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Resolving reticulate evolutionary histories of polyploid species of *Azorella* (Apiaceae) endemic to New Zealand

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ABSTRACT

Genera with species of multiple ploidy levels provide models to understand successive rounds of whole genome duplication leading to intricate reticulate relationships of polyploid plant species. Here, we studied 17 polyploid taxa (species, subspecies, or varieties) in *Azorella* (Apiaceae) sections *Schizeilema* and *Stilbocarpa* that are mostly endemic to New Zealand. Using phylogenomic approaches, our goals were to resolve species relationships, determine the origins of the higher-level polyploids (6*x* and 10*x*), and assess the biogeography of the New Zealand *Azorella* species. Phylogenomic analysis of Anigosperms353 baits-captured Hyb-Seq data, together with comparison of phylogenies reconstructed using genome-skimming retrieved nrDNA and plastome sequences, showed that species diversification within New Zealand may relate to multiple origins from South America, which has been further shaped by additional rounds of polyploidy as well as hybridization or introgression. The two *Azorella* sections in New Zealand likely resulted from different biogeographic events from South America – one to the subantarctic islands (section *Stilbocarpa*) and a second to the South Island (section *Schizeilema*). In addition, within section *Schizeilema*, species have dispersed from the South Island (New Zealand) to Australia, the subantarctic islands, and the North Island (New Zealand). Our combined approach of phylogenomic analyses of plastome and nuclear locus-based data, together with SNP-based network approaches allowed us to determine the origins of some higher-level polyploids in New Zealand *Azorella* and revealed a more complex picture of historical and ongoing polyploidy and hybridization within these lineages.

1. Introduction

Whole genome duplication (WGD) or polyploidization is an important evolutionary process in plant species radiations and diversification (Clark and Donoghue, 2018; Jiao et al., 2011; Soltis et al., 2009). A polyploid species may originate from the duplication of the genome from a single species (creating an autopolyploid with multiple homologous gene copies) or the combination of genomes from different species (creating an allopolyploid with multiple homeologous gene copies) (reviewed by Otto and Whitton, 2000). When multiple rounds of WGD occur in closely-related species, these events can lead to the formation of a polyploid-rich genus (Meudt et al., 2021). Such genera, which comprise species with differing numbers of chromosome sets, and thereby ploidy levels, are valuable systems to investigate species diversification in light of WGD (Oxelman et al., 2017; Van de Peer et al., 2020).

Understanding the origins and species relationships of polyploid genera with phylogenetic approaches, which is the first step to examining their diversification patterns, is challenging (Ning et al., 2024; Rothfels, 2021). Traditionally, a handful of frequently selected markers

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from plastid DNA (cpDNA) or nuclear regions [e.g., the internal and external transcribed spacer (ITS and ETS) regions within nuclear ribosomal DNA (nrDNA)] have been used for reconstructing phylogenetic relationships of polyploid plants (Osuna-Mascaró et al., 2022; Xu et al., 2017; Fernández et al., 2017a). However, these markers may be of limited value to reflect relationships between closely related polyploids, including those that have experienced multiple WGD events. For cpDNA, the main limitation is that in flowering plants the plastid is typically inherited from one parent, most often maternal (Birky, 1995). Further complications with interpreting plastid-based phylogenies can arise when local hybridization or introgression occurs (Alvarez and Wendel, 2003). A benefit to using nuclear markers that are single copy or low copy is that their biparental inheritance should be maintained and therefore parental origins of polyploids may be revealed. More recently, genome-wide single copy nuclear genes (SCNGs) have proven informative among closely related species, including polyploids (Karbstein et al., 2022; Nicol et al., 2024; Thomas et al., 2021), but interpreting the nature of duplicated nuclear genomes remains challenging when using traditional phylogenetic methods that reconstruct bifurcating trees. In addition to WGD events, reticulation events, such as hybridization or introgression, as well as post-WGD genomic reorganization processes can further increase the levels of genomic complexity in polyploids and their phylogenetic inference (Li et al., 2021; Meudt et al., 2025).

New Zealand has high levels of endemic plant diversity and its species have been well documented for chromosome numbers (Dawson, 2000; Murray et al., 2011), providing strong evidence that the country is host to numerous polyploid-rich lineages (Meudt et al., 2021). One such genus is *Azorella* (Apiaceae), which in New Zealand comprises two well-defined sections: section *Schizeilema* and section *Stilbocarpa*, with 16 perennial species, subspecies, and varieties (hereafter, taxa; Table 1). These taxa vary in chromosome number [$2n = 32$, $2n = 48$ and $2n = 80$ (Beuzenberg and Hair, 1983; Hair, 1980)], leaf morphological traits (Fig. 1; Table 1; Fig. S1) (Allan, 1961), habit, and geographical distributions (Plunkett and Nicolas, 2017). The 14 taxa in section *Schizeilema* (Fig. 1) are all small, rhizomatous, rosulate herbs with diverse ploidy levels (4*x*, 6*x* or 10*x*) that are mostly endemic to the main New Zealand islands and Stewart Island. The two exceptions are *A. schizeilema* (4*x*), endemic to the subantarctic Auckland Islands and Campbell Island, and *A. fragosea* (?*x* = *unknown ploidy level*), which is endemic to Australia (New South Wales and Victoria). The three so-called subantarctic megaherbs in section *Stilbocarpa* (Fig. S1) (Saldivia et al., 2022), *A. polaris* (6*x*), *A. robusta* (?*x*) and *A. lyallii* (?*x*), are endemic to Stewart Island and

Table 1
Taxa of *Azorella* included in this study, their ploidy levels and chromosome number (where known), geographic distribution and habitat. Geographic distribution was determined by herbarium records at AK, CHR, MPN, OTA and WELT. Ploidy levels were determined by published chromosome numbers (http://www.tropicos.org/project/ipcn); unknown ploidy level and chromosome number are indicated by "?". All chromosome numbers and ploidy levels were confirmed by checking specimen identification in CHR by W.N., and additional genome sizes (Ning unpub. data) were estimated to predict the ploidy level. An exception was for *Azorella pallida*, where the original chromosome voucher at CHR could not be located; therefore flow cytometry was used to estimate its ploidy level (via genome size) (Ning, 2023; Murray Dawson et al., unpubl. data) as a proxy. Sectional taxonomy follows Plunkett and Nicolas (2017) and subgenus classification follows Fernández and Calviño (2019). Habitat for New Zealand species is summarised from Allan (1961).

Species	Subgenus	Section	ploidy level	Chromosome number	Geographical distribution	Habitat
Azorella lycopodioides Gaud.	*Andinae*	*Glabratae*	2*x*	16	South America	
Azorella ranunculus d'Urv.	*Azorella*	*Ranunculus*	2*x*	16	South America	
Azorella burkartii (Mathias & Constance) G.M.Plunkett & A.N. Nicolas	*Azorella*	*Ranunculus*	?*x*	?	South America	
Azorella polaris (Hombr. & Jacquinot ex Hook.f.) G.M.Plunkett & A.N. Nicolas	*Azorella*	*Stilbocarpa*	6*x*	48	New Zealand and Australian subantarctic islands – multiple	lowland to subalpine; rocky places
Azorella robusta (Kirk) G.M.Plunkett & A.N.Nicolas	*Azorella*	*Stilbocarpa*	?*x*	?	New Zealand subantarctic islands – Snares Islands	lowland; coastal and shrubland
Azorella lyallii (Armstr.) G.M.Plunkett & A.N.Nicolas	*Azorella*	*Stilbocarpa*	?*x*	?	New Zealand – Stewart Island	lowland; coastal and forest
Azorella roughii (Hook.f.) Kirk	*Azorella*	*Schizeilema*	4*x*	32	New Zealand – South Island	montane to subalpine; grassland and herbfields
Azorella allanii (Cheeseman) G.M. Plunkett & A.N.Nicolas	*Azorella*	*Schizeilema*	4*x*	32	New Zealand – North Island	subalpine; open shrubland
Azorella cockaynei Diels	*Azorella*	*Schizeilema*	6*x*	48	New Zealand – South Island	lowland; damp sites
Azorella hookeri Drude var. *hookeri*	*Azorella*	*Schizeilema*	6*x*	48	New Zealand – North & South Islands	lowland to subalpine; wet sites
Azorella hookeri var. *tripartita* (Hook. f.) G.M.Plunkett & A.N.Nicolas	*Azorella*	*Schizeilema*	6*x*	48	New Zealand – North & South Islands	lowland
Azorella nitens Petrie	*Azorella*	*Schizeilema*	6*x*	48	New Zealand – North & South Islands	lowland to subalpine
Azorella colensoi (Domin) G.M. Plunkett & A.N.Nicolas	*Azorella*	*Schizeilema*	10*x*	80	New Zealand – North & South Islands	montane to subalpine; rocky places
Azorella hydrocotyloides (Hook.f.) Kirk	*Azorella*	*Schizeilema*	4*x*	32	New Zealand – South Island	montane to subalpine; forest margins, shrubland, grassland, herbfield and fellfield
Azorella haastii (Hook.f.) Drude subsp. *haastii*	*Azorella*	*Schizeilema*	4*x*	32	New Zealand – South Island	montane to subalpine; rocky places and fellfield
Azorella haastii subsp. *cyanopetala* (Domin) G.M.Plunkett & A.N. Nicolas	*Azorella*	*Schizeilema*	4*x*	32	New Zealand – South Island	montane to subalpine; rocky places and fellfield
Azorella schizeilema G.M.Plunkett & A.N.Nicolas	*Azorella*	*Schizeilema*	4*x*	32	New Zealand – subantarctic islands – Auckland and Campbell Islands	lowland to subalpine; rocky places
Azorella exigua (Hook.f.) Drude	*Azorella*	*Schizeilema*	4*x*	32	New Zealand – South Island	subalpine; herbfield
Azorella pallida (Kirk) Kirk	*Azorella*	*Schizeilema*	4*x**	?	New Zealand – South Island	montane to subalpine; forest, shrubland, grassland or herbfield
Azorella fragosea (F.Muell.) Druce	*Azorella*	*Schizeilema*	?*x*	?	Australia	alpine; rocky, damp herbfields

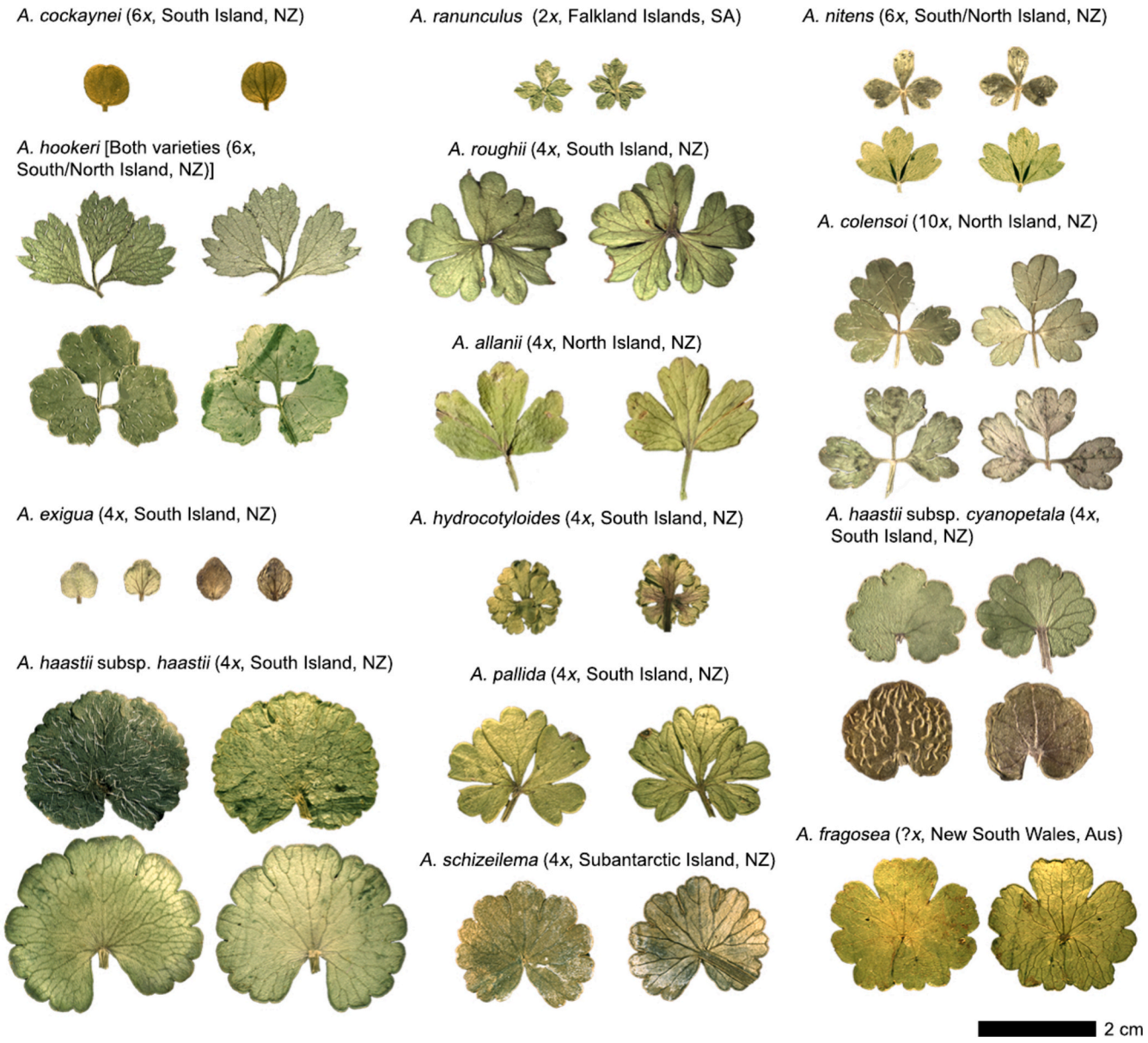

Fig. 1. Comparison of leaf morphology, ploidy level (where known) and geographic distribution of *Azorella* taxa included in the current study. Depicted are the 14 described *Azorella* species in section *Schizeilema* that are endemic to New Zealand (NZ; including two varieties of *A. hookeri*) and Australia (Au; *A. fragosea*), as well as one South American (SA) relative *A. ranunculus* from section *Ranunculus*.

the subantarctic islands of New Zealand (Beuzenberg and Hair, 1983; McGlone, 2002; Mitchell et al., 1999).

In the most recent taxonomic classification of *Azorella* based on molecular phylogenies by Plunkett and Nicolas (2017), with updates from Fernández and Calviño (2019), the genus currently comprises ~58 species from ten sections throughout the southern hemisphere. Of these, the 23 herbaceous species from New Zealand, Australia and South American Patagonia from Plunkett and Nicolas' (2017) sections *Schizeilema* (12 species, New Zealand and Australia), *Stilbocarpa* (3 species, New Zealand including subantarctic), *Azorella* (3 species, Tierra del Fuego and Patagonia), *Huanaca* (2 species, southern Chile and Argentina), and *Ranunculus* (3 species, Andes of Chile and Argentina) belong to the monophyletic subgenus *Azorella* (the "Andean-Patagonian lineage", see Fernández et al., 2017a; Fernández and Calviño, 2019). By contrast, the remaining 35 woody species from the high-elevation South American Andes comprise sections *Spinosae, Pectophytum, Glabratae, Laretia* and *Cirrhosae,* forming the subgenus *Andinae,* which is sister to subgenus *Azorella* (the "Austral lineage" of Fernández et al., 2017a; Fernández and Calviño, 2019). Previous phylogenetic studies using nuclear or plastid data sets have resolved the relationships of most of these monophyletic sections with strong support (Andersson et al., 2006; Fernández et al., 2017a, Plunkett and Nicolas, 2017; Nicolas et al., 2025). Relationships between the two monophyletic sections from New Zealand and Australia, sections *Schizeilema* and *Stilbocarpa*, and of these sections to the other three sections were not resolved when comparing the phylogenies using nuclear or plastid data sets of Plunkett and Nicolas (2017) and Nicolas et al. (2025). Notably, the most recent phylogenetic analysis of *Azorella* revealed section *Schizeilema* to be paraphyletic based on ITS + ETS sequence data (Nicolas et al., 2025). While most species in this section were reconstructed in one strongly supported clade, a second clade of section *Schizeilema* species was reconstructed as sister to section *Ranunculus* with strong support. The authors suggested that this could be explained by an allopolyploid origin for the ancestor of section *Schizeilema* based on the differences between the plastid and nuclear-based trees, as well as considering variable chromosome numbers within the genus and section *Schizeilema* in particular (Nicolas et al.,

2025). This ancestor was suggested to have formed between species from section *Ranunculus* and section *Stilbocarpa*, the latter of which was the sister group to section *Ranunculus* in both nuclear and plastid phylogenies (Nicolas et al., 2025). Additionally, several species within section *Schizeilema* were not monophyletic based on either plastid or nuclear data, indicating that additional undescribed species may exist in New Zealand or that other evolutionary factors, such as hybridization or introgression, may be influencing species evolution. A previous study of South American *Azorella* revealed multiple reticulations among species (Fernández et al., 2017a) – particularly in the more heavily sampled Andean-Patagonian subgenus *Andinae* – indicating that species in *Azorella* as a whole may be prone to hybridization and introgression. As mentioned earlier, the diverse habits and chromosome numbers of the species in section *Schizeilema* make this group particularly intriguing to investigate higher-level polyploid evolution.

In the current phylogenomic study, we focus on section *Schizeilema* by including all 14 taxa of *Azorella* from Australia, New Zealand, and the subantarctic islands, as well as three taxa of section *Stilbocarpa* from the subantarctic islands and two South American species from section *Ranunculus* (*A. ranunculus* and *A. burkartii*) as close relatives (based on Nicolas et al., 2025). Using locus-based phylogenomic analyses of nuclear (Angiosperms353 and nrDNA) and plastome data, as well as comparative network analyses of SNPs derived from Angiosperms353 data, we aimed to (1) test species monophyly and assess interspecific relationships, (2) identify the lineages that comprise the origins and sources of reticulation of the polyploid species, and in particular the higher-level polyploids (6*x* and 10*x*), and (3) analyze the biogeographical history of polyploid species in section *Schizeilema*, with an emphasis on its relationship to sections *Stilbocarpa* and *Ranunculus*.

2. Methods

2.1. Taxon sampling

As our main focus was *Azorella* sections *Schizeilema*, *Stilbocarpa,* and *Ranunculus* (Plunkett and Nicolas, 2017), samples from these sections were the priority to include for phylogenomic analysis. All species, subspecies and varieties from these sections were represented (except *A. boelckei* (Mathias & Constance) G.M. Plunkett & A.N. Nicolas in section *Ranunculus*) from field collections or existing herbarium specimens. Comprehensive collections of each species, including multiple individuals from different sites in New Zealand, were made during 2018–2023, based on locality data from previous herbarium specimens (AK, CHR, MPN, WELT, CANB, NY) and iNaturalist observations (Table S1; Fig. 2). Our identifications followed the descriptions in Allan (1961) and the taxonomy and nomenclature followed Plunkett and Nicolas (2017) (Table 1). For each field collection, leaves from multiple individuals in a population were preserved separately in silica gel and a representative voucher specimen was made and deposited in CHR, MPN, or WELT (Table S1). Two individual specimens collected from one population of a putatively new (unnamed) species that does not fit the taxonomy of Allan (1961) or Plunkett and Nicolas (2017) were also

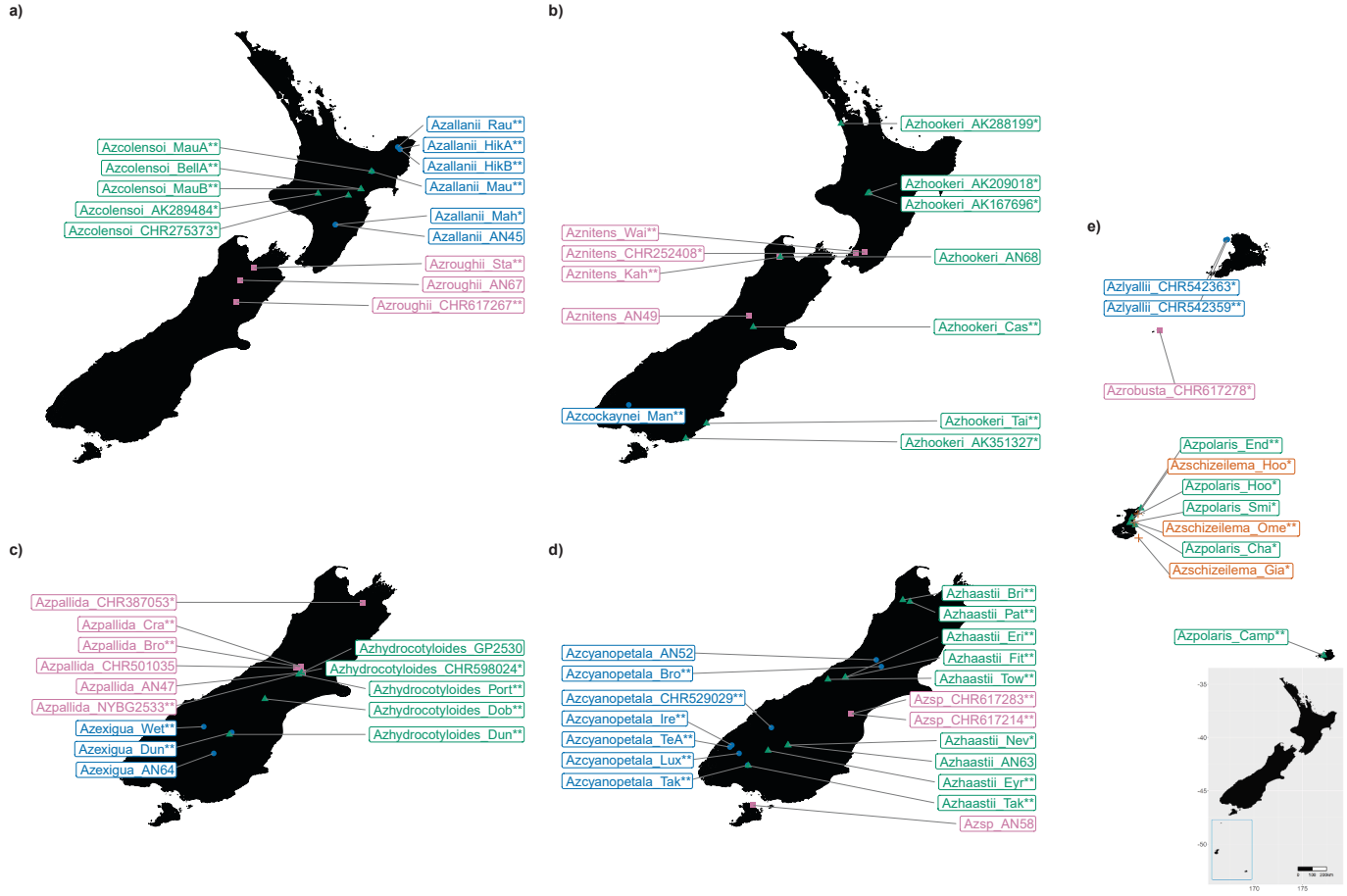

Fig. 2. Taxon sampling of New Zealand *Azorella* species, including 14 *Azorella* species in section *Schizeilema* (a-e), and three megaherbs in section *Stilbocarpa* (e). Each taxon is represented by a shape and colour in each subplot, and each individual is labelled with a species code ("Az" and the species name) and the locality (if field-collected) or herbarium specimen accession number/collection number (if sampled from a specimen) (see Table S1 for more details about individuals, populations and full species names). Two asterisks (**) represent individuals with data from both genome-skimming and Hyb-Seq sequence data available, one asterisk (*) represents individuals with data from Hyb-Seq sequence only, and no asterisks represent individuals with data from genome-skimming only.

included in our sampling (*Azorella* sp.: Azsp_CHR617214 and Azsp_CHR617283; Canterbury, New Zealand) (PB Heenan, pers. obs.). Additional herbarium specimens were sampled for three *Azorella* species that occur outside of New Zealand, including *A. fragosea* (Australia; Au) from section *Schizeilema*, and *A. ranunculus* and *A. burkartii* (South America; SA) from section *Ranunculus* (Table S1), bringing the total number of species from subgenus *Azorella* sampled here to 17 of 23. For the outgroup, we selected the South American species *A. lycopodioides* from section *Glabratae* (subgenus *Andinae*), which is more distantly related to sections *Schizeilema*, *Stilbocarpa* and *Ranunculus* (Plunkett and Nicolas 2017). For each species, ploidy level was estimated using published chromosome numbers (Beuzenberg and Hair, 1983; Hair, 1980) or genome size data (Ning, 2023; Murray Dawson et al., unpubl. data) as a proxy.

2.2. Genomic library preparation and sequencing

DNA was extracted from the leaf tissue using a standard CTAB method (Doyle and Doyle, 1987) or the DNeasy® Plant Mini Kit (QIAGEN). Genomic libraries were constructed using the NEBNext® UltraTM II Library Prep kit (New England Biolabs) following the manufacturer's protocol but with half of the recommended volumes for all steps (Workflow see Fig. 3; Note S1).

A total of 131 genomic libraries from 127 samples from 67 sites representing 20 taxa (including one undescribed taxon, *Azorella* sp., and four individuals with two technical replicates each; Table S1) were divided into four batches for Hyb-Seq with the Angiosperms353 (i.e., A353) universal baits (Johnson, et al., 2018) (Table S1). The myBaits® Kit Manual V4 and V5 protocols were followed to capture the targeted DNA fragments (incubation at 65 °C for at least 20 h). In total, 31 of the bait capture-enriched genomic libraries were sequenced on Illumina Miseq™ (Massey Genome Service, New Zealand) and 101 were sequenced on Illumina Hiseq™ 2000 (Novogene, Singapore), respectively, to produce 150 bp paired-end reads.

To recover nrDNA and plastomes, we re-sequenced 93 genome libraries that had a sufficient quantity of DNA remaining, originally used for bait capture, to produce 150 bp genome-skim paired-end reads on Illumina Hiseq™ 2000 (Novogene, Singapore). Additional genome-skimming data of 12 individuals (in section *Schizeilema*) were generated independently by A.N.N. and G.M.P. at Cold Spring Harbor Laboratory. TrueSeq libraries were generated and sequenced using Illumina HiSeq2500™, including another potentially undescribed species, Azsp_AN58 sampled from Stewart Island, New Zealand (Table S1). In total, we obtained genome-skim data for 105 *Azorella* individuals collected from 57 sites and representing 20 taxa (Table S1).

2.3. Hyb-Seq targeted gene recovery

All retrieved sequence reads were trimmed using Trimmomatic v.0.39 (Bolger et al., 2014) to remove adaptor sequences and low-quality bases (for all bioinformatic pipelines and settings, see: https://github.com/WeixuanPlant/NZAzorella). To retrieve the target-enriched exon sequences from the Hyb-Seq data, we used HybPiper v.2 (Johnson et al., 2016) with the reference file 'mega353.fasta' from McLay et al. (2021). In addition to exons, we extracted the supercontigs (partial introns or flanking regions) of targeted genes using the command ' -run_intronerate' in HybPiper (Johnson et al., 2016).

The occurrence of homeologs among the recovered genes was calculated using 'paralog_retriever' in HybPiper. A number of factors may result in inaccurate estimation of the number of extracted homeologs from *de novo* assembled contigs, including the divergence between subgenomes, the genomic library quality, and the sequencing depth of each sample (Johnson et al., 2016). Allele divergence (SNP percentage within targeted loci), an indication of homeolog variation, has lower requirements for assembling 'paralogs' in HybPiper, which can be more useful to determine the allele variation among targeted genes.

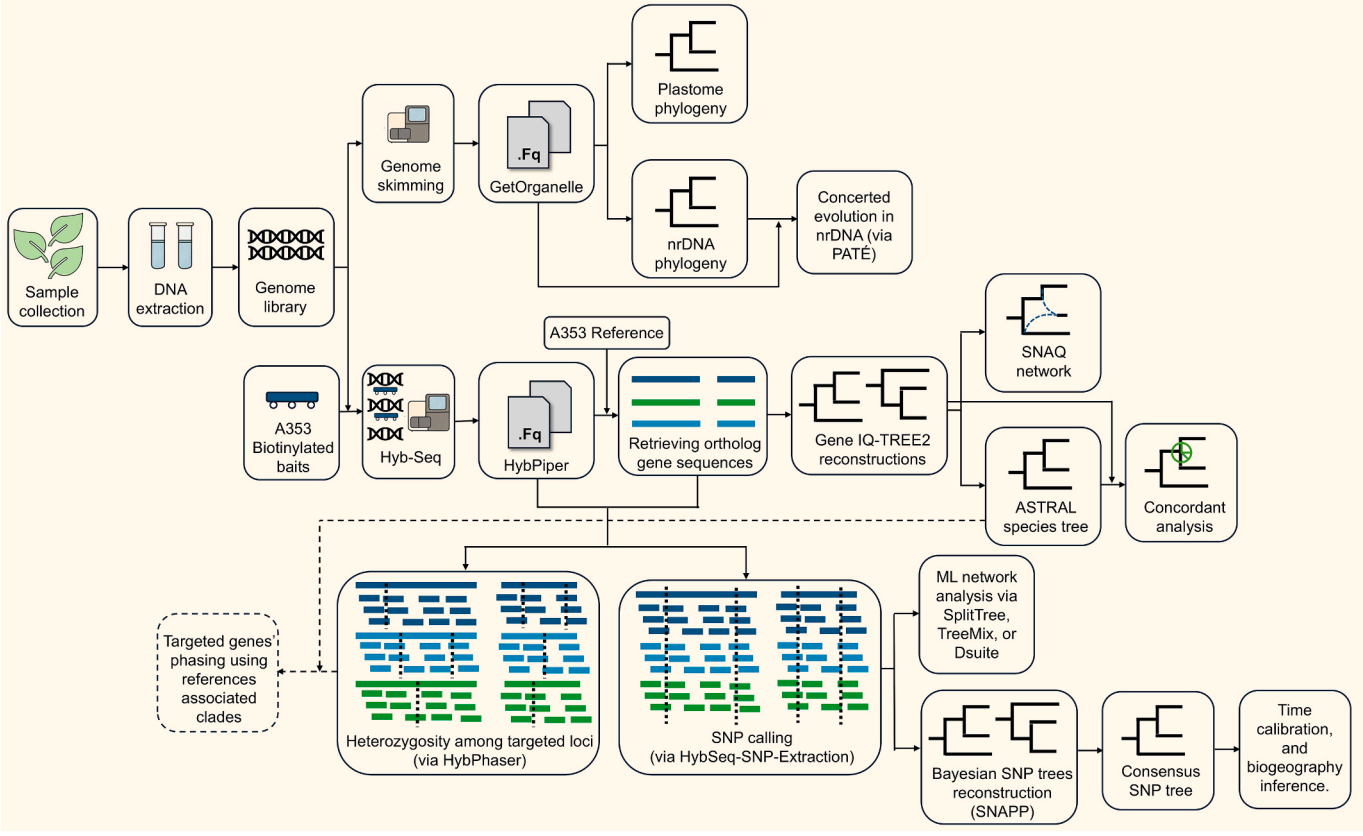

Fig. 3. Workflow for this paper illustrating sampling, genome sequencing, data partitioning, processing, and final phylogenetic tree reconstructions.

Therefore, we mapped the trimmed reads to the extracted supercontigs of each gene in HybPhaser v.2.0 (Nauheimer et al., 2021), and calculated the proportion of single nucleotide polymorphisms (SNPs) among each target gene for all sequenced samples to estimate the overall allele divergence among loci (see https://github.com/WeixuanPlant/NZAzorella for scripts).

2.4. Reconstructing targeted gene trees and performing concordance analysis for Hyb-Seq data

To reconstruct gene trees from target-enriched genes, we first aligned the supercontigs of each locus with the '-auto' option in MAFFT v.7.429 (Katoh and Standley, 2013). After removing the gap sites present in 30 % or more of the alignments (-gt 0.7) using trimAl v.1.4 (Capella-Gutiérrez et al., 2009), we filtered alignments with fewer than 15 individuals and those lacking the outgroup *A. lycopodioides* (~11 % of alignments). There were thus 345 locus alignments for which gene trees were reconstructed using IQ-TREE2 (Minh et al., 2020b) with automatic model detection via ModelFinder (Kalyaanamoorthy et al., 2017) and 1000 bootstrap (bs) replicates ('-B 1000').

We collapsed the internodes among gene trees with bootstrap values less than 10 % using the script 'i & b<=10′ in 'nw_ed' (Junier and Zdobnov, 2010) to improve the accuracy of the final species tree reconstruction (Zhang et al., 2017). Next, these gene trees were rerooted using the outgroup *A. lycopodioides* and the script 'reroot_trees.py' (https://github.com/mossmatters/phyloscripts/tree/master/phypartspiecharts). Finally, 345 gene trees were used to generate the species tree with a multispecies coalescent model for 132 individuals in ASTRAL v.5.7.7 (Mirarab et al., 2014; Zhang et al., 2017), and a local posterior probability was calculated for each node in the species tree.

Gene concordance factor (gcf) measures the proportion of gene trees with support for each internode in the species tree (Minh et al., 2020a). The proportion of genes concordant or discordant at each node of the final ASTRAL species tree was calculated in PhyParts [https://bitbucket.org/blackrim/phyparts; (Smith et al., 2015)] using 'phypartspiecharts.py' (https://github.com/mossmatters/phyloscripts/tree/master/phypartspiecharts), and visualised as a pie chart using the ggtree package (Yu et al., 2017) in R v.4.0.1 (R. Team, 2014).

2.5. Phylogenies of nrDNA and whole plastomes

After trimming the low quality bases of the genome-skimming reads with Trimmomatic, the nrDNA region and whole plastome markers were *de novo* assembled for 105 individuals, using the default settings of 'get_organelle_from_reads.py' in GetOrganelle v.1.7.5 (Jin et al., 2020). Using one annotated nrDNA sequence (Azallanii_AN45; Table S1), we manually selected only the cistronic region (ETS-18S-ITS1-5.8S-ITS2-26S) of nrDNA for phylogenetic reconstruction. The intergenic spacer (IGS) region was excluded due to excessive variable sites that were difficult to align. For six samples for which a complete plastome could not be assembled in GetOrganelle, we used consensus sequence calling via SAMtools (Li et al., 2009) and BCFtools (Li, 2011) by mapping their genome-skimming reads to a selected individual that had the longest *de novo* assembled plastome (Azcyanopetala_Ire6; Table S1).

The plastome and nrDNA cistron sequences for 105 individuals were aligned individually with MAFFT, and phylogenetic trees of each partition were reconstructed using IQ-TREE2 with 1000 bootstrap replicates. The homogenization levels among bi-parentally inherited nrDNA were confirmed by mapping the genome-skimming reads to the *de novo* assembled nrDNA cistron and calculating the proportion of SNPs for each sample using PATÉ (Tiley et al., 2024).

2.6. Network estimation of A353 locus data for a subset of Azorella taxa

To reduce computational running time and minimize conflicting intraspecific signals, one individual from each *Azorella* taxon that was monophyletic in both Hyb-Seq and plastome phylogenetic trees was selected to represent each taxon (see Results). Using similar thresholds and settings (https://github.com/WeixuanPlant/NZAzorella), the ASTRAL tree of the Hyb-Seq data for this smaller subset of taxa was reconstructed for 23 selected representative individuals using 219 gene trees that had no missing individuals. The concordance level among gene trees was calculated using PhyParts as described earlier. In addition, we concatenated the trimmed Hyb-Seq gene alignments for the 219 loci using AMAS.py (Borowiec, 2016) and visualized the conflicting signals among gene sequences using a network approach (Neighbor-Net method) in SplitsTree4 (Huson and Bryant, 2006). The result was plotted using ggsplitnet (Schliep et al., 2016) in R.

We also applied a maximum pseudo-likelihood approach to estimate the Hyb-Seq network among 23 individuals via SNaQ in PhyloNetworks v.0.14.2 (Solís-Lemus et al., 2017). Using the 219 Hyb-Seq gene trees as input and the 23-individual ASTRAL tree as the starting tree, we calculated a list of unrooted quartet concordance factors (qcf) in SNaQ and searched for reticulation events (−h) from 0 to 10. Each reticulation event (−h) used 15 parallel searches for the best model that had the lowest negative log pseudo-likelihood value. The final network was visualized using ggevonet in the R package tanggle (https://klausvigo.github.io/tanggle/).

2.7. Homeolog phasing of nrDNA data in PATÉ

To test for evidence of hybridization or allopolyploid origin in the nrDNA data, the assembled nrDNA cistrons of 22 of the same 23 representative individuals (Table S4) were used as a reference for homeolog phasing using PATÉ with default settings (*A. robusta* was excluded as that taxon had no genome-skimming data, see Results). We realigned the PATÉ phased sequences in MAFFT, and added the copy number for each phased homeologous sequence, then the nrDNA multi-labelled (MUL) tree was reconstructed in IQ-TREE2.

2.8. Network estimation using A353-derived SNPs

In addition to the gene-tree based network inference, we also performed network inference using SNP data. To extract SNPs from the Hyb-Seq data for all 23 selected individuals, we assigned the supercontigs of the individual 'Azschizeilema_Hoo' (Table S2) as the reference sequences for joint-calling SNPs with the pipeline 'HybSeq-SNP-Extraction' (Slimp et al., 2021). All SNPs were filtered using a hard-filtering threshold ("QD < 5.0 || FS > 60.0 || MQ < 40.0 || MQRankSum < -12.5 || ReadPosRankSum < -8.0″), and only SNP sites without missing data ('F_MISSING<=0′) and with biallelic sites ('-m2 −M2 −v snps') were retained using BCFtools. As chromosomal locations of the targeted genes are unknown, we thinned the SNP positions with a window size of 20 bp ('–thin 20′) using VCFtools (Danecek et al., 2011), which resulted in 9,182 sites. We estimated the network relationships with TreeMix (Pickrell and Pritchard, 2012) by specifying the migration ('-m') from 0 to 6, and selected the best model with OptM (Fitak, 2021) in R.

2.9. D-statistic test of genomic introgression signals in A353-derived SNP data

Patterson's D-statistic test, also known as the ABBA-BABA test (Malinsky et al., 2021), was used to identify genomic introgression signals within the Hyb-Seq SNP data. We applied Dsuite (Malinsky et al., 2021) to calculate the D-statistics, the related f4-ratio (admixture fraction f; an intermediate step in the calculation of F-branch values) from all possible species trios among filtered 9,182 SNPs. Finally, we used the F-branch metric (Fbranch) (Malinsky et al., 2018) to plot the phylogenetic tree nodes on the 23-individual SNAPP consensus tree (see section 2.10), which identified those nodes with high probabilities of introgression.

2.10. Bayesian inference of species relationships using A353-derived SNPs

The phylogeny and divergence times of 23 individuals were estimated using the 9,182 filtered SNPs under the Bayesian based multispecies coalescent approach of SNAPP (Bryant et al., 2012) as implemented in BEAST2 (Bouckaert et al., 2014). We used 'snapp_prep.rb' (https://github.com/mmatschiner/snapp_prep) to convert the VCF format as input format for SNAPP. The Markov Chain Monte Carlo (MCMC) chain length was specified to 5,000,000 and the output trees were saved every 250 iterations.

We extracted the previous estimated divergence times between *A. lycopodioides* with sections *Schizeilema*, *Stilbocarpa*, *Ranunculus*, *Huanaca* and *Azorella* [28.723 Ma (million years ago) with 95 % HPD (highest posterior density) between 19.99 and 37.766 Ma] from a time-calibrated tree of the order Apiales (Nicolas and Plunkett, 2014) as the crown age for our SNAPP trees. The final effective sample size (ESS > 200) for the Bayesian posterior distribution was confirmed in Tracer (Rambaut et al., 2018). After specifying the burn-in percentage to 10 % and setting the mean heights as node heights, the final consensus SNAPP tree was generated using TreeAnnotator (Drummond and Rambaut, 2007). The concordance level between SNAPP trees and the consensus tree was visualized in DensiTree (Bouckaert, 2010).

2.11. Biogeographic history of New Zealand Azorella based on A353-derived SNP data

The biogeographic history of New Zealand *Azorella* was analysed using the SNAPP consensus tree for 23 representative individuals via BioGeoBEARS package (Matzke, 2013) in R. From the known distribution of selected *Azorella* species, we defined six geographically distinct regions to determine biogeographic connections between South America and New Zealand, as well as to estimate the species diversification within New Zealand. The defined regions were South America (A); subantarctic islands, New Zealand (B); Australia (C); North Island, New Zealand (D); South Island, New Zealand (E); and Stewart Island, New Zealand (F). The maximum number of areas in which a species could occur was set to two based on known occurrence records. After comparing the result of six models (DEC, DEC + J, DIVALIKE, DIVALIKE + J, BAYAREALIKE, and BAYAREALIKE + J) in BioGeoBEARS, we selected the model with the highest AICc weight (AICc_wt) value to infer the biogeographical history of the sampled *Azorella* species.

3. Results

3.1. Angiosperms353 baits sequencing efficiency and gene recovery

Using outputs from HybPiper and HybPhaser (Fig. 4; Fig. 5; Table S2), we compared the Hyb-Seq sequencing efficiency and targeted gene recovery rates of Angiosperms353 (A353) baits for all 131 samples of *Azorella* based on the type of collected leaf material (field-collected vs herbarium specimens) and the sequence platform (MiSeq vs HiSeq). For each of the 131 sequenced individuals, the number of genes with successfully assembled exons varied from 103 to 349, and the average supercontig recovery lengths ranged from 435 to 3,468 bp per individual (Table S2). One targeted locus (6514) had no sequence assembled for any of the 131 individuals.

Our results showed at least 1.3 million mapped reads were necessary for assembling the targeted genes with supercontigs (over 300 loci) for both field-collected and specimen-based *Azorella* samples (Fig. 4a). Given the different ploidy levels in New Zealand *Azorella*, we also assessed the gene assembly quality by comparing the number of mapped reads vs conflicting signals from the homeologous copies. The higher number of mapped reads in the 101 samples sequenced on HiSeq compared to those sequenced on MiSeq (Fig. 4b) also lead to the larger number of genes (over 200 loci per sample) with stitched contigs (i.e., genes with sequence derived from multiple SPAdes assembled contigs), and a higher number of paralogs were also detected (over 25 loci per sample; Fig. 4c). In addition, for those individuals sequenced on HiSeq with a sufficient number of mapped reads (Fig. 4d; Fig. 5), the average

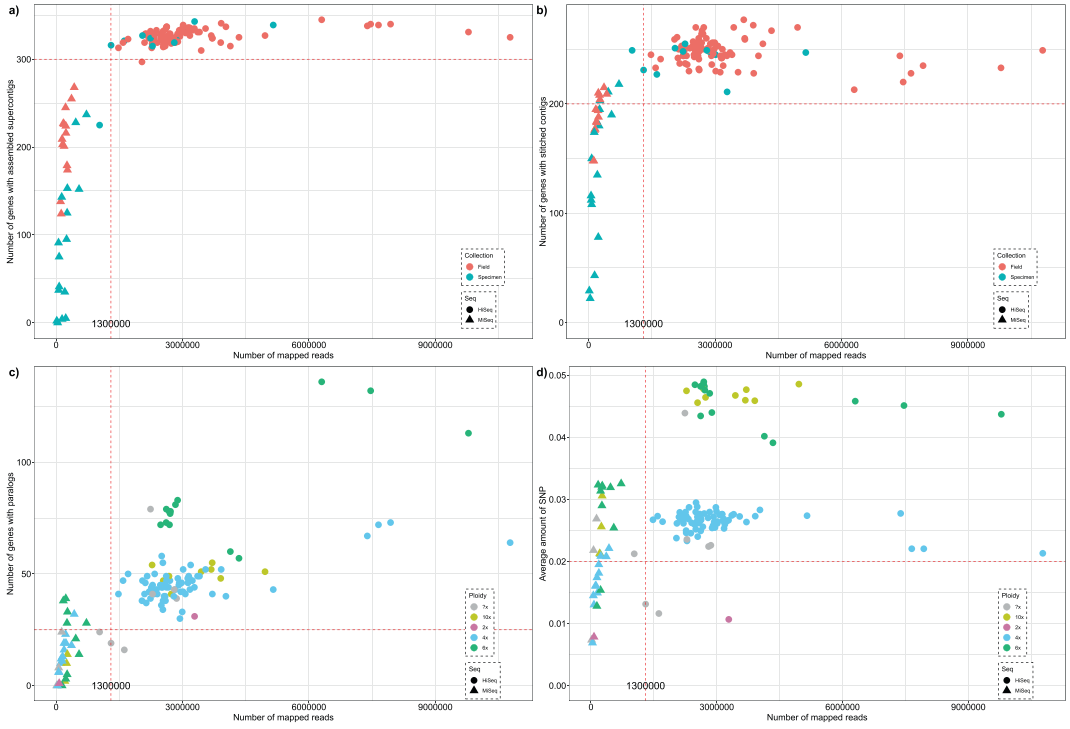

Fig. 4. The efficiency of target-enrichment sequencing as measured by plotting the number of mapped reads against a) the number of genes with assembled supercontigs ('GenesAt150pct' in Table S2); b) the number of genes with stitched contigs ('GenesWithStitchedContigs'); c) the number of genes with assembled paralogs ('ParalogWarningsLong'); and d) allele divergence as calculated by the proportion of SNPs among Hyb-Seq remapped reads ('allele_divergence').

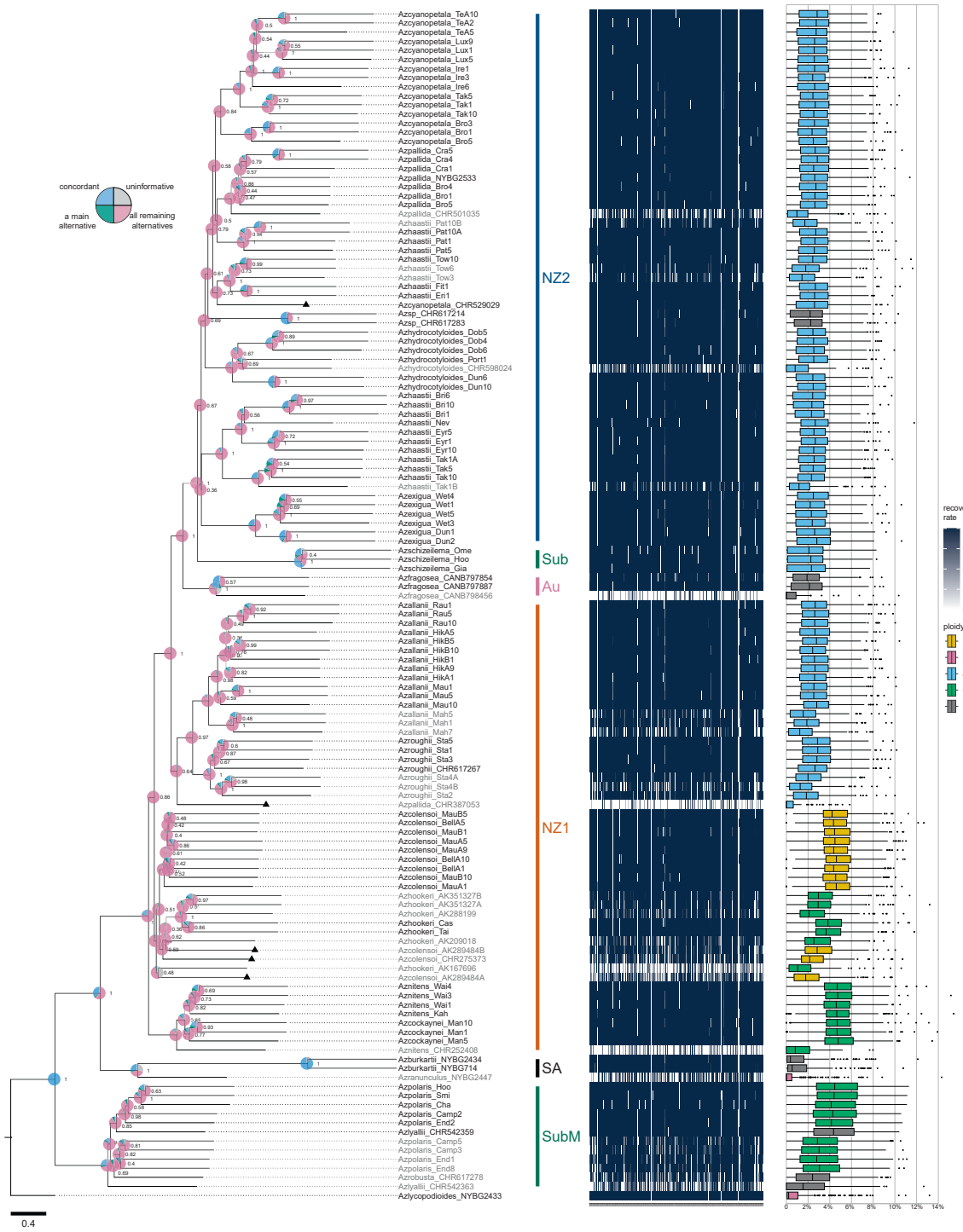

Fig. 5. The multispecies coalescent ASTRAL species tree of 131 *Azorella* individuals using 345 single-copy nuclear genes captured using Angiosperms353 baits. All the terminal branch lengths were set to 1 in the ASTRAL tree. The phylogeny shows six main groups, including five clades and one grade: New Zealand 1 (clade NZ1), New Zealand 2 (grade NZ2), Australia (clade Au), subantarctic islands (clade Sub), South America (clade SA) and the megaherbs in subantarctic islands (clade SubM). The individual sample names represent the species name and the sampling locality, i.e., the name of the field site or the herbarium specimen accession number, plus a number when more than one individual was collected from the same locality (see Table S1 for full species names). Five individuals with biological replicates were annotated as A and B at the end of each sample name (e.g., the replicate pair Azhaastii_Pat10A and Azhaastii_Pat10B). Each node is supported by a local posterior probability (maximum = 1.0) and a gene concordant pie chart (blue = concordant, green = discordant with a main alternative, pink = discordant with all remaining alternatives, and grey = uninformative). For each individual, the supercontig recovery rates of the targeted 353 genes are shown in the heatmap with colour gradient from 0 (white) to 100 % (dark blue). Each column of the heatmap represents one targeted gene and the y-axis is correlated with the tree tips. Boxplots show the allele divergence (0 to 14 %) for the corresponding tip and the color represents ploidy level [?*x* = unknown (grey), 2x (pink), 4*x* (blue), 6*x* (green) and 10*x* (yellow)] of each species, based on published chromosome numbers (Table 1). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

percentage of SNPs (i.e., allele divergence) was correlated with ploidy, i.e. ~ 1 % for the 2*x* outgroup, ~2 % for 4*x* ingroup individuals, and > 4 % for higher polyploids (6*x* and 10*x*).

3.2. Angiosperms353 ASTRAL species tree reconstruction

Among all 345 filtered single-copy genes for 131 *Azorella* samples, the trimmed alignment length varied from 222 to 7,188 bp (average 1,812 bp), and on average, each locus contained 456 parsimony informative sites (Table S3). The final Angiosperms353 (A353) ASTRAL species tree generated from 345 gene trees showed high local posterior probabilities (1.0) at the backbone nodes, whereas most nodes showed high discordance levels among gene trees (pink pie charts). Interestingly, for samples even without a sufficient number of recovered gene sequences (e.g., Azfragosea_CANB798456 or Aznitens_CHR252408), in the ASTRAL species tree, these individuals were nevertheless assigned to the same clade as other individuals of the same taxon. However, a low number of recovered genes also led to a lack of resolution regarding intraspecific relationships for closely related taxa, such as the three megaherbs in section *Stilbocarpa* (*A. polaris*, *A. lyallii*, *A. robusta*; Fig. 5).

The reconstructed ASTRAL species tree (Fig. 5) comprised six main clades that corresponded with taxonomy and geographical distribution. First, section *Stilbocarpa*, comprising three megaherbs from the New Zealand subantarctic islands, was monophyletic (hereafter, clade SubM). Similarly, section *Schizeilema* was also monophyletic, comprising three subclades and one grade, including one grade and one clade of six New Zealand taxa each (grade NZ1 and clade NZ2), a clade of Australian *A. fragosea* (clade Au), and a clade of subantarctic *A. schizeilema* (clade Sub) (Fig. 5). The final clade comprised the two South American species from section *Ranunculus* (clade SA). Overall, the ASTRAL tree showed that the three sampled sections (*Stilbocarpa, Schizeilema* and *Ranunculus*) formed three monophyletic groups (Fig. 5), and section *Stilbocarpa* was more distantly related to the sister clades of sections *Ranunculus* and *Schizeilema*.

Grade NZ1 comprised species of three different ploidy levels, including two sister tetraploid species (*A. allanii* and *A. roughii*), and a paraphyletic group of higher polyploids, i.e., *A. hookeri* (6*x*), *A. nitens* (6*x*), *A. cockaynei* (6*x*) and *A. colensoi* (10*x*). Clade NZ2 comprised species that were mostly tetraploids, including *A. exigua* (4*x*), *A.* sp. (?*x*; Azsp_CHR617214 and Azsp_CHR617283), *A. hydrocotyloides* (4*x*), *A. haastii* subsp. *cyanopetala* (4*x*; samples labelled Azcyanopetala) and *A. pallida* (6*x*). Furthermore, *A. haastii* subsp. *haastii* (4*x*, samples labelled Azhaastii) formed three different lineages.

Overall, several section *Schizeilema* taxa represented by individuals collected from multiple populations were monophyletic in the A353 ASTRAL tree (*A. pallida*, *A. allanii*, *A. hydrocotyloides*, *A. exigua*, *A. schizeilema*, *A. fragosea*, *A. allanii* and *A. roughii*). In addition, some *Azorella* taxa were challenging to identify using the key in Allan (1961), given their similar morphological characteristics and phenotypic plasticity. These individuals that were difficult to identify (shown with black triangles in Fig. 5) were found in mixed clades or grades (Fig. 1; Fig. 5), including three *A. colensoi* (Azcolensoi_AK289484A and B, and Azcolensoi_CHR275373) that group with *A. hookeri*, and one *A. haastii* subsp. *cyanopetala* (Azcyanopetala_CHR529029) with *A. haastii* subsp. *haastii*. In addition, Azpallida_CHR387053 did not cluster with the other sampled *A. pallida* individuals, but instead was in an unresolved position within the NZ1 clade near *A. roughii* and *A. allanii*. As this individual was sampled from a locality within the range of *A. roughii* (Nelson, South Island, New Zealand; Fig. 2), it is possible it was misidentified.

3.3. Topological incongruence between nrDNA- and plastome-based phylogenies

Whole plastomes and the nrDNA cistrons (ETS-18S-ITS1-5.8S-ITS2-26S) were *de novo* assembled for 105 *Azorella* individuals from genome-skimming sequences. The trimmed alignment length for the nrDNA cistron was 7,619 bp and contained 828 parsimony informative sites. The aligned plastome data set was 155,710 bp and contained 3,469 parsimony informative sites (Table S3).

The plastome phylogenetic tree reconstructed using IQ-TREE2 (Fig. 6a) had some of the same clades as the A353 ASTRAL species tree (NZ1, SA, Au and SA), but some of the species relationships differed (Fig. 5). In the plastome tree, SubM and SA formed a clade that was sister to a clade of section *Schizeilema* (Fig. 6a), which comprised all taxa in the NZ1, NZ2, Au and Sub A353 groups (Fig. 5). In addition, the NZ2 clade reconstructed from A353 data (Fig. 5) was split into two clades (NZ2_cp1 and NZ2_cp2) that were not each other's closest relative (Fig. 6a). Some NZ2 taxa that were monophyletic in the A353 ASTRAL species tree (Fig. 5), such as *A. haastii* subsp. *cyanopetala* and *A. pallida*, were split across the two NZ2 clades in the plastome tree. The sole individual of *A. schizeilema* sampled in this tree, Azschizeilema_Ome (Sub), was in the NZ2_cp2 clade (Fig. 6a). Most taxa within the NZ1 clade of the A353 tree (Fig. 5) were also not monophyletic. For example, all individuals of the two higher polyploids *A. colensoi* (6*x*) and *A. hookeri* (10*x*) were nested within a paraphyletic grade of all *A. allanii* (4*x*) and *A. roughii* (4*x*) (Fig. 6a).

The nrDNA tree (Fig. 6b) displayed yet another topology compared to the plastome tree (Fig. 6a) and the A353 ASTRAL tree (Fig. 5). The six species previously reconstructed in a single clade NZ1 (in the A353 and plastome trees) were reconstructed in two different clades in the nrDNA tree (NZ1_nr1 and NZ1_nr2). Interestingly, NZ1_nr2 included the four higher polyploids (10*x A. colensoi,* and 6*x A. hookeri, A. cockaynei* and *A. nitens*) that were sister to SA, and NZ1_nr2 plus SA were in turn sister to SubM. By contrast, the NZ1_nr1 clade (all 4*x*) was sister to the NZ2 clade (all 4*x*), and these were in turn sister to *A. schizeilema,* and then a clade of Au (*A. fragosea*). Although NZ2 was reconstructed as a clade, most taxa were not monophyletic, except *A. exigua* (Fig. 6b).

The homogenization levels among biparentally inherited nrDNA cistrons were confirmed by calculating the allele divergence (proportion of SNPs) from genome-skimming reads using PATÉ (Table S4). Across all 105 individuals, allele divergence was on average 0.13 %, i.e. there were on average only nine sites per individual that were heterozygous in a nearly 7 kbp region for the entire nrDNA cistron.

3.4. Analysis of reticulation in Azorella species using A353 locus and SNP data

Based on the number of assembled genes with supercontigs in the Hyb-Seq data, one individual per taxon was selected to test for reticulation events among New Zealand *Azorella* species. We included additional individuals for species that had more than one lineage in the A353 locus tree (Fig. 5) or with more than one chloroplast type (Fig. 6a). In total, 23 representative individuals were selected, including three individuals of *A. haastii* subsp. *haastii* from each of the three polyphyletic groups (Fig. 5), and two individuals each of *A. pallida* and *A. haastii* subsp. *cyanopetala* to represent their two plastome types (Fig. 6a).

We first reconstructed the ASTRAL species tree of the 23 selected individuals from 219 filtered A353 loci that had no missing taxa and calculated the gcf between gene trees. On average, each trimmed gene alignment was 2,144 bp in length and had 237 parsimony informative sites. The topology of the 23-individual A353 ASTRAL species tree (Fig. 7a) showed similar species relationships as the 131-individual A353 ASTRAL species tree (Fig. 5), including *A. haastii* subsp. *haastii* which consistently exhibited the same topology of three separate lineages.

By excluding the conflicting intraspecific signals and reducing the missing taxa among targeted genes, the 23-individual ASTRAL species tree (Fig. 7a) showed improvements in gene concordance levels in clade NZ1 (blue pie charts), except two nodes which showed a high proportion of genes that supported a main alternative topology (green pie charts), e.g., the nodes leading to individuals Azcolensoi_BellA10 and Azhookeri_Tai, respectively. Similarly, the three megaherbs in clade SubM also

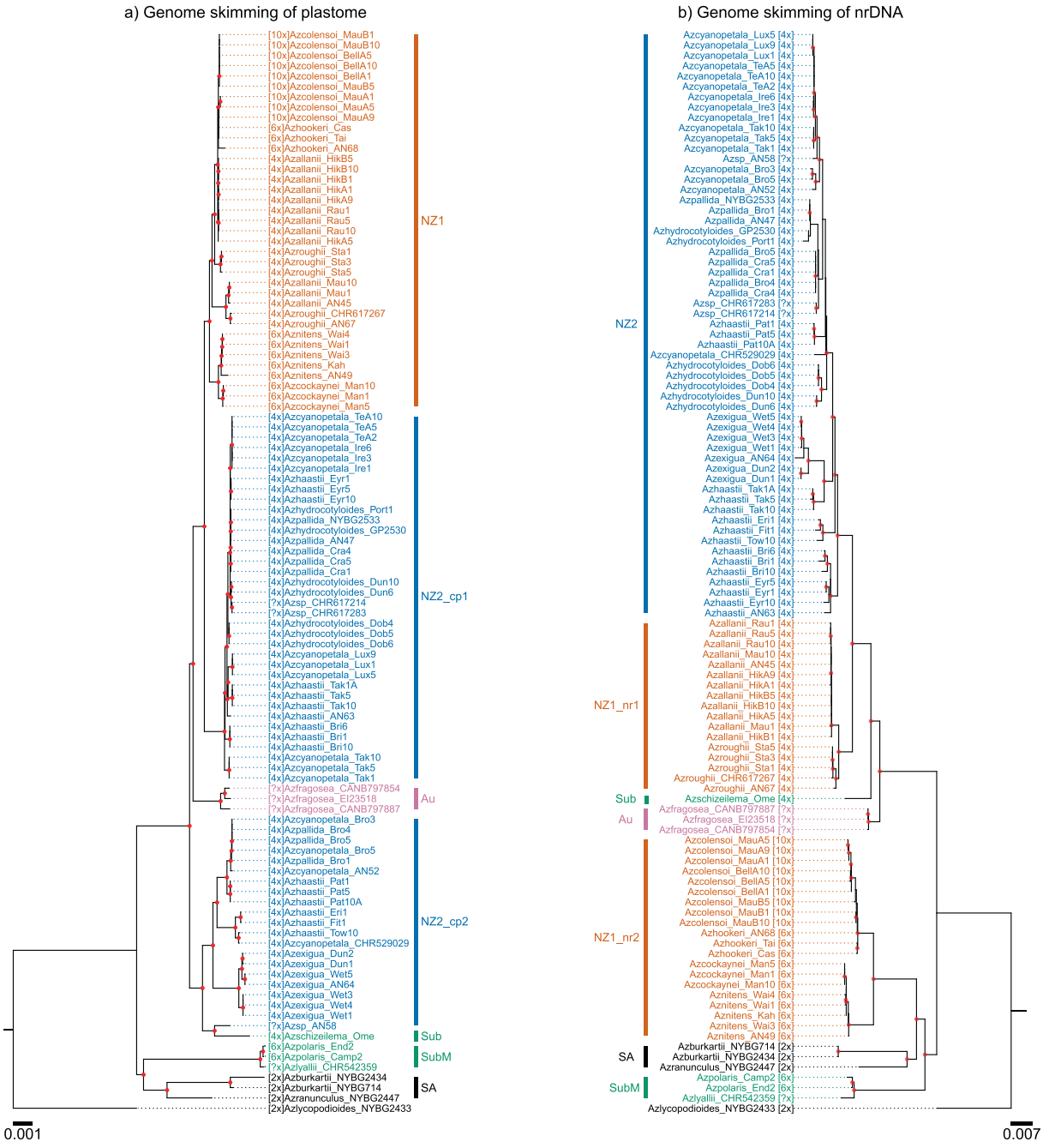

Fig. 6. Comparison of phylogenetic relationships derived from a) plastome and b) nrDNA data for 105 samples of *Azorella* sections *Schizeilema*, *Stilbocarpa* and *Ranunculus*. Nodes with bootstrap support values higher than 90% are indicated with red dots. The individual names represent the species name, and accession ID (see Table S1 for full species names). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

supported a main alternative topology (green pie charts). This indicates the alternative species relationships at these nodes should be considered. By contrast, the interspecific discordance levels between NZ2, Sub and Au remained high.

SplitsTree estimated the network from the concatenated sequences of 219 filtered Hyb-Seq loci (with a total of 51,796 parsimony-informative sites) using the Neighbor-Net method (Fig. 7b). The network supported clear sectional relationships between SubM (section *Stilbocarpa*), SA (section *Ranunculus*) and section *Schizeilema*, whereas four other groups (Au, Sub, NZ1, NZ2) within New Zealand *Azorella* showed unresolved, conflicting signals (as indicated by the box-like relationships among species).

The optimal model for the SNaQ network of 23 individuals supported four hybridization events (h = 4) (Fig. S2; Fig. 7c). These include (1) a hybrid origin of 10*x* individual Azcolensoi_BellA10 from 6*x* Azhookeri_Tai, and a hybrid origin of the ancestral lineage to the NZ1 tetraploids, NZ2, Au, and Sub. In addition, there were two hybridization events within NZ2, which led to the formation of individuals Azhaastii_Eri1 (2) and Azhydrocotyloides_Dob6 (3), respectively (Fig. 7c). Finally, individual Azburkartii_NYBG714 also exhibited a hybrid origin (4) with Azranunculus_NYBG2447 as one of the parental species (Fig. S2; Fig. 7c).

The homeologs of the nrDNA cistron were extracted for the 22 selected individuals using their phasing results in PATÉ (Table S4). However, the result showed the copy number of homeologs varied from a minimum of zero to a maximum of seven, which were not correlated

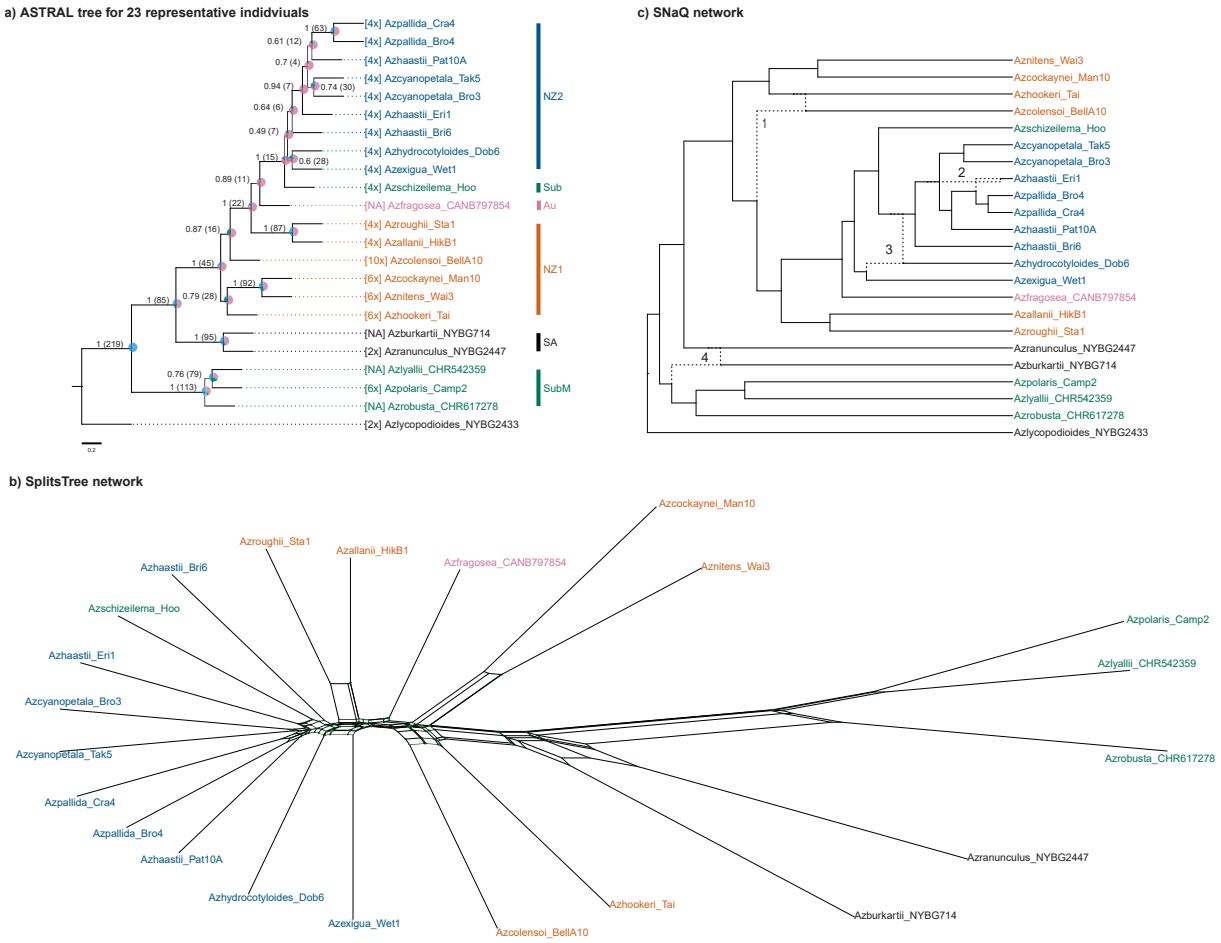

Fig. 7. Network analysis of 23 *Azorella* individuals representing all 18 taxa in the 131-individual A353 ASTRAL species tree (Fig. 5). a) The 23-individual ASTRAL species tree of selected *Azorella* individuals using 219 filtered Angiosperms353 loci. Each node is labelled with a local posterior probability (0 to 1) and the number of concordant genes (0 to 219), plus a gene concordance pie chart (i.e., blue = concordant, green = discordant with a main alternative, pink = discordant with all remaining alternatives, and grey = uninformative). b) SplitsTree network from Neighbor-Net for 23 individuals (the outgroup species *A. lycopodioides* is not shown due to a long branch). The box nodes are highlighted by green dots. c) SNaQ estimated network for the 23-individuals dataset when h = 4. The dashed lines represent four hypothesized hybridization events between species or lineages. See Table S1 for full species names. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with species ploidy levels. Moreover, the multi-labelled tree showed no reticulation signals were found in nrDNA (Fig. S3).

3.5. Phylogenetic inference of Hyb-Seq data using SNPs

Phylogenies were constructed using 9,182 biallelic SNP sites without missing data, which were selected from a total of 187,165 joint-called SNPs. The best model for the TreeMix network was at m = 2 (Fig. S4). The model supported two gene flow events from sister tetraploids (*A. allanii* and *A. roughii*) in NZ1 to hexaploid *A. hookeri* and decaploid *A. colensoi*, respectively (Fig. 8a).

We estimated the Bayesian tree of 23 individuals using SNAPP with the same selected 9,182 biallelic SNPs. The consensus SNAPP tree was calculated from 17,518 SNAPP trees after 10% burn-in (total 19,464 trees). The backbone of the SNAPP tree (Fig. 8c) was supported by high posterior probabilities (PP = 1.0), and overall the topology was similar to the 23-individual ASTRAL species tree (Fig. 7a), except that Sub was further nested within the NZ2 clade, and NZ1 formed a monophyletic group. However, the discordance levels within the NZ2 and SubM clades remained high, and both exhibited a high proportion of alternative topologies (Fig. 8b).

3.6. Genomic introgression test using A353-derived SNPs

From the 9,182 SNPs filtered from the A353 Hyb-Seq data, genomic introgression signals among all 23 selected individuals were calculated using Patterson's D-statistic and the related f-branch (fb) values. The highest fb values corresponding to the SNAPP tree lineages (Fig. 9) can be found between individual Azcolensoi_BellA10 and the sister NZ1 tetraploid individuals Azallanii_HikB1 (fb = 0.34) and Azroughii_Sta1 (fb = 0.21). In addition, the ancestral node to the NZ1 sister tetraploids also showed introgression signals across all NZ2, Au, and Sub groups (fb > 0.13). One individual of *A. haastii* subsp. *haastii* (Azhaastii_Bri6) showed gene flow with the two selected *A. haastii* subsp. *cyanopetala* (average fb = 0.10) and two *A. pallida* (average fb = 0.12) individuals, and a low fb introgression value (0.05) from *A. exigua* that likely resulted from similar ancestral polymorphisms. Moreover, an individual from the SubM lineage, Azrobusta_CHR617278, exhibited gene flow signals across all sampled individuals, except the two other SubM megaherbs (average fb = 0.09), which may likely be due to insufficient sequencing data of this sample.

3.7. Biogeographical histories of New Zealand Azorella

The BioGeoBEARS result supported DEC + J as the most likely model for biogeographical inference (Table S5). The model showed South

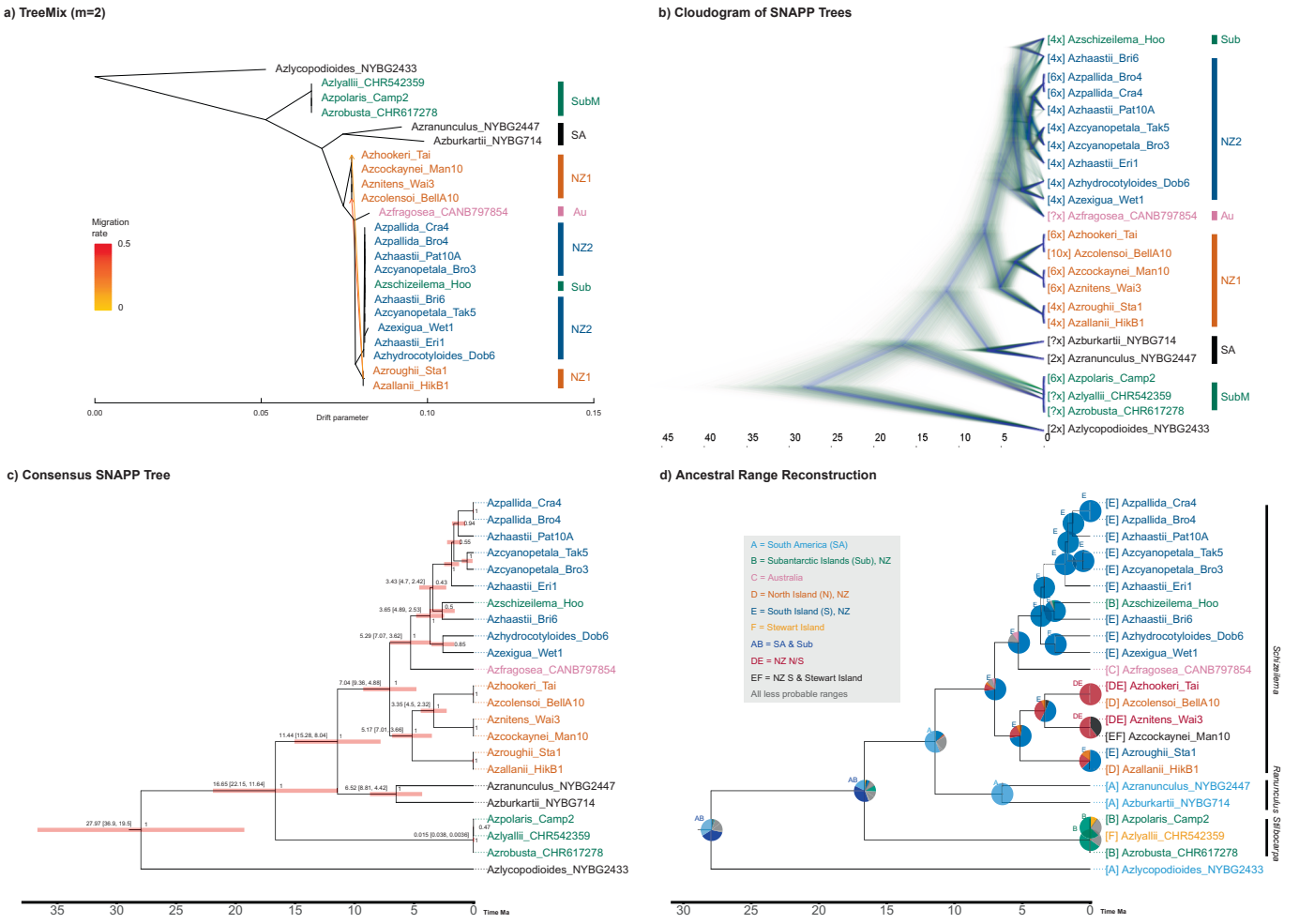

Fig. 8. Network, phylogeny, divergence time and biogeographic history estimation of *Azorella* species with Hyb-Seq A353 SNPs from 23 representative individuals. a) A TreeMix network shows two introgression events (represented by orange arrows) between sampled individuals. The colour of individuals (tree tips) represents the previously identified genetic groups (Fig. 5) in a to c panels. b) Cloudogram of species relationships for 23 individuals inferred from Bayesian phylogenetic SNAPP trees. The main consensus SNAPP tree is shown in blue with the alternative topologies represented by green or red. The x-axis shows the predicted divergence time (40 Ma to 0). c) The consensus Bayesian SNAPP time-calibrated consensus phylogenetic tree of selected 23 individuals. The posterior probabilities are annotated on the right side of each node (maximum = 1.0), and the time scale bar is labelled on the x-axis. The red bars and numbers in brackets show the 95 % highest posterior density (HPD) corresponding to the divergence time on the x-axis. d) Biogeographic inference of selected 23 individuals with sectional names labelled on the right side. The nodes and individuals (tree tips) are annotated and coloured with their distribution or estimated ancestral ranges before and after cladogenetic events, corresponding to the labels in the upper left box. The pie charts at each node are proportional to the posterior probability of the estimated ancestral range for that node and coloured based on the present species distribution ranges, with the less probable ranges represented in grey. See Table S1 for full species names. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

America (SA) and the subantarctic islands (Sub) were the most likely origins for section *Schizeilema* (NZ1, Au, Sub, NZ2), section *Ranunculus* (SA), and section *Stilbocarpa* (Sub) (Fig. 8d; Fig. S5). Furthermore, independent dispersal events were supported for the two New Zealand *Azorella* sections, suggesting two different origins. The ancestor to the split between section *Stilbocarpa* and sections *Schizeilema* + *Ranunculus* originated around 16.65 Ma (with 95 % highest posterior density (HPD) 22.15 to 11.64 Ma) with a more recent origin for the subantarctic islands taxa (~15,000 years ago). By contrast, the ancestor to section *Schizeilema* dispersed to New Zealand around 7.04 Ma (with 95 % HPD between 9.36 and 4.88) from South America. In addition, within section *Schizeilema*, the model also supported diversification of the species from the South Island of New Zealand to the North Island (NZ), Australia (to Au *c*. 5.29 Ma) or subantarctic islands (to Sub *c*. 2.64 Ma).

4. Discussion

Resolving relationships of polyploid-rich genera can provide insight into the origins and diversification of polyploid lineages (Ning et al., 2024; Rothfels, 2021). In this study, we used the universal Angiosperms353 baits (Johnson et al., 2019) and successfully captured over 300 loci for phylogenetic, biogeographical, and network inferences of New Zealand *Azorella* polyploids (4*x*, 6*x* and 10*x*) and their close relatives. Our results also highlight the benefits of combining locus-based phylogenomic analyses of nuclear and plastome data with SNP-based analyses of nuclear loci to provide a more comprehensive understanding of the origins and reticulate relationships of polyploid species. We acknowledge two caveats that could increase phylogenetic uncertainty: hybridization and polyploidization events (Rothfels, 2021), which can affect the biogeographical inference using a bifurcating tree, and limited outgroup sampling at the root of the tree (sections *Huanuca* and *Azorella* were not sampled). Using different phylogenetic approaches and comprehensive ingroup sampling throughout New Zealand, these results nevertheless provide a clear picture of the patterns of evolution and diversification in New Zealand *Azorella*. In particular, although the A353 loci were not phased into separate parental homeologs for each species, we recovered meaningful species relationships for most taxa and were able to identify some polyploid origins as discussed below.

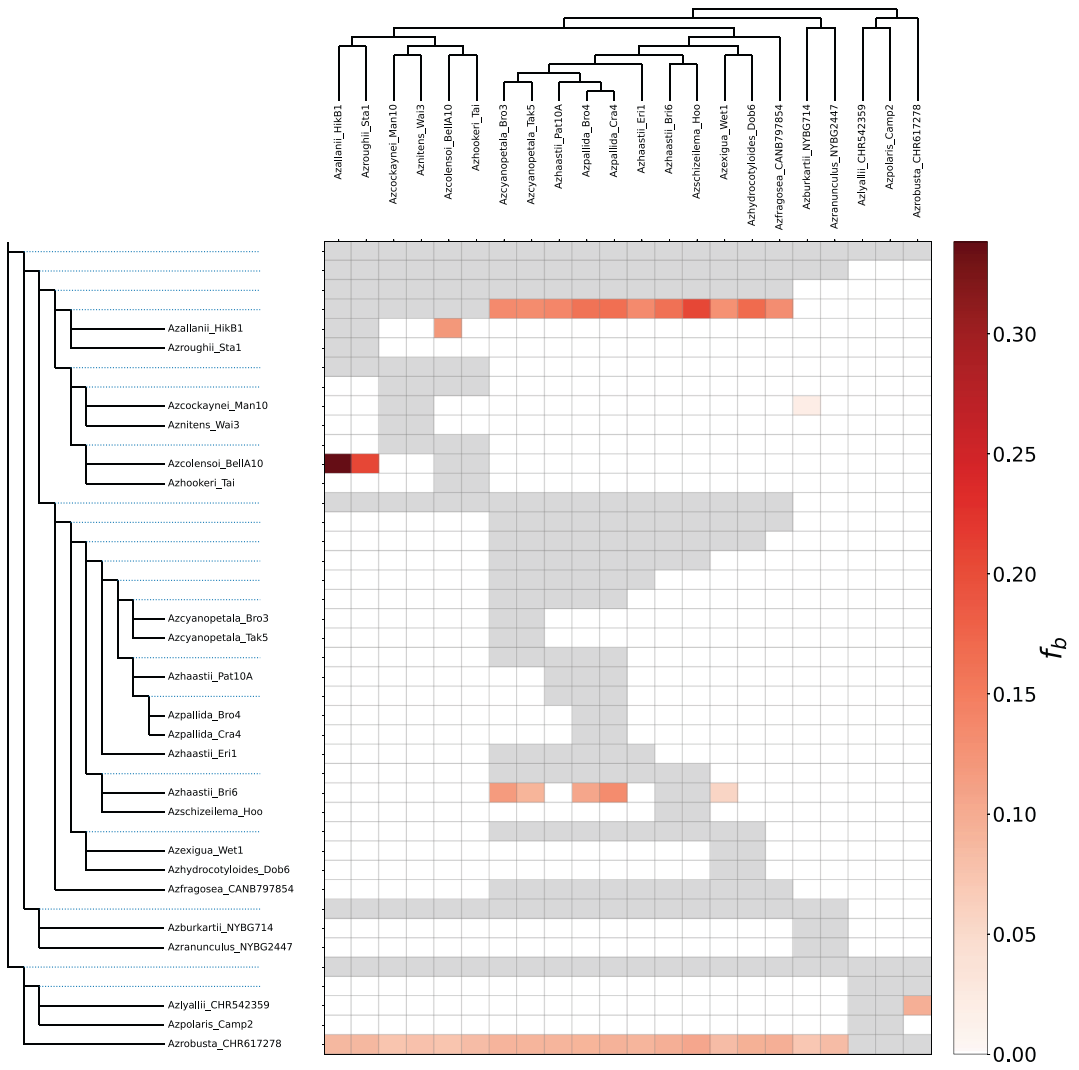

Fig. 9. Genomic introgression signals identified by the ABBA-BABA test using Hyb-Seq SNPs extracted from 23 representative individuals. The x-axis corresponds to the SNAPP tree topology and the y-axis represents all pairwise correlated nodes or tips to the SNAPP tree. The colour gradient shows f-branch values (0 to 0.35) for each species-trio combination.

4.1. Phylogenomics of polyploid Azorella

Phylogenetic inferences of non-model plant genera have been significantly improved by the development of new sequencing techniques over the last decade (Hibbins and Hahn, 2022; McKain et al., 2018; Rothfels, 2021). Taking polyploid species (4*x*, 6*x* and 10*x*) in New Zealand *Azorella* sections *Schizeilema* and *Stilbocarpa* as an example, without having reference genome sequences, we recovered over 300 nuclear loci with sufficient sequencing depth from Angiosperms353 baits target-capture sequence reads (Fig. 5). Even for individuals with limited starting material (such as field-collected *A. exigua* that only yielded 5 ng total DNA; Fig. 1) or herbarium specimens that had low-quality, degraded DNA (Fig. 5), the robust laboratory and bioinformatic pipelines have nevertheless recovered sufficient sequence reads for gene recovery.

Single-copy genes have been shown to be reliable for polyploid phylogenetic inference (Naranjo et al., 2024) and a reliable species phylogeny can be reconstructed when considering the phylogenetic signals from genome-wide markers (Maddison and Knowles, 2006). However, the diverse and dynamic nature of polyploid diversification can generate additional challenges to fully resolve the interspecific relationships using only Hyb-Seq data (Ning et al., 2024). Similar to other studies on polyploid genera (Nauheimer et al., 2021; Nicol et al., 2024; Thomas et al., 2021), individuals of New Zealand *Azorella* also exhibited a high allele divergence (average percentage of SNPs in each sample > 1 %; Fig. 4d; Fig. 5), which indicated potentially unsorted homeologous gene copies that could be used for phylogenetic reconstruction. Although the phasing of target loci has been developed for Hyb-Seq data (Mendez-Reneau et al., 2023; Nauheimer et al., 2021; Tiley et al., 2024), this is difficult to achieve with the universal Angiosperms353 baits, especially for higher-level polyploids (i.e., greater than 4*x*). This is because these baits mostly target the discontinuous exonic regions within each locus, and it is a major challenge to assemble the complete gene sequences without gaps from the short-read Illumina data.

Consequently, when sequencing depth and coverage is sufficient, Hyb-Seq can be particularly useful to resolve the origins of allopolyploid or hybrid species by recovering all homeologous sequences for each targeted gene (Tiley et al., 2024). However, phasing is also just the first step towards resolving the phylogenomic complexity of polyploid species, i.e., assigning each homeologous copy to its correct subgenomic/parental copy. Especially for higher polyploids, determining how many potential subgenome donors are involved may eventually require a completely assembled whole genome or at least reference sequences of related diploids. If only using recovered genes with low allelic divergence (i.e., no paralogs) to obtain a robustly supported backbone, the resulting phylogeny may lack resolution of intra- or interspecific

relationships (Zhou et al., 2022). Such is the case for New Zealand *Azorella* species, for which a related diploid lineage is not known. In addition, the difficulties encountered to correctly identify New Zealand *Azorella* species using current keys indicate that a taxonomic revision is needed, as has been done recently for some of the South American species in sections *Laretia* and *Spinosae* from subgenus *Andinae* (Fernández et al., 2016, 2017b, 2017c).

In spite of these challenges, our results nevertheless showed the utility of Angiosperms353 loci in providing resolved and meaningful species relationships within the polyploid-rich section *Schizeilema* (see below), especially when viewed in a comparative framework together with the phylogeny based on Hyb-Seq SNP data only, and together with the nrDNA and plastome phylogenies (Fig. 5; Fig. 6; Fig. 7c). Therefore, for polyploid genera, it is important to compare biparentally inherited nuclear markers with maternally inherited plastid markers, and to incorporate data on chromosome counts and ploidy level, where available (Meudt et al., 2021; Ning et al., 2024; Fernández et al., 2017a). Moreover, increasing Hyb-Seq read length using a third-generation sequencer (e.g., PacBio) and developing taxon-specific bait sets, may help capture full-length homeologous sequences and reduce conflicting signals among stitched exons.

4.2. Species relationships and the origins of the higher polyploids in *Azorella* section *Schizeilema*

A comparison of the topologies reconstructed using different phylogenomic approaches of the Hyb-Seq data provides information on species relationships and the likely origins of the higher-order polyploids of New Zealand mainland *Azorella*. Moreover, the incongruence between plastome, nrDNA or A353 loci phylogenies, which can be expected for polyploid species due to hybridization and introgression events (de Lima Ferreira et al., 2022; Kandziora et al., 2021; Rose et al., 2020; Fernández et al., 2017a), provides evidence to confirm the reticulate origin of the relationships of these taxa.

Within the NZ1 clade, the species relationships between the two tetraploids (*A. allanii*, *A. roughii*), three hexaploids (*A. hookeri*, *A. nitens*, and *A. cockaynei*) and the decaploid (*A. colensoi*) varied in the different bifurcating phylogenetic trees (Fig. 7a; Fig. 8c). By contrast, all network approaches (Fig. 7c,d; Fig. 8a) separated the two tetraploids from the higher polyploids and grouped them with other tetraploids in section *Schizeilema*. In addition, the networks and D-tests (Fig. 7c,d; Fig. 8a; Fig. 9) also consistently identified the allopolyploid origin of *A. colensoi* (10*x*) from maternal parent *A. hookeri* (6*x*), given these two species shared nearly identical plastome sequences (Fig. 6a). By contrast, the paternal lineage of *A. colensoi* was related to the sister species *A. roughii* and *A. allanii* (both 4*x*), and is most likely to be *A. allanii* (Fig. 8a; Fig. 9), because *A. colensoi* is a North Island endemic whose geographic distribution overlaps with that of *A. allanii* (Fig. 2). Therefore, strictly bifurcating trees in themselves are not fully informative regarding polyploid origins, but when accompanied by network methods can improve resolutions substantially (Kong et al., 2025). This was also shown to be the case in South American (Andean-Patagonian) *Azorella,* where a hybridization network showed six different reticulation events resulting in both homoploid and allopolyploid species, including five in subgenus *Andinae*, with most of these in the "Diversifolia" group, where hybridization is hypothesized to be an important evolutionary force in that similarly rapidly radiating lineage (Fernández et al., 2017a).

The homogenization processes of the nrDNA sequences reconstructed here (Fig. 6b; Fig. S3) means that all tetraploids in New Zealand (as well as the Australian *A. fragosea* and the subantarctic *A. schizeilema*) share a similar nrDNA type which may have originated from the same South American ancestor, whereas hexaploids that contain a similar rDNA type to sections *Ranunculus* and *Stilbocarpa* may have a different South American origin. On the other hand, similar plastome types for all six species in the NZ1 clade (Fig. 6a) may indicate the ancestor to New Zealand tetraploids was also involved in the formation of higher hexaploids. However, due to incomplete sampling from South America [i.e., the taxa from sections *Azorella* and *Huanaca* in subgenus *Azorella*, which are in a polytomy with section *Schizeilema* in previous studies (Fernández et al., 2017a; Plunkett and Nicolas, 2017)], it is difficult to fully determine whether the formation of the hexaploids happened before or after the dispersal of *Azorella* to New Zealand.

Within the NZ2 grade, the ancestors of *A. fragosea*, *A. roughii* and *A. allanii* may have played important roles regarding the diversification of *Azorella* tetraploids. In the plastome trees (Fig. 6a), instead of showing geographical patterns or patterns similar to nuclear data (A353 ASTRAL tree and nrDNA tree) (Fig. 2; Fig. 5), the NZ2 group displayed two different plastome types but only one type of nrDNA, which most likely resulted from a hybridization event between the ancestor of *A. fragosea*, and the ancestor of the sister species *A. roughii* and *A. allanii*, followed by speciation via ecological differentiation and perhaps further hybridization (Joly et al., 2014). Indeed, the NZ2 grade exhibited substantial introgression and complex network relationships as evidenced by the discordance levels of Hyb-Seq gene trees and SNP trees (Fig. 7a; Fig. 8b), as well as the reticulation signals in the network (Fig. 7c; Fig. 9). The single nrDNA type of the NZ2 group could have also resulted from concerted evolution, accelerated by reticulation events to promote the homogenization process in NZ2 taxa (Alvarez and Wendel, 2003; Garcia-Jacas et al., 2009; Hillis and Dixon, 1991).

As allopolyploidy has been an integral part of the origin of species in section *Schizeilema*, species circumscription remains challenging. For example, we sampled multiple populations of the two subspecies of *A. haastii*, which were polyphyletic in the ASTRAL tree as well as in the plastome and nrDNA trees (Fig. 5; Fig. 6a, b). These subspecies were similarly not monophyletic in the analyses of Nicolas et al. (2025), and field identification can sometimes be difficult. We also included samples that possibly represent new taxa and are in need of formal naming and description because they do not align with current descriptions of species in New Zealand (e.g., Azsp_CHR617214, Azsp_CHR617283, and Azsp_AN58).

4.3. Dynamic dispersal patterns of *Azorella* section *Schizeilema*

The BioGeoBEARS biogeographical model based on SNAPP data indicated the ancestor of the 14 New Zealand *Azorella* taxa in section *Schizeilema*, which is closely related to the South American section *Ranunculus* (also in subgenus *Azorella*), dispersed to the South Island of New Zealand during the Late Miocene, c. 7.04 Ma (Fig. 8d; Fig. S5). In comparison, the age of this clade in Nicolas et al. (2025) based on plastid sequence data was 10.09 Ma and the ancestor inferred from the subantarctic or Stewart Islands. These minor differences are likely explained by the distinct data sets and differences in taxon sampling. The circumpolar westerly winds and ocean currents formed during the Miocene (23 to 5 Ma) (Barker and Burrell, 1982) provided opportunities for plant propagules to disperse from South America to New Zealand (Sanmartín et al., 2007; Sanmartín and Ronquist, 2004; Winkworth et al., 2002). Species diversification within section *Schizeilema* started from the Late Miocene (7 to 5 Ma) and continued throughout the Pliocene-Pleistocene (5 to 0 Ma), which also fits the predicted time of active vascular plant evolution in New Zealand, beginning in the Late Miocene and thereafter (10 to 0 Ma) (Heenan and McGlone, 2019).

Rapid diversification of South Island endemic alpine *Azorella* started by *c.* 3.65 Ma (NZ2, blue clade in Fig. 8c) and may be correlated with late Pliocene-Pleistocene (5 to 0 Ma) geological events in the New Zealand Southern Alps, especially their uplift and glaciation cycles (Winkworth et al., 2005; Heenan and McGlone, 2013). Species diversification was influenced by reticulation and polyploidization events, as has been the case in the South American species of *Azorella,* especially in subgenus *Andinae* (Fernández et al., 2017a), or reticulation and polyploidization events, as well as many other New Zealand plant genera, e.g., *Veronica* (Plantaginaceae) (Thomas et al., 2021; Wagstaff and Garnock-Jones, 1998), *Ranunculus* (Ranunculaceae) (Lockhart et al., 2001),

Ourisia (Plantaginaceae) (Meudt and Simpson, 2006), *Pachycladon* (Brassicaceae) (Joly et al., 2009), *Plantago* (Plantaginaceae) (Tay et al., 2010), *Lepidium* (Brassicaceae) (Smissen et al., 2024), and *Leptinella* (Asteraceae) (Himmelreich et al., 2014).

A perennial life-form, vegetative reproduction (Fig. S1), and small seed size (Friedman, 2020; Meudt et al., 2021) are all characteristics that may have helped New Zealand *Azorella* become a transoceanic traveller to colonize new lands. Indeed, hybridization may have also played a role in such colonizations, as it did in two homoploid hybrid South American species in subgenus *Andinae*, *A. selago* and *A. filamentosa,* which were hypothesised to colonise harsher habitats (including in the subantarctic) due to hybrid origins facilitating their range expansion (Fernández et al., 2017a). The facilitation of gene exchange, recombination, and an increased adaptive potential through hybridization and allopolyploidy in *Azorella*, may have been a mechanism enabling adaptation to climate and environmental change in New Zealand through the late-Cenozoic (Becker et al., 2013). Although the NZ2 grade showed high topological uncertainties likely due to interspecific hybridization (Fig. 7; Fig. 9), *A. schizeilema* (Sub) – endemic to the New Zealand subantarctic islands (Auckland Islands and Campbell Island) – most likely diverged *c.* 2.6 Ma from within this lineage, and dispersed to the subantarctic islands from the South Island (Fig. 6; Fig. 8c,d). Similarly, *A. fragosea* (Au) dispersed from the South Island of New Zealand to Australia c. 5.3 Ma (Fig. 8c,d). Despite the strong westerly winds limiting dispersal from New Zealand to Australia, dispersal via easterly winds in the lower-atmosphere or via bird migration is still possible (Wardle, 1978; Winkworth et al., 2002). Indeed, several New Zealand plant genera show similar biogeographical patterns of South American origin and long-distance dispersal to New Zealand, where they subsequently underwent diversification and range expansion, particularly in the South Island, from which they dispersed again to Australia, subantarctic islands or other areas, e.g., *Ranunculus* (Lockhart et al., 2001), *Pachycladon* (Heenan and Mitchell 2003), *Ourisia* (Meudt and Simpson, 2006), and *Veronica* (Meudt and Bayly, 2008; Wagstaff et al., 2002).

Species dispersal from the South Island to the North Island of New Zealand may have a more recent history in section *Schizeilema*. Although the species relationships within clade NZ1 are not well resolved due to reticulate evolutionary histories (Fig. 7c; Fig. 8a; Fig. 9) (see below), the recent divergence time of the well supported sister species, *A. allanii* (from southern North Island) and *A. roughii* (from northern South Island), was *c.* 40,900 years ago. The speciation of this sister species pair and their allopatric distribution across the Cook Strait may have been facilitated by two geologically recent events (middle Quaternary, c. 1 Ma), i.e., a land bridge that connected southern areas of the North Island to northern parts of the South Island, e.g., between the Wellington and Nelson regions (Trewick and Bland, 2012), and the formation of the North Island mountains and alpine habitats. Similarly, the dispersal of *A. cockaynei* from the South Island to Stewart Island may also have been assisted by a land bridge that existed during the Pleistocene (Lockhart et al., 2001).

4.4. Biogeographical history of subantarctic Azorella megaherbs (section Stilbocarpa)

Section *Stilbocarpa*, which includes the subantarctic megaherbs, was strongly supported as monophyletic in all analyses and more distantly related to the two smaller rhizomatous herbaceous sections *Schizeilema* and *Ranunculus* (Fig. 5; Fig. 7a; Fig. 8c). Moreover, the topology of the SNAPP consensus tree (Fig. 8d) showed that section *Stilbocarpa* may have a different biogeographical origin compared to sections *Schizeilema* and *Ranunculus*. Our result is consistent with Nicolas et al. (2025) in reconstructing the ancestor of section *Stilbocarpa* from the subantarctic islands.

The BioGeoBEARS biogeographical analysis indicated a date of *c.* 16.65 Ma for the ancestor of the split between sections *Stilbocarpa* (SubM) and *Ranunculus* + *Schizeilema* (Fig. 8d), with its most likely ancestral area in South America or subantarctic islands. This timing could coincide with a hypothesis of dispersal via Antarctica from South America (e.g., Wagstaff et al., 2007; Lehnebach et al., 2017; Sancho et al., 2015) before the ice sheet covered the whole Antarctic continent and eliminated all the flowering plants estimated to have occurred 14 to 3.9 Ma (Convey et al., 2008; Lewis et al., 2008; Sancho et al., 2015; Sanmartín et al., 2007; Winkworth et al., 2015). Such a hypothesis would mean that section *Stilbocarpa* must have survived the glaciation of the last glacial maximum (LGM: 20,000 years ago), when glaciers covered most of the subantarctic islands, including all of the landmass of the Auckland Islands, a large portion of Campbell Island, and part of Stewart Island, but not Macquarie Island (Hodgson et al., 2014; McGlone, 2002; Wagstaff et al., 2011). Until 15,000 years ago, the retreat of LGM glaciers provided open space for plant recolonization (Fraser et al., 2009; McGlone, 2002; Suggate, 1990). On the other hand, Rainsley et al., (2019) suggested glaciation was much more restricted on the Auckland Islands and Campbell Island during the LGM. Similarly, the megaherb genus *Pleurophyllum* (Asteraceae) also had a post-glacial dispersal history on the New Zealand subantarctic islands and subsequently survived the LGM (Wagstaff et al., 2011).

Nevertheless, the SNAPP analysis also suggests the three megaherb species within section *Stilbocarpa* likely diversified and dispersed across different regions of subantarctic islands only after the LGM around 15,000 years ago (Fig. 8c). *Azorella polaris* is currently widely distributed throughout the subantarctic, including Macquarie Island (Australia), Auckland Islands, Campbell Island, Stewart Island, and Antipodes Island. By contrast, the other two megaherbs have more restricted distributions: *A. lyallii* is endemic to Stewart Island and *A. robusta* can only be found on Snares Island (Table 1).

Although the clade containing all three megaherb species was always monophyletic in the A353 ASTRAL, plastome and nrDNA trees (and also sister to section *Ranunculus*), the species relationships among them were not fully resolved (Fig. 5; Fig. 6). They also formed a polytomy in a previous phylogenetic study based on ITS (Mitchell et al., 1999) and showed varying relationships in plastid and nuclear trees in Nicolas et al. (2025). Furthermore, our results showed large portions of discordant Hyb-Seq gene trees (Fig. 7a) and discordance in the SNAPP tree (Fig. 8b), which means alternative topologies among the three species must be considered. Therefore, a more comprehensive sampling within each species, only one of which has a known ploidy level (*A. polaris,* 6*x*), may be necessary to determine the species boundaries and population structure among the megaherbs.

5. Conclusion

Azorella section *Schizeilema* is an excellent system to study the evolution of polyploidy, especially the origin and diversification of higher-level polyploids. By incorporating data from hundreds of nuclear loci with plastome data, the topological incongruence between organellar and nuclear-based trees highlighted the importance of considering network approaches when inferring the phylogeny of polyploid-rich groups. However, current network methods have limited power when multiple polyploid species are involved in the network. Nonetheless, such an approach would be useful to undertake for other genera within Apiaceae to reveal relationships that may be obscured by the use of a single-genome data set (e.g., Wen et al., 2021, Clarkson et al., 2021). Species in section *Schizeilema* have diversified in New Zealand into a variety of habitats, which may have been facilitated by their allopolyploid origins. As several species were reconstructed as not monophyletic, future studies should investigate species boundaries, with likely new taxa to be described. Similarly, future sequencing approaches that can retrieve full-length homeologous loci and pipelines that can distinguish among higher level polyploids would facilitate evolutionary studies of complex flowering plant genera.

CRediT authorship contribution statement

Weixuan Ning: Writing – original draft, Formal analysis, Data curation, Conceptualization. **Heidi M. Meudt:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization. **Antoine N. Nicolas:** Writing – review & editing, Data curation. **Gregory M. Plunkett:** Writing – review & editing, Data curation. **Peter B. Heenan:** Writing – review & editing, Data curation. **William G. Lee:** Writing – review & editing, Funding acquisition, Conceptualization. **Jennifer A. Tate:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2025.108469.

The target enrichment sequencing data and genome-skimming data are available at NCBI BioProject PRJNA885464 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA885464/; Table S1). The NCBI accession numbers for extracted nrDNA and plastome sequences can be found in Table S1. All supplementary figures, supplementary tables, retrieved gene sequence data, gene alignments, phylogenetic trees and scripts for conducting all analyses are available at: https://github.com/WeixuanPlant/NZAzorellaAngiosperms353.

Data availability

Data are available at https://github.com/WeixuanPlant/NZAzorella.

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