Botanical Journal of the Linnean Society, 2024, **205**, 292–312
DOI: https://doi.org/10.1093/botlinnean/boad069
Advance access publication 6 December 2023
Original Article

OXFORD

Original Article

Genomic data inform taxonomy and conservation of Critically Endangered shrubs: a case study of *Zieria* (Rutaceae) species from eastern Australia

Harvey K. Orel[1], Todd G.B. McLay[1,2], Lydia K. Guja[3,4], Marco F. Duretto,[5], and Michael J. Bayly[1]

[1]School of BioSciences, The University of Melbourne, Parkville, Victoria 3010, Australia
[2]National Herbarium of Victoria, Royal Botanic Gardens Victoria, South Yarra, Victoria 3141, Australia
[3]National Seed Bank, Australian National Botanic Gardens, Parks Australia, Canberra, ACT 2601, Australia
[4]Centre for Australian National Biodiversity Research, CSIRO, Canberra, ACT 2601, Australia
[5]National Herbarium of New South Wales, Australian Institute of Botanical Science, Botanic Gardens of Sydney, Locked Bag 6002, Mount Annan, NSW 2567, Australia

Corresponding author: Harvey K. Orel, School of BioSciences, The University of Melbourne, Parkville, Victoria 3010, Australia. E-mail: horel@student.unimelb.edu.au

ABSTRACT

Zieria buxijugum, *Z. formosa*, and *Z. parrisiae* are three closely related, Critically Endangered species of questionable taxonomic validity that occur within six kilometres of each other on the south coast of New South Wales, Australia. We investigated genetic relationships and diversity of these species, along with two related but taxonomically distinct congeners, *Z. granulata* and *Z. tuberculata*, and a possible undescribed taxon, *Z. aff. tuberculata*. Double-digest restriction-site associated sequencing (ddRADseq) was used to generate anonymous genomic loci that were used for phylogenetic, network, and genetic structure analyses, and for estimating genetic diversity of the threatened species. Our results support the current taxonomic status of *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae*, and suggest that *Z. aff. tuberculata* warrants recognition as a distinct species. We detected no evidence of inbreeding in the three Critically Endangered species, and found their genetic diversity to be similar to that of the more widespread species *Z. granulata* and *Z. tuberculata*. Comparison of plant material held in *ex situ* collections at the Australian National Botanic Gardens with wild plants highlighted several genotypes of the Critically Endangered species that are not represented in the *ex situ* collection, and we provide suggestions for the future inclusion of those unrepresented genotypes.

Keywords: conservation genetics; ddRADseq; ex situ; genetic diversity; hierarchical structure, inbreeding; species delimitation

INTRODUCTION

Species are used as discrete units for measuring and managing biodiversity because they provide a simple means of categorization for managers of threatened biodiversity (Coates *et al.* 2018). In practice however, defining discrete conservable units can be difficult as the line between species and populations is often blurred by numerous factors, including protracted speciation, varying genetic uniformity across species, labile species treatments, and inconsistent application of taxonomic ranks (Coates *et al.* 2018, Stanton *et al.* 2019). Large genomic datasets provide powerful insights into fine scale, indiscrete patterns of genetic diversity that can help evaluate species and population boundaries to inform and refine conservation efforts (Rossetto *et al.* 2021). The value of evaluating recognized species and species complexes using genomic data has been previously highlighted in instances where morphology has failed to correctly delimit species and units for conservation (Spano *et al.* 2018, Reyes-Velasco *et al.* 2020, Rossetto *et al.* 2021, Binks and Byrne 2022). In these cases, genomic studies can reduce the potential for well-intentioned but misguided conservation actions. For instance, after being described and managed as a formally listed Critically Endangered species based on morphology (Stimpson *et al.* 2014), genomic data have since demonstrated that *Banksia vincentia* Stimpson & P.H.Weston is actually a geographically outlying population of *B. neoanglica* (A.S.George) Stimpson & J.J.Bruhl (Rossetto *et al.* 2021).

Received 20 July 2023; revised 9 October 2023; accepted 5 November 2023

Incongruence between taxonomic hypotheses derived from morphology and genetics has been previously demonstrated in *Zieria* Sm. (e.g. Hogbin and Crisp 2003), a genus with a centre of diversity in eastern Australia (George *et al.* 2013, Duretto 2019, Forster 2020). Here, we seek to evaluate the status of three *Zieria* species, *Z. formosa* J.D.Briggs & J.A.Armstr., *Z. buxijugum* J.D.Briggs & J.A.Armstr., and *Z. parrisiae* J.D.Briggs & J.A.Armstr., that are listed as Critically Endangered in New South Wales (NSW), and known only from single populations in close geographic proximity to one another, west of Pambula, NSW (Fig. 1). The species are very similar morphologically (Armstrong 2002), and several molecular phylogenies of *Zieria* have reported that they are closely related genetically (Barrett *et al.* 2014, 2018, Morton 2015). The most recent of these studies sequenced 1093 bases from the internal transcribed spacer and external transcribed spacer and found these regions to be identical in *Z. formosa*, *Z. parrisiae*, and *Z. buxijugum* (Barrett *et al.* 2018). Barrett *et al.* (2014) also found very similar, but not identical, chloroplast sequences between the three species from four cpDNA markers (*rpl*32–*trn*L, *trn*L–*trn*F, *trn*Q–*rps*16, *trn*S–*trn*G; 23 variable sites out of 4260). The combination of similar morphologies, geographic proximity, and highly similar DNA has raised questions as to whether the current morphology-based classification of *Z. formosa*, *Z. parrisiae*, and *Z. buxijugum* accurately reflects genetic relationships between plants in these taxa (Barrett *et al.* 2018), and whether they each warrant separate conservation management.

More broadly, the relationships of *Zieria buxijugum*, *Z. formosa*, and *Z. parrisiae* to other species are not fully resolved. In a comprehensive monograph on the genus, Armstrong (2002) placed the three species in a group of 14 species characterized by the possession of distinctly tuberculate younger branches, petioles, midveins, peduncles, and fruits. Based on morphology and phytochemical analyses, Armstrong (2002) believed the three species to be most closely related to *Z. granulata* C.Moore ex Benth., *Z. obcordata* A.Cunn., *Z. floydii* J.A.Armstr., *Z. verrucosa* J.A.Armstr., *Z. tuberculata* J.A.Armstr., and *Z. collina* C.T.White. Molecular phylogenies have placed *Z. formosa*, *Z. parrisiae*, and *Z. buxijugum* in a clade with *Z. granulata* and *Z. tuberculata* (Morton 2015, Barrett *et al.* 2018), two threatened species that also occur on the NSW south coast. However, issues with the methodological approach of Morton (2015), pertaining to a combined analysis of cpDNA and nrDNA sequence data despite extensive cytonuclear discordance in *Zieria* (Barrett *et al.* 2014, 2018), along with equivocal support for this clade in the phylogenetic tree of Barrett *et al.* (2018) mean that this relationship is not certain. Besides *Z. granulata* and *Z. tuberculata*, another probable close relation of *Z. buxijugum*, *Z. formosa*, and *Z.*

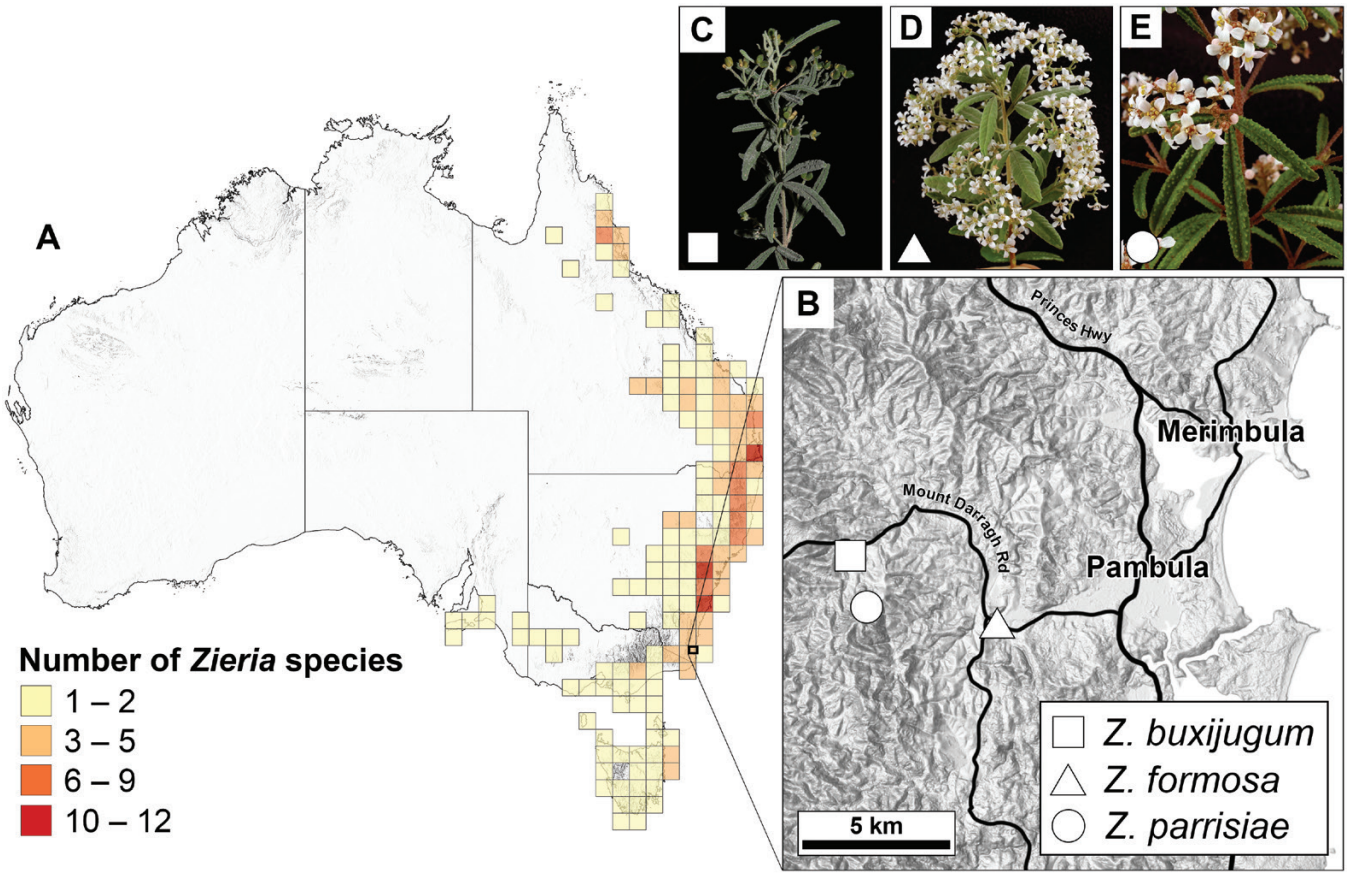

Figure 1. Information on distribution and morphology of *Zieria*. A, Map of *Zieria* species richness across Australia based on 'cleaned' collection records from the Australasian Virtual Herbarium (AVH 2022). B, Distribution of single populations of *Z. formosa*, *Z. parrisiae*, and *Z. buxijugum* (inset). Grid squares are 100 by 100 km. Records with geographic uncertainty greater than 1 km or with erroneous geographic coordinates were excluded. C, *Z. buxijugum*; D, *Z. formosa*; E, *Z. parrisiae*. Photographs: © Botanic Gardens of Sydney, attributed to C, A. Orme; D, L. Lee; and E, L. Elkan.

parrisiae is a potentially undescribed taxon from Cambewarra Mountain Range that was alluded to by Armstrong (2002: 368) in a footnote under the treatment of *Z. granulata*. Plants of this entity have been grown from cuttings and incorporated into the living collections at the Australian National Botanic Gardens (ANBG), where they have been referred to as '*Zieria aff. tuberculata*'. As *Z. aff. tuberculata* has not been included in any recent molecular phylogenetic studies on *Zieria*, its relationships to described species are unknown and its worthiness of taxonomic description is untested by genetic data.

The Critically Endangered status of *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae* means taxonomic classification of these species is significant in guiding conservation strategies and spending. Since 2001, the species have been jointly managed under the 'National Recovery Plan for *Zieria formosa*, *Zieria buxijugum*, and *Zieria parrisiae*' (NPWS 2002). One component of the management of the species is the maintenance of plants *ex situ* in living collections. *Ex situ* living collections act as safeguards to extinction for threatened species and help to preserve genetic diversity, which is the basis for evolutionary change and is intrinsically linked to the adaptability and fitness of populations (Booy *et al.* 2000). For this reason, maximizing genetic diversity in seed banks and *ex situ* living collections is a priority for the conservation of threatened species. Despite this, most *ex situ* collections do not sufficiently capture the genetic diversity of wild populations (Hoban *et al.* 2020). In particular, living plant collections may be limited by spatial and higher maintenance requirements relative to seed collections. An evaluation of genetic diversity in wild populations and *ex situ* living collections of *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae* would provide valuable insight into the current adequacy of *ex situ* plant collections in terms of their representation of the overall genetic diversity of each species.

The current study sought to investigate genetic relationships and genetic variation in *Z. buxijugum*, *Z. formosa*, *Z. parrisiae*, and related species using genomic data generated by double-digest restriction-site associated DNA sequencing (ddRADseq). Our primary aims were to: (i) test the current classification of *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae*; (ii) investigate genetic relationships between *Z. aff. tuberculata* and other species thought to be closely related; and (iii) provide insight into the genetic diversity of wild populations and *ex situ* living collections at the ANBG.

MATERIAL AND METHODS

Study system

Zieria Sm. is a genus of 63 described species of shrubs or small trees in the family Rutaceae that, except for one species (*Z. chevalieri* Virot; New Caledonia), is endemic to eastern Australia. In Australia, the genus is most species-rich in NSW and the Wet Tropics of Queensland (Fig. 1). A large proportion of species in the genus are rare and threatened, and these are usually restricted to small geographic ranges, often growing on or around rocky outcrops and mountain peaks along eastern Australia's Great Dividing Range (Armstrong 2002, George *et al.* 2013, Duretto 2019, Forster 2020). Half of the species (17/34) that occur in New South Wales are listed in the state threatened species register, provided in the NSW Biodiversity Conservation Act 2016 (BCA 2016; see https://www.environment.nsw.gov.au/threatenedSpeciesApp/).

With the exception of two species (*Z. minutiflora* Domin and *Z. pilosa* Rudge), *Zieria* flowers are entomophilous and mostly pollinated by flies and beetles (Armstrong 2002). *Zieria granulata*, which possesses very similar flowers to those of *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae*, is pollinated mainly by flies and bees (Lopresti *et al.* 2023). *Zieria* seeds are elastically ejected from mature fruit to the ground and are secondarily dispersed by ants with the aid of elaiosomes (Armstrong 1991, 2002). Across *Zieria*, self-incompatibility appears to be plesiomorphic, with self-compatibility arising multiple times homoplasiously (Armstrong 2002). Multiple species have a mixed mating system, whereby individuals may exhibit both self-compatibility and self-incompatibility (Armstrong 2002), but the breeding systems of *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae* are unknown. Three cytotypes are known in *Zieria* (n = 18, 27, and 36), with n = 18 by far the most common, and x = 18 probably being the ancestral diploid cytotype (Stace *et al.* 1993, Armstrong 2002). Chromosome numbers of *Z. buxijugum*, *Z. formosa* and *Z. parrisiae* are unknown, although n = 18 in *Z. tuberculata* (Armstrong 2002). The three species display an overlap in flowering periods, with flowers recorded for *Z. buxijugum* in September, in *Z. formosa* from September to November and in *Z. parrisiae* from late September to early November (Armstrong 2002). Comprehensive surveys conducted in 2001 for the formulation of the species' joint recovery plan (NPWS 2002) listed 32 adult individuals (> 1 metre in height) of *Z. buxijugum* (305 total individuals of all ages), 38 adult individuals of *Z. formosa* (737 total individuals of all ages), and 36 adult individuals of *Z. parrisiae* (221 total individuals of all ages). Recent estimates of population numbers for *Z. buxjiugum* and *Z. formosa* report ~400 and 300 total individuals for each species, respectively, in 2021 (see https://www.environment.nsw.gov.au/threatenedspeciesapp/profile.aspx?id=10 851; https://www.environment.nsw.gov.au/threatenedspeciesapp/profile.aspx?id=10 855).

Sampling

In total, 94 samples were included in the study. For each of *Zieria buxijugum*, *Z. formosa*, and *Z. parrisiae*, we sampled 14 plants, including 10 newly collected wild individuals and four individuals, derived from different wild parent cuttings, established in the *ex situ* living collections at the ANBG. Samples from ANBG represented almost all of the *ex situ* genotypes for the species, with plants in the ANBG living collections being derived from a total of five different wild parents for *Z. buxijugum*, five different parents for *Z. formosa*, and six different parents for *Z. parrisiae* (T. Golson, pers. comm.). Collections from wild populations were also made for *Z. granulata* (seven individuals each from two populations, and three individuals from ANBG; 17 total) and *Z. tuberculata* (five individuals from one population, four each from two populations, and three from ANBG; 17 total); these taxa were included to allow comparison of genetic variation in the taxonomically questionable species (*Zieria buxijugum*, *Z. formosa*, and *Z. parrisiae*) with that seen in related but taxonomically distinct species. Four individuals (grown by vegetative propagation from different wild parent cuttings) of *Z. aff. tuberculata* were sampled from the ANBG Living Collection.

To supplement these collections, we sampled six species thought to be more distantly related to *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae* (based on Armstrong 2002 and Barrett *et al.* 2018); these additional species were *Z. eungellaensis* Duretto & P.I.Forst. (one individual), *Z. obcordata* (two individuals), *Z. verrucosa* (two individuals), *Z. collina* (two individuals), *Z. baeuerlenii* J.A.Armstr. (two individuals), and *Z. littoralis* J.A.Armstr. (five individuals). Five individuals were included from *Z. littoralis* to provide a comparative measure of intraspecific genetic variation in a species with a disjunct geographic distribution (*Z. littoralis* is disjunct between south-eastern NSW and eastern Tasmania). From each sampled wild population, one voucher was collected and lodged at the National Herbarium of NSW. Samples from plants in the ANBG living collections are represented by herbarium vouchers from the original parent plant. Details of all samples used in the study are provided in Appendix 1.

DNA extraction, library preparation, and sequencing

Total DNA was extracted from 25–100 mg of recently collected (< 6 months old) silica-dried leaf tissue following the modified CTAB protocol of McLay (2017) with a 2-hour CTAB incubation. Isolated DNA was suspended in 50 µL of dH_2O. DNA quality was assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific), and quantity was assessed using a Qubit 2.0 fluorometer (Thermo Fisher Scientific). To prepare isolations for library preparation, 15 µL of each sample was aliquoted to a well in a plate and made up to 20 µL with dH_2O. Three samples (*Zieria formosa* PAM75.1, *Z. parrisiae* PAM82.2, and *Z. buxijugum* Orme 1693.1) were made up to 25 µL with 10 µL of dH_2O for testing enzyme combinations. Samples were then sent to the Australian Genome Research Facility (Melbourne, Victoria, Australia) for double-digest restriction-site associated DNA (ddRAD) library preparation. Samples were quality-checked using QuantiFluor (Promega) and visualized on a Genomic DNA ScreenTape (Agilent Technologies). Libraries were prepared following Peterson *et al.* (2012). To assess enzyme combinations, restriction enzyme double-digests were conducted on a pool of the three samples submitted with a 25 µL volume, testing eight different restriction enzyme combinations that included a six-base cutter (*PstI* or *EcoRI*) and a four-base cutter (*MspI*, *MseI*, *NlaIII*, or *HpyCH4IV*). Following digestion, libraries were prepared without size selection and evaluated with gel electrophoresis using a TapeStation (Agilent Technologies) to select the enzyme combination with the best amplification and lowest likelihood of yielding repetitive sequences. From this, the enzymes *PstI* and *NlaIII* were determined to be the optimal combination. Libraries for all samples were then prepared using the *PstI-NlaIII* restriction enzyme combination for digestion and a 95 bp wide size selection (selecting fragments between 280 and 375 bp). Libraries were indexed, combined into two pools, and sequenced in single-end 150 bp runs across four lanes of a single flow cell on a NextSeq500 platform (Illumina).

Quality checking, data filtering, and data assembly

Raw sequence reads were quality-checked using FastQC v.0.11.9 (Andrews 2010). Sequences were demultiplexed, trimmed, and assembled into loci using the ipyrad pipeline v.0.9.81 (Eaton and Overcast 2020). Optimal parameter settings for ipyrad were determined by running a subset of 24 samples (including all sampled species) through the pipeline under three clustering threshold values (clust_threshold: 0.85, 0.90, and 0.95). All other parameters were set to default except for the following: filter_adapters = '2', datatype = 'ddrad', restriction_overhang = 'TGCAG'. The optimization runs identified 0.90 as the best value for the clust_threshold parameter, as it provided the highest number of recovered loci across all samples; the 0.95 value for this parameter recovered more loci from ingroup samples but fewer loci from outgroups. Following clustering optimization, the full set of samples was run through ipyrad with the same settings, a clust_threshold value of 0.90, and five values for the 'min_samples_locus' parameter [4 (ipyrad default; ~4% of samples required to have data at a given locus for the locus to be retained), 24 (~25%), 48 (~50%), 72 (~75%), 94 (100%)] to produce five datasets varying in the minimum number of samples present per locus in each dataset (Table 1). From these, the ~75% dataset was selected for use in network and phylogenetic analyses as it was considered to strike the best balance between containing a relatively low amount of missing data and not overbiasing conserved regions. Similar filtering thresholds have been identified as optimal in other studies (e.g. Crotti *et al.* 2019, Fahey *et al.* 2021).

Following the network and phylogenetic analyses, we separately ran three subsets of samples, varying in closeness to *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae*, through ipyrad to investigate possible hierarchical structuring and obtain datasets with improved locus recovery and more data for more accurately estimating summary statistics and genetic structure in taxa of interest. The three subsets comprised: a distant ingroups subset (including all seven taxa represented by ≥ 4 samples: *Z. tuberculata*, *Z. littoralis*, *Z. aff. tuberculata*, *Z. granulata*, *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae*), a close ingroups subset (including five taxa that formed a clear clade in our network and phylogenetic analyses: *Z. aff. tuberculata*, *Z. granulata*, *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae*) and a target species set (including only the three Critically Endangered species: *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae*) (Table 2). These subsets were run through step 7 of ipyrad with the same settings as the full sample set (i.e. min_samples_locus set to ~75%). Parameter settings for ipyrad runs that produced datasets used in downstream analyses are detailed in Supplementary File S1 in the Supporting Information.

Network analyses

To investigate relationships and reticulation among our samples, we generated phylogenetic networks from the concatenated supermatrix alignment (i.e. the '.phy' file) and unlinked single-nucleotide polymorphism (SNP) matrix (i.e. the '.usnps' file). Networks were constructed in SplitsTree4 v.4.17.1 (Huson and Bryant 2006) using default settings; implementing Uncorrected P/Hamming Distances (Hamming 1950) to obtain distance matrices, NeighborNet (Bryant and Moulton 2004) to obtain splits, and the Equal Angle Convex Hull algorithm (Dress and Huson 2004) to obtain splits networks.

Phylogenetic tree reconstruction

Phylogenetic relationships were inferred from the concatenated supermatrix alignment using maximum likelihood (ML) in the

software IQ-TREE v.2.1.3 (Minh *et al.* 2020), employing ultrafast bootstrap (UFB) (Hoang *et al.* 2018) and SH-aLRT (Guindon *et al.* 2010) with 1000 replicates to estimate branch support. Branches with UFB support ≥ 95% and SH-aLRT ≥ 80% are considered robust (Minh *et al.* 2013, Schmidt *et al.* 2022). As bootstrap-like support measures are biased by dataset size, we calculated site concordance (sCF, the percentage of decisive alignment sites supporting a branch in the species tree) using the '--scfl' option in IQ-TREE v.2.2.2 (Mo *et al.* 2022) as an additional measure of branch support. Best-fit models for the data were estimated in IQ-TREE using ModelFinder (Kalyaanamoorthy *et al.* 2017).

We also reconstructed phylogenetic relationships using a site-based coalescent approach. For this, we used SVDquartets (Chifman and Kubatko 2014, 2015), in PAUP* v.4.0a169 (Swofford 2003) to infer a species tree from the unlinked SNP matrix, treating each sample as a terminal (i.e. no species assignments). All quartets (= 3 049 501) were evaluated employing Quartet FM (Reaz *et al.* 2014) for quartet assembly, and node support was inferred with 100 nonparametric bootstrap (NPB) replicates.

Measures of genetic diversity

To assess genetic variation within species and between species, we calculated several standard metrics used to assess genetic variation from SNP data: interspecies and interpopulation pairwise F_{ST}, inbreeding (F_{IS}), and observed and expected heterozygosity (H_o and H_e). These genetic diversity measures are accurate for small sample sizes (e.g. 5–10 individuals per population) when analysed using large SNP datasets (Nazareno *et al.* 2017, Schmidt *et al.* 2021). To calculate these measures the '.usnps' file output by the ipyrad run of the distant ingroups subset was used as input. This file consisted of a matrix of 8899 unlinked SNPs that included only one SNP from each locus. F_{ST} estimates are generally robust to missing data (Arnold *et al.* 2013, Shafer *et al.* 2017), while estimates of inbreeding and heterozygosity are sensitive to missing data (Shafer *et al.* 2017, Schmidt *et al.* 2021). Because of this, all sites with missing data were removed to produce a dataset of 2409 SNPs from which F_{ST}, F_{IS}, H_o, and H_e were calculated. The R packages adegenet (Jombart 2008, Jombart and Ahmed 2011) and hierfstat (Goudet 2005) were used to calculate mean pairwise F_{ST} values according to Nei (1987) with 95% confidence intervals based on 100 bootstrap replicates. F_{ST} values were estimated for each species pair, and subsequently again for populations within species using the 'genet.dist' function in *hierfstat*, employing the method of Nei (1987). Because different distance measures may produce different results (Takezaki and Nei 1996, Georges *et al.* 2023), we also calculated chord distances, using the same function in hierfstat, following the method of Cavalli-Sforza and Edwards (1967). SNP H_o and H_e were obtained from the summary of an adegenet genind object, and F_{IS} was calculated using the 'basic.stats' function in hierfstat.

As heterozygosity estimates based on SNP datasets are biased by sample size, we also calculated autosomal heterozygosity (*sensu* Schmidt *et al.* 2021; i.e. including both polymorphic and monomorphic sites) from the 1 310 922 bp long concatenated supermatrix alignment of the distant ingroups subset. Following the recommendation of Schmidt *et al.* (2021), sites with missing data were removed to produce a final matrix 183 813 bp long. Autosomal H_o and H_e were obtained from the summary of an adegenet genind object.

To provide a summary comparison of genetic diversity in wild populations versus that held in *ex situ* living collections at the ANBG, the R package pofadinr was used to calculate distance matrices between sampled individuals in each of the three Critically Endangered species, *Z. buxijugum*, *Z. formosai*, and *Z. parrisiae*. For this, the 2409 SNP dataset was used as input and distances were calculated using the genpofad model (Joly *et al.* 2015). To supplement this comparison using an alternate approach, we performed principal component analyses (PCAs) for each species using the dudi.pca function in ade4 from the same dataset (Dray and Dufour 2007).

Assessing genetic structure

We used a hierarchical approach (Vähä *et al.* 2007) to evaluate genetic structure and admixture in our data. To do so, the 'close ingroups' and 'target species' datasets were assessed using the Bayesian Markov Chain Monte Carlo (MCMC) method implemented in STRUCTURE v.2.3.4 (Pritchard *et al.* 2000) via the ipyrad-analysis toolkit (structure.py script). For each subset, the '.snps.hdf5' file output by ipyrad was used as input and sites with missing data were excluded. Analyses were run with default ipyrad-analysis toolkit settings; sampling unlinked SNPs represented in at least 75% of samples (mincov = 0.75), and assuming correlated allele frequencies (FREQSCORR = 0, with FPRIORMEAN and FPRIORSD 0.01 and 0.05, respectively), admixture (NOADMIX = 0), and no prior population information (USEPOPINFO = 0). To analyse each subset, we conducted 10 runs for each value of K = 1–7 (for the 'close ingroups' subset) and K = 1–5 (for the target species subset) using 200 000 MCMC cycles with a burn-in of 20 000 cycles. Initially, the optimal value of K for each subset was evaluated using a standard approach in Structure Harvester v.0.6.94 (Earl and vonHoldt 2012). For both datasets, Structure Harvester identified K = 2 as optimal based on deltaK. As deltaK is sensitive to runs that have not converged (Evanno *et al.* 2005) and is biased towards K = 2 (Janes *et al.* 2017), we used python and ipyrad functions to filter out runs that had not converged (see Supplementary File S2 in the Supporting Information for a description of these methods). Following filtering of unconverged runs, the remaining STRUCTURE runs were combined using CLUMPP v.1.1.2 (Jakobsson and Rosenberg 2007) via the 'get_clumpp_table' function of ipyrad.

Genetic structure was also assessed using TESS3 (Caye *et al.* 2016) in the R package tess3r (Caye *et al.* 2018), a program that implements a spatially explicit Bayesian clustering model with admixture. Bayesian clustering models that take geographic information into account better capture the relationship between genetic barriers and spatially structured variables, and can provide more accurate results than models without spatial information (Guillot *et al.* 2009, François and Durand 2010). The '.ugeno' file from the target species subset was used as input for TESS3 analysis in tess3r. Cultivated collections were excluded if they did not possess geographic information, and SNPs with > 75% missing data were excluded to produce a matrix of 7823 SNPs. TESS3 was run 10 times using the alternating projected least squares algorithm for each value of K = 1–10, treating all samples

as diploids. The most likely value for *K* was selected based on interpretation of the cross-validation plot, where the best choice for *K* corresponds to a plateau or increase in the curve (Frichot and François 2015, Caye *et al.* 2016).

RESULTS

An average of 1 247 662 (σ = 393 165) reads per sample passed ipyrad read filtering. Ipyrad output statistics for the full sample dataset filtered under five thresholds for minimum percentage of samples required per locus (~4%, 25%, 50%, 75%, 100%) are provided in Table 1. Ipyrad output statistics for the three subsets of samples filtered with ~75% of samples required per locus are provided in Table 2.

Phylogenetic analyses

Comparison of the two NeighbourNet networks revealed that the network produced from the concatenated supermatrix alignment was slightly less well-resolved than that produced from the SNP matrix, although the topologies of the two networks were congruent; only the network constructed from the unlinked SNP dataset is presented here (Fig. 2). Of the approaches used to reconstruct phylogenetic trees, the ML analysis produced a well-resolved and highly supported phylogenetic tree (Fig. 3A), while the tree produced by the SVDquartets analysis was less well supported (Fig. 3B). The relationships recovered by all the phylogenetic methods that we employed (networks and trees) were congruent with one another. Across all analyses, *Zieria eungellaensis* and *Z. obcordata* were found to be highly genetically divergent from all other sampled species; these species were treated as outgroups in the phylogenetic trees. All species were resolved as monophyletic, with no recent or ongoing reticulation observed between the focus species of this study (Figs 2, 3; following support for the monophyly of all species, tips were subsequently grouped into species post-analysis to aid visualization in Fig. 3B). *Zieria formosa*, *Z. buxijugum*, and *Z. parrisiae*

Table 1. Output statistics for ipyrad runs of the full set of samples under five tested 'min_samples_locus' values. Numbers in brackets denote the percentage of samples required to have data for a locus to be retained. The USNPs matrix (unlinked SNPs) contains one SNP sampled from each locus; for loci with multiple SNPs, sites with the least missing data are sampled; if amounts of missing data are equal then sites are sampled randomly. A value of ~75% was subsequently employed in the generation of all datasets used for downstream analyses.

	Minimum samples required for locus to pass filtering				
	4 (~4%)	**24 (~25%)**	**48 (~50%)**	**72 (~75%)**	**94 (100%)**
Total filtered loci (retained)	40 915	17 839	11, 019	8655	1558
Total variable sites (SNPs)	239 329	141 243	103 778	83 537	13 468
Total parsimony informative sites (PIS)	126 223	83 809	61, 108	48 669	7662
Concatenated supermatrix (bp)	5 559 297	2 532 697	1 568 772	1 232 398	221 176
SNPs matrix (bp)	239 329	141 243	103 778	83 537	13 468
USNPs matrix (bp)	33 481	17 115	10 866	8591	1549
Percentages of missing data (USNPs matrix, SNPs matrix, Supermatrix)	1.4, 53.81, 64.62	2.1, 28.37, 33.99	2.6, 14.68, 14.67	2.7, 8.54, 7.70	1.5, 2.14, 2.43

Table 2. Output statistics for ipyrad runs conducted on three different subsets of species. The 'Distant Ingroups' dataset was used for estimating F_{ST}, F_{IS}, H_o, and He. The 'Close Ingroups' and 'Target Species' datasets were used to assess genetic structure in STRUCTURE and TESS3. The USNPs matrix (unlinked SNPs) contains one SNP sampled from each locus; for loci with multiple SNPs, sites with the least missing data are sampled; if amounts of missing data are equal then sites are sampled randomly.

Subset	**Distant ingroups**	**Close ingroups**	**Target species**
Species included	*Z. tuberculata* *Z. littoralis* *Z. granulata* *Z. aff. tuberculata* *Z. parrisiae* *Z. buxijugum* *Z. formosa*	*Z. granulata* *Z. aff. tuberculata* *Z. parrisiae* *Z. buxijugum* *Z. formosa*	*Z. parrisiae* *Z. buxijugum* *Z. formosa*
Total filtered loci (retained)	9235	9614	9943
Total variable sites (SNPs)	49 934	37 623	25 829
Total Parsimony informative sites (PIS)	38 725	31 860	21 924
Concatenated supermatrix (bp)	1 310 922	1 362 560	1 406 418
SNPs matrix (bp)	49 934	37 623	25 829
USNPs matrix (bp)	8899	8806	7995
Percentage of missing data (USNPs, SNPs, Supermatrix)	3.2, 9.67, 6.79	3.6, 9.61, 6.04	3.6, 9.17, 5.05

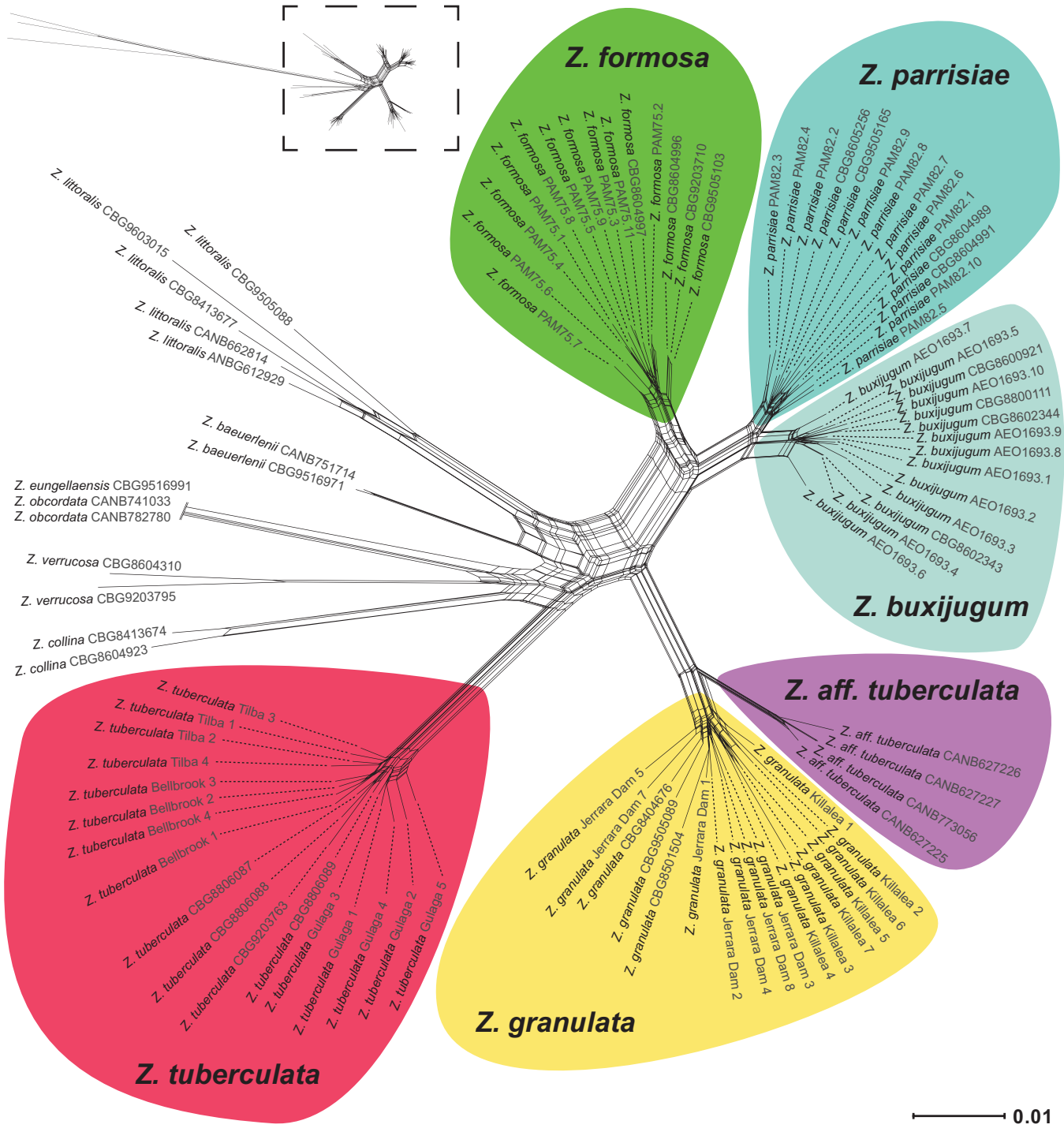

Figure 2. SplitsTree network constructed from the alignment of 8591 unlinked SNPs. Taxa of major focus are labelled and represented by coloured clouds. The main figure includes trimmed branches for *Z. eungellaensis* and *Z. obcordata*. The relative scale for these branches is evident in the original network, inset at the top left. Samples taken from the living collection at the ANBG are labelled according to their plant number in that collection (accessible at https://www.anbg.gov.au/cgi-bin/stock). Samples collected from multiple wild populations are labelled according to population and plant number, and samples collected from only one wild population (i.e. *Z. formosa*, *Z. parrisiae*, and *Z. buxijugum*) are labelled by collector number.

were found to constitute a distinct clade, in which *Z. formosa* was resolved as sister to the remaining two species. *Zieria granulata* and *Z. aff. tuberculata* formed a well-defined group, with both taxa resolved as distinct. Together, these two taxa were resolved sister to the clade of *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae*, with high support in both phylogenetic trees (Fig. 3A: UFB: 100%, SH-aLRT: 100%, sCF: 41%, supported by ~1652 of 4030 total decisive sites [sCF_N/sDF1_N/sDF2_N/sN = 16 52.63/1301.48/1076.67/4030.78]; Fig 3B: NPB: 98%). Within *Z. granulata*, samples from the two wild populations, located at

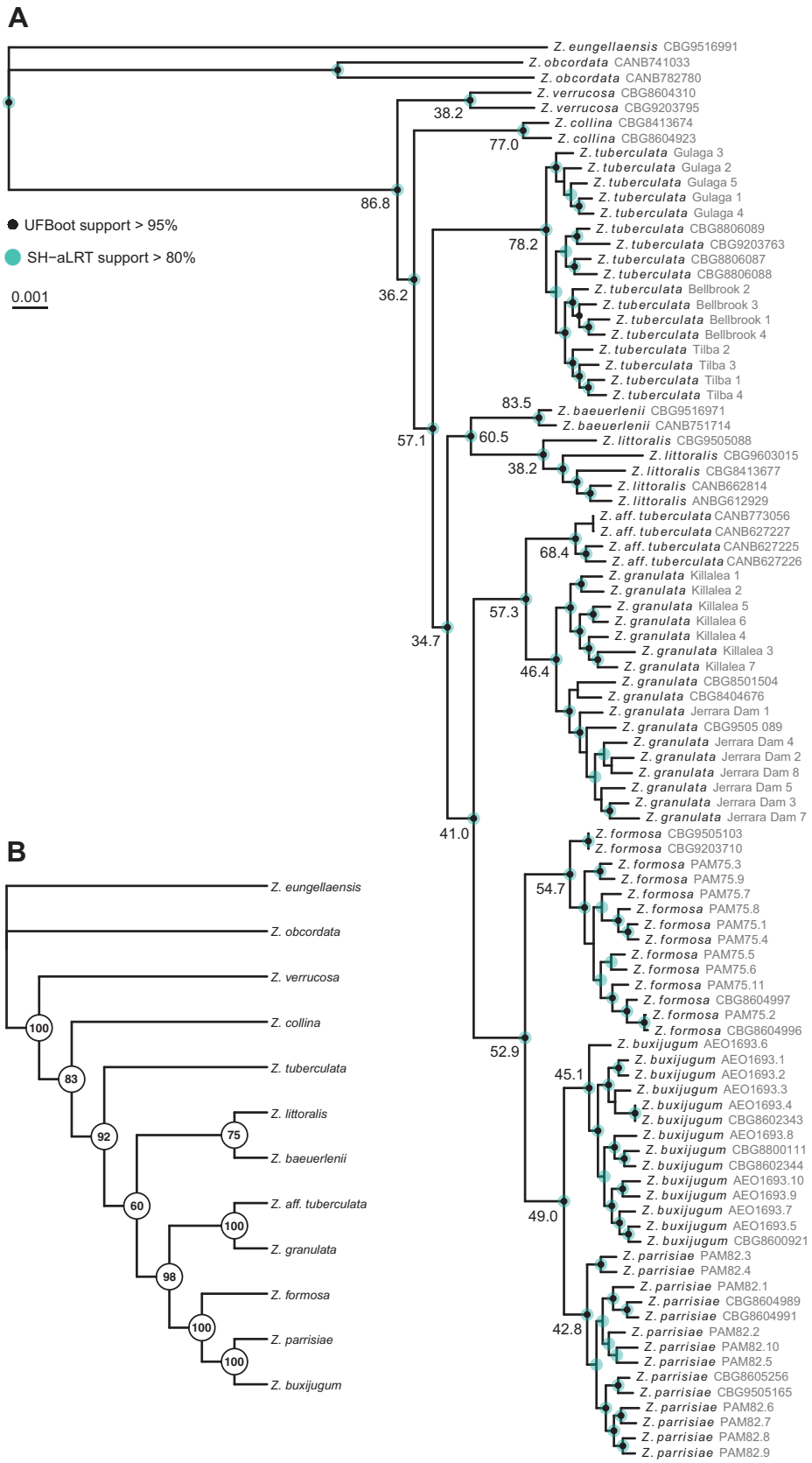

Figure 3. Trees produced from phylogenetic analyses. A, IQ-TREE phylogenetic tree generated from ML analysis of the 1 232 398 bp concatenated supermatrix alignment. Nodes with high ultrafast bootstrap (UFBoot) support (> 95%) are denoted with a small black circle. Nodes with high SH-aLRT support (> 80%) are denoted with larger light blue, semi-transparent circles. Site concordance factors (sCF %) are labelled near nodes for clades at species rank and above. Samples taken from the living collection at the ANBG are labelled according to their plant number in that collection (accessible at https://www.anbg.gov.au/cgi-bin/stock). Samples collected from multiple wild populations

Jerrara Dam and Killalea, formed two well-supported groups in the ML tree (Fig. 3A), however, the boundary between these populations was unclear in the phylogenetic network (Fig. 2). Among the hypothesized distantly related taxa (to *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae*), both phylogenetic trees resolved a grade of successive divergences of three species (in order): *Z. verrucosa*, *Z. collina*, and *Z. tuberculata*, followed by the divergence of a clade containing *Z. baeuerlenii* and *Z. littoralis*, sister to the clade of *Z. buxijugum*, *Z. formosa, Z. parrisiae, Z. granulata*, and *Z. aff. tuberculata* (Fig. 3). These backbone relationships were well supported in the ML tree (Fig. 3A), but not the SVDquartets tree (Fig. 3B). The relationships of *Z. tuberculata*, *Z. littoralis*, *Z. baeuerlenii*, *Z. verrucosa*, and *Z. collina* were not clear in the phylogenetic network (Fig. 2).

Genetic diversity

Across species, autosomal H_e ranged from 0.0021 to 0.0028 and autosomal H_o ranged from 0.0017 to 0.0026 (Table 3). Species SNP H_e ranged from 0.070 to 0.096 and SNP H_o ranged from 0.053 to 0.089 (Table 3). The four species that are restricted to small, single populations (*Z. aff. tuberculata*, *Z. formosa*, *Z. parrisiae*, *Z. buxijugum*) displayed H_o estimates consistently higher than H_e. *Zieria granulata* and *Z. tuberculata* both displayed marginally lower H_o than H_e. In *Z. littoralis*, H_o was considerably lower than H_e for both the autosomal and SNP heterozygosity estimates. Species F_{IS} estimates (Table 3) ranged from −0.069 to 0.488, with *Z. littoralis* having a very high F_{IS} value (F_{IS} = 0.488) that was ~4.5 times higher than the next highest F_{IS} estimate (*Z. granulata*; F_{IS} = 0.109). In general, comparisons between autosomal H_o and H_e followed the same patterns as SNP H_o and H_e except for *Z. buxijugum*, which displayed autosomal H_o higher than H_e but SNP H_e higher than H_o. Averaged across all species, SNP heterozygosity estimates were ~33 times higher than autosomal heterozygosity estimates (for both H_e and H_o).

Interspecies pairwise F_{ST} values were high (mean 0.484, standard deviation 0.112), with all mean F_{ST} values > 0.1 and no confidence intervals overlapping zero (Table 3). The lowest pairwise interspecies average F_{ST} occurred between *Z. buxijugum* and *Z. parrisiae* (0.166), with the next lowest average value occurring between *Z. aff. tuberculata* and *Z. granulata* (0.284). *Zieria tuberculata* and *Z. littoralis* displayed average F_{ST} estimates above the total mean F_{ST} for all pairs. To estimate pairwise F_{ST} for populations of *Z. granulata* and *Z. tuberculata*, these species were grouped into three and four populations, respectively [*Z. granulata*: two wild populations from Jerrara Dam and Killalea, and one population from the ANBG Living Collection (including three individuals from different wild populations); *Z. tuberculata*: three wild populations from Bellbrook, Gulaga and Tilba, and one population from the ANBG Living Collection (including four individuals from the same wild population)]. Average F_{ST} estimates between all populations of these two species were low (Table 4; all ≤ 0.095), and no confidence intervals overlapped zero. Interspecies F_{ST} values were higher than interpopulation F_{ST} values for species that were represented by more than one population (i.e. *Z. tuberculata* