small genomes and shorter genes have a general advantage as a result of faster replication and transcription (Kraaijeveld, 2010), cannot be excluded at the moment. The lack of genomic data for *Veronica* and adaptive evolution subsequent to the polyploid event in the respective lineages prevent us from testing this hypothesis in this genus. One alternative hypothesis, that (parallel) genome downsizing would follow a radiation, is refuted by cases such as subgenera *Pentasepalae* (European lineage), *Beccabunga* and *Veronica*. These are essentially radiations at the diploid level with multiple independent polyploid lineages and relatively low diversification rates. They also show little or no genome downsizing, or even show genome upsizing (at least in the case of the European lineage of subgenus *Pentasepalae* for which we have better sampling here; Figs 2, 5). To demonstrate the wider applicability of this correlation, we would need to compare multiple polyploid lineages with varying degrees of genome up- and downsizing. It would be of great interest to increase sampling in several subgenera, e.g. the polyploid Asian species of subgenus *Veronica*, to further test the putative link between polyploidy and species radiations in *Veronica*, although these lineages may be too young to show a significant diversification rate change. Indeed, this may also be the reason why a significant rate change was not found in subgenera *Pseudolysimachium* and *Pentasepalae*, the crown ages of which are much more recent than that of subgenus *Pseudoveronica* (Table S3). The only older polyploid lineage in Veroniceae available for comparison is *Picrorhiza* Royle which has just three species and a relatively large genome. Therefore, it will be important to investigate this pattern comprehensively in other genera.

Evolution of genome size in Southern Hemisphere *Veronica*

Southern Hemisphere *Veronica* is a polyploid species radiation of 158 species (Garnock-Jones, 1993b; Wagstaff *et al*., 2002; Bayly & Kellow, 2006; Albach & Briggs, 2012) that shows strong evidence of genome downsizing. 1C-values in Southern Hemisphere *Veronica* spp. range from 0.73 to 3.19 pg, including some of the highest values in the genus, but with 1Cx-values ranging from 0.24 to 0.52 pg (2.2-fold variation), including some of the lowest. The low 1Cx-values found in subgenus *Pseudoveronica* (the only insular radiation in the genus) are in line with studies showing that insular endemic radiations in the Canary, Hawaiian and Marquesas Islands have smaller genome sizes relative to the mainland flora (Suda, Kyncl & Jarolímová, 2005; Kapralov & Filatov, 2011). The extent to which the New Zealand endemic flora follows this pattern is unknown, as phylogenetic studies of genome size are lacking, and indeed very few (< 10%) native angiosperms have had their genome sizes estimated.

Veronica subgenus *Pseudoveronica* originated at 11.43 Mya (95% HPD, 14.85–8.39 Mya), with the crown lineage beginning diversification soon after at 10.21 Mya (95% HPD, 13.33–7.21 Mya) (Table S3, Fig. 3). In the subgenus, all five (ITS) or four of five (*trnL-trnL-trnF*) sampled individuals of section *Labiatoides* (Australia) form a sister lineage to section *Hebe* (New Zealand + Papua New Guinea), congruent with previous phylogenetic studies with fewer species sampled (Wagstaff *et al*., 2002; Albach *et al*., 2005b; Albach & Meudt, 2010; Müller & Albach, 2010). The stem (10.21 Mya; 95% HPD, 13.33–7.21 Mya) and crown (section *Labiatoides*: 6.63 Mya; 95% HPD, 9.53–3.98 Mya; section *Hebe*: 7.62 Mya; 95% HPD, 10.26–5.29 Mya) ages fall in the late Miocene and are in line with those from several other genera in New Zealand, which, at the time, supported a low-altitude, warm-temperate, high-rainfall rainforest and sclerophyll

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vegetation (Lee, Tanentzap & Heenan, 2012; Heenan & McGlone, 2013). It has been hypothesized previously that subgenus *Pseudoveronica* ($x = 20/21$) is derived from a palaeohexaploid ancestor that had a base number of $x = 8$ (Albach *et al*., 2008). Therefore, it is possible that some genomic downsizing may have occurred in concert with dysploidy early in the evolutionary history of the subgenus. Nevertheless, the significant genomic downsizing events detected in our analyses probably occurred later, with the first occurring in section *Hebe* at ~6.4 Mya, but prior to major diversification in New Zealand, which occurred in concert with cooling and mountain building during the Pliocene to Pleistocene.

Furthermore, the results from the BAMM diversification and trait analyses indicate that this genomic downsizing in subgenus *Pseudoveronica* (predominantly in section *Hebe*) preceded an increased diversification rate (Fig. 6A, Table S3), and there is also some evidence for an increased macroevolutionary rate (data not shown; see Results). In this way, subgenus *Pseudoveronica* fits the 'whole genome duplication radiation lag-time model', in which the polyploid ancestor evolved into a species-rich crown radiation (section *Hebe*, 135 species) and a species-poor sister clade (section *Labiatoides*, 23 species) (Schranz, Mohammadin & Edger, 2012). The lower than expected 1C- and 1Cx-values for the New Zealand hexaploids also suggest such a scenario (Fig. 4). For the $12x$ and $18x$ species, which have gone through one and two additional rounds of polyploidization, respectively, there has been little additional downsizing, as can be seen by the relatively stable 1Cx-values (Fig. 4B) and the strongly linearly correlated 1C-values (Fig. 4A). Nevertheless, there are exceptions; for example, the sun hebes (red colour in Fig. 5B; crown age, ~2.1 Mya) are perhaps the most extreme example of genomic downsizing in this lineage. The sun hebes are a clear case of genomic downsizing accompanying morphological change, as this group of six species is one of the most morphologically distinctive in section *Hebe* (Garnock-Jones, 1993a, as *Heliohebe*). The mean 1C-values of the five Australian species (section *Labiatoides*, $2n = 6x = 36$–42; Briggs & Ehrendorfer, 2006) sampled here are lower than expected and intermediate between the New Zealand and Northern Hemisphere mean values (Table S1); data from the remaining 18 Australian species would be valuable.

Evolution of genome size in Northern Hemisphere *Veronica*

Even without considering the new estimates in subgenus *Pseudoveronica*, our data broadened the range of genome sizes in (Northern Hemisphere) *Veronica* relative to the earlier study (Albach & Greilhuber, 2004). 1C-values now range from 0.26 to 2.44 pg and 1Cx-values from 0.26 to 1.08 pg, the upper limit being a slight decrease from the earlier study because of the reinterpretation of ploidy in *V. ciliata*. The species with the lowest genome size in the genus is now *V. javanica*, an annual species of isolated phylogenetic position from tropical regions. Although most of the main lineages had already been sampled in the earlier study (Albach & Greilhuber, 2004), our increase in genome size data stems mainly from species-rich subgenera which have been the subject of more intense investigation in recent years. In contrast, our sampling of less species-rich clades of annuals has only increased slightly. Whether or not this may have biased the analyses remains to be studied. Our results indicate that genomic downsizing has occurred in subgenera *Pentasepalae* and *Pseudolysimachium*; these two subgenera also have slightly higher diversification rates relative to other Northern Hemisphere subgenera (but only by 0.1–0.2; Table S3).

Data from the current study allow us to provide a baseline for ploidy level analyses in the genus. In the following, we discuss patterns of 1Cx-value evolution in the larger Northern Hemisphere subgenera, putative errors in estimates and species in need of further analysis.

Veronica* subgenus *Beccabunga (1Cx-value = 0.32–0.64 pg; 2.0-fold variation) has a large number of polyploid species (Albach *et al*., 2008), but a low diversification rate (Table S3). It includes three sections, with the largest genomes and the largest variation being found in the annual section *Acinifolia* (Römpp) Albach (1Cx-value = 0.54–0.70 pg). Further chromosome counts will be relevant to firmly establish the monoploid genome size of the section. If annual life history has indeed evolved within the section and is not ancestral for the subgenus, it originated at ~6.3–3.2 Mya. In section *Beccabunga* (Hill) Dumort., the hexaploid *V. peregrina* has the smallest monoploid genome (0.29–0.33 pg) and has been inferred to be tetraploid by Castro *et al*. (2012), which would give it a 1Cx-value more in line with related species. However, also given the considerable age of the lineage (up to ~4.7 Mya), we consider genome downsizing to be more likely as the hexaploid level has been universally inferred in 13 different chromosome counts and it is known that dysploidy has occurred in this species, resulting in fewer chromosomes (Albach *et al*., 2008). All measurements in *V. anagallis-aquatica* L. and its close relatives are remarkably homogeneous (0.53–0.54 pg), which parallels the low genetic diversity in this group (F. Yousef & D. C. Albach, unpubl. data) and allows easy inference of ploidy. The third section *Serpyllifolia* G.Don includes

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one of the most complex polyploid groups, *V. gentianoides*, with all even and odd ploidies, ranging from 2*x* to 10*x*, having been reported (Albach *et al*., 2008) and 1C-values ranging from 0.91 to 2.35 pg. For two specimens (*Albach 318*, *Albach 350*), chromosome counts and genome size are available. However, because of the unclear chromosome base number in the species complex, ploidy is still difficult to determine. Associating the counts of $2n = c.$ 72 (Albach *et al*., 2008) with the 12*x* level would allow the other samples to be 4*x*, 6*x* and 8*x* with 1Cx-values ranging from 0.38 to 0.47 pg, a scenario which fits with the monoploid genome size of the sister species, *V. serpyllifolia*, at 0.44 pg. Nevertheless, this species complex is one that will require more genome size and chromosome number estimates.

Veronica* subgenus *Veronica (1Cx-value = 0.31–0.85 pg; 2.6-fold variation) has 44 species with few polyploids and a low diversification rate (Table S3). Chinese species of subsection *Canae* (T.Yamaz.) Elenevsky are poorly sampled for genome size and chromosome number, and so an analysis for this subgenus can only be preliminary. 1Cx-value estimates for subgenus *Veronica* are rather high, but deserve more attention. For example, *V. urticifolia* was assumed to be strictly diploid based on nine chromosome counts from across its geographical range (Albach *et al*., 2008). Genome size estimates by Pustahija *et al*. (2013), however, indicated the presence of diploid and tetraploid cytotypes. This means that the Tyrolian sample used in Albach & Greilhuber (2004) should be reinterpreted to be a tetraploid (instead of a diploid) and its 1Cx-value of 0.31 pg is the lowest in the subgenus. The second species in the subgenus with multiple estimates is tetraploid *V. officinalis* with 1Cx-value estimates ranging from 0.45 to 0.59 pg. There could be multiple methodological or biological reasons for this intraspecific variation, including the use of Feulgen (0.59 pg; Vidic *et al*., 2009) vs. flow cytometry (0.45–0.55 pg), the use of different standards or buffers, and the possibility of intraspecific variation related to the wide distribution area.

Veronica* subgenus *Cochlidiosperma (1Cx-value = 0.32–0.70 pg; 2.2-fold variation) is the oldest purely annual subgenus. Its crown age (8.55 Mya; 95% HPD, 12.12–5.41 Mya) coincides roughly with the onset of the Messinian salinity crisis, which has frequently been associated with the evolution of annual life history in the Mediterranean area (e.g. Jabbour & Renner, 2011). It has a low diversification rate (Table S3), but two small polyploid complexes: the blue-flowered subsection *Cochlidiosperma* (Rchb.) Albach with monoploid genome sizes between 0.61 and 0.70 pg, and the white-flowered subsection *Cymbalariae* Benth. with monoploid genome sizes between 0.35 and 0.46 pg (excluding the even lower value of one Feulgen estimate). In the latter subsection, there is a further difference between the self-incompatible diploid *V. lycica* (0.44 pg) and the selfing diploids *V. trichadena* and *V. panormitana* (0.35–0.40 pg), supporting the suggestion by Albach & Greilhuber (2004) that selfers have lower genome sizes than outcrossers. In the polyploid *V. cymbalaria*, we did not find a large difference (< 1%) between the results from Feulgen and flow cytometry, but rather a trend for the hexaploids to have a higher 1Cx-value (0.40–0.46 pg) than the tetraploids (0.37–0.42 pg), which, if anything, shows slight genome upsizing rather than downsizing. Of the two groups of *V. cymbalaria* of different origin (Albach, 2007), tetraploids from the southern group have a lower 1Cx-value (0.37–0.38 pg) than the specimen from the western group (0.40 pg), but there is no similar pattern discernible between hexaploids of different origins.

Veronica* subgenus *Chamaedrys (1Cx-value = 0.44–1.08 pg; 2.5-fold variation). This is a small, little diversified subgenus with just three polyploids. The most notable patterns within it are the detection of significant genome upsizing (Fig. 5B) and the difference between life histories. Excluding the low Feulgen estimates of *V. arvensis* L. and *V. chamaedrys* (see below), annual selfers have smaller 1Cx-values (0.41–0.54 pg) than perennial outcrossers (0.87–1.08 pg). The monoploid genome size of the perennials is indeed large compared with most other species in the genus, which led Castro *et al*. (2012) to suggest that *V. micrantha* is a tetraploid and their sample of *V. chamaedrys* is hexaploid as they have the same 1C-value and 1.5 times the 1C-value, respectively, of tetraploid *V. officinalis* (*V.* subgenus *Veronica*). However, our estimates fit well with diploid chromosome counts estimated for *V. micrantha* (Albach *et al*., 2008) and the consistently diploid and tetraploid levels found for *V. chamaedrys* on the Balkan Peninsula (Bardy *et al*., 2010). Thus, only an extensive sampling at both ploidies allowed the inference that genome upsizing at the diploid level rather than polyploidy is responsible for the large genome size in this group.

Veronica* subgenus *Pentasepalae (1Cx-value = 0.28–0.92 pg; 3.29-fold variation) comprises a large number of Asian and European dry meadow and alpine species, including many polyploid complexes (e.g. Martínez-Ortega *et al*., 2004; Sonibare *et al*., 2014). With a diversification rate of 0.23 speciation events per million years, it exhibits the third fastest diversification rate in the genus (Table S3). Despite the large number of species with known chromosome numbers in *V.* subgenus *Pentasepalae*, it is extremely difficult to determine the 1Cx-value in this subgenus. Chromosome numbers are urgently needed for species in the Asian lineage, which is one of three lineages in the genus in which significant genome downsizing

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was detected (Fig. 5B). *Veronica* subgenus *Pentasepalae* has a large variation in 1C-values (0.32–2.44 pg) which comes close to encompassing that of the whole genus (0.26–3.19 pg). Thus, it is difficult to calibrate ploidy for those species for which the chromosome number is not yet known (e.g. *V. oltensis* 6*x* or 8*x*; *V. vendetta-deae* 2*x* or 4*x*). There are currently two ways in which the genome size data could be interpreted for ploidy determination in *V.* subgenus *Pentasepalae*. First, assuming a subgenus-wide monoploid genome size, the subgenus would have a 1Cx-value range of 0.28–0.48 pg, which is in line with published diploid chromosome counts for several of these taxa (*V. cinerea* Boiss. & Balansa, *V. armena* Boiss. & Heut.), but would also suggest the first report for a diploid *V. orientalis*. Even worse, this interpretation would also mean that the individuals with the largest genomes (i.e. *V. teucrium* L.) are 12*x* rather than the commonly reported 8*x* (Albach *et al.*, 2008). In contrast, another way to interpret the data is to assume different monoploid genome size ranges in the European and Turkish/Near Eastern Asiatic species. Thus, the individuals of *V. teucrium* investigated here for genome size would have the same chromosome number as that regularly reported previously ($2n = 64$; Albach *et al.*, 2008), which would mean that the European species of the subgenus would have a larger and more variable 1Cx-value (0.56–0.92 pg, 1.64-fold variation) than the Asian species. This is also in better agreement with previous chromosome number reports for the other species (reviewed in Albach *et al.*, 2008), and would assign *V. prostrata* L. and *V. crinita* Kit. ex Schult. to the diploid level, *V. orbiculata* A.Kern. to the tetraploid level, *V. jacquinii* Baumg. to the diploid and hexaploid levels, *V. austriaca* L. to the hexaploid level and *V. teucrium* to the octoploid level. This is also in perfect agreement with the genome size estimates of a more detailed analysis of the European members of subgenus *Pentasepalae* (B. M. Rojas-Andrés *et al.*, unpubl. data). Thus, this European clade is a second example for genome upsizing at the diploid level prior to polyploidization. A lower 1Cx-value of 0.28–0.38 pg for the Turkish/Near Eastern samples of the subgenus is difficult to support above the diploid level because of the lack of chromosome number reports for these species. Nevertheless, *V. pectinata*, previously considered as diploid (Fischer, 1970), seems to be tetraploid, as inferred for *V. polifolia* and *V. vendetta-deae*. *Veronica leiocarpa* and *V. oltensis* are here inferred to be octoploid. However, this should be considered as tentative and needs to be further supported by chromosome numbers and genome size estimates made on the same individual. *Veronica thymifolia*, considered to be diploid based on chromosome counts, fits karyologically and geographically into the European lineage of the subgenus with its 1C-value = 0.57 pg (1Cx-value = 0.57 pg), but phylogenetically it belongs to the Asian species (Muñoz-Centeno *et al.*, 2006; Sonibare *et al.*, 2014; Fig. S3).

Veronica* subgenus *Stenocarpon (1Cx-value = 0.46–0.98 pg; 2.1-fold variation) is one of two subgenera in which significant genome upsizing was detected (Fig. 5B). Plants in this subgenus are long-lived alpines (with the exception of some populations of apparently annual plants of *V. ciliata* on the Tibetan Plateau; D. C. Albach pers. observ.) with overall high genome sizes. Subgenus *Stenocarpon* was previously believed to be uniformly diploid with the only tetraploid chromosome count belonging to an aberrant probable autotetraploid of a diploid species (Albach *et al.*, 2008). Nevertheless, 1C-values differ enormously (0.75–1.68 pg), suggesting that, in *V. macrostemon*, *V. densiflora* and probably also *V. ciliata*, tetraploid populations exist. However, it is still possible that genome upsizing has occurred at the diploid level without polyploidization in alpine habitats, which may not select against genome upsizing (Albach & Greilhuber, 2004).

Veronica* subgenus *Pseudolysimachium (1Cx-value = 0.28–0.48 pg; 1.7-fold variation) evolved from a polyploidy event, probably with subsequent dysploidy leading to a chromosome base number of $x = 17$. The subgenus is one of the clearest examples of genome downsizing (Fig. 5B). The age of this polyploidization event was inferred to be ~3.2 Mya, which is slightly older than the age inferred on the basis of general plastid DNA substitution rates (~2.4 Mya; D. C. Albach & P. Kosachev, unpubl. data). With a diversification rate of 0.24 speciation events per million years, it exhibits the second fastest diversification rate in the genus (Table S3). The subgenus seems to follow the lag-time pattern in diversification of polyploid lineages (Schranz *et al.*, 2012) with a species-poor lineage [section *Schmidtianae* (Boriss.) Assejeva, two species] sister to a species-rich lineage (section *Pseudolysimachium* W.D.J.Koch, 26 species) that is much more widely distributed (Japan vs. across northern Eurasia) and morphologically and ecologically (alpine vs. swamp to steppes) diverse.

Methodological issues

In our study, genome size measurements from silica gel-dried material stored for 6 weeks and 5 months were generally similar to those of fresh material of the same individual, although a statistically significant difference was found between them ($N = 6$, see Results). On average, measurements from silica gel-dried material were slightly higher with worse CVs (see Results; Table S1), and good estimates could not be obtained from silica gel-dried material from some

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individuals ranging in storage times from only 2 weeks (e.g. *V. jovellanoides* Garn.-Jones & de Lange) to 2 years (data not shown). Our inability to obtain good quality measurements of such samples could be a result of a combination of silica gel storage with the presence of cytosolic compounds (such as phenolics) that act as fluorescence inhibitors (Greilhuber, Temsch & Loureiro, 2007). Indeed, the nuclear suspensions of some samples turned brown and/or showed precipitation, which could be a sign of such inhibition (D. C. Albach, B. M. Rojas-Andrés & H. M. Meudt, pers. observ.). As we measured 46% of silica gel-dried samples within 6 weeks of collection and 66% within 3 months, our results suggest that most of the degradation occurs in the early stages of desiccation.

Our results are congruent with previous studies which also showed small increases in genome size and CV when using silica gel or herbarium material (Cires *et al*., 2009; Bainard *et al*., 2011; Dyer *et al*., 2013), and which also showed that high-quality genome size data can be successfully acquired (at least in the short term) from such material. However, Sánchez-Jiménez *et al*. (2012) found considerably more variation than we did here (including both higher and lower genome size measurements) between fresh and silica gel-dried leaves of < 6 months old from the same populations of *Echinops* L. (Asteraceae). Thus, comparisons within and between studies with regard to measurements made with silica gel-dried vs. fresh material must be considered carefully. For *Veronica*, fresh material is clearly better than silica gel-dried material for flow cytometry measurements, but, in many cases, high-quality estimates using silica gel-dried material of < 5 months old can still be obtained. Nevertheless, the measurements we obtained from silica gel-dried material with CV values between 5–8% should be considered preliminary until additional measurements using fresh material can confirm them.

There are now 19 samples representing 17 *Veronica* spp. for which genome size measurements using Feulgen densitometry and flow cytometry are available, allowing a differentiated comparison to be made, which showed no statistically significant difference between them (see Results). Feulgen densitometry is considered to be disadvantageous relative to flow cytometry for leaf material rich in phenolic compounds (Greilhuber, 2008) or when rigid cell walls prevent the stain from entering the cells (Temsch *et al*., 2010). If cells are not stained properly, estimates using Feulgen densitometry should be markedly lower than those using flow cytometry. Of our 19 comparisons, there are four possible examples in which staining may have been suboptimal, as Feulgen estimates are 17.5–30.5% lower than those of flow cytometry, and which should be used with care [*V. beccabunga* L., *V. arvensis*, *V. triphyllos* L. and *V. chamaedrys* (4*x*)]. In two other cases (*V. persica* Poir. and *V. syriaca* Roem. & Schult.), Feulgen estimates are 8.7–9.1% higher than those using flow cytometry. Here, phenolic compounds, as possibly indicated by large standard deviations, may have caused problems. In all other cases, Feulgen estimates are similar to those from flow cytometry (< 7% difference) and within the range of intraspecific variation (see above).

Conclusions and future directions

The genome size data and molecular dating analyses presented here provide a further understanding of the evolution of *Veronica*, and especially of the Southern Hemisphere lineage. First, we have increased the number of species with known genome sizes in *Veronica* by nearly three-fold to 128 species, or *c*. 30% of the genus. The 54 Southern Hemisphere species sampled had 1C-values ranging from 0.73 to 3.19 pg and 1Cx-values ranging from 0.24 to 0.52 pg. Second, through phylogenetic analyses, we have shown that genome downsizing has occurred in three subgenera and, through BAMM, that macroevolutionary rate shifts and differences in diversification rate among subgenera have taken place. Genome downsizing was strongly linked to an increased diversification rate in the polyploid Southern Hemisphere subgenus. Third, we have shown that polyploidy and life history (but not alpine habitat) are important in the evolution of genome size in *Veronica*. Future studies that are focused on certain lineages of *Veronica* will benefit from our estimates of ancestral 1C- and 1Cx-values and clade ages. Finally, we have shown that most genome size measurements using Feulgen densitometry vs. flow cytometry, and fresh vs. silica gel-dried material, are comparable.

More generally, our analyses suggest a relationship of genome downsizing with species diversification. However, despite some work being performed on this question (e.g. Kraaijeveld, 2010), we do not know how general this pattern might be or what causes it. One potential caveat is that we know nothing about the relative rates of DNA loss and gain among lineages of *Veronica*. Hawkins *et al*. (2009) argued that the overall trend in plant genome size evolution is towards gain, and that 'genome downsizing' events could instead be interpreted as being a difference in relative rates of DNA loss and gain among lineages, i.e. small genomes are at stasis (or expanding very slowly) relative to large genomes (which are expanding rapidly). Another potential caveat of our analysis is that we do not know exactly the ancestral diploid species of subgenera *Pseudoveronica* and

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Pseudolysimachium, as is the case in any older polyploid lineage including the angiosperms as a whole. We therefore need to rely on comparison with the closest extant relative to determine what aspect of genome downsizing facilitates diversification and whether it is related to consequences regarding meiosis, genetic changes or nucleotypic effects of lower genome size. Answers to these questions will require either an analysis of similar patterns in genera with more genomic information or the generation of more genomic information in *Veronica*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Best RAxML phylogenetic tree with bootstrap values for *Veronica* and selected outgroups for internal transcribed spacer (ITS).

Figure S2. Best RAxML phylogenetic tree with bootstrap values for *Veronica* and selected outgroups for *trnL-trnL-trnF*.

Figure S3. Best RAxML phylogenetic tree with bootstrap values for *Veronica* and selected outgroups for combined ITS + *trnL-trnL-trnF*.

Table S1. *Veronica* vouchers for genome size and DNA sequence data used in this study and in previous studies. Individuals with # and a number in the 'Method' column represent 13 individuals for which multiple measurements were taken. A '?' in the 'Material' column means the type of material is unknown because it was not stated in the publication. Publications listed in this table are cited in 'References' section of the paper.

Table S2. Flow cytometry measurements of genome size of new internal standard *Hedychium gardnerianum* (1C-value = 2.01 pg).

Table S3. Crown and stem ages, including maximum and minimum of the 95% highest posterior density (HPD) intervals, for *Veronica* and selected subgenera and sections based on BEAST analysis of the expanded internal transcribed spacer (ITS) dataset (time tree is supplied in Fig. 3). Number and percentage of species (total, sampled and polyploid) and diversification rate are also shown.

Table S4. Results of phylogenetic generalized least-squares (PGLS) analysis for *Veronica* and outgroup genome size and character data on the *trnL-trnL-trnF* and ITS + *trnL-trnL-trnF* phylogenies. Significant P values are indicated in bold type.

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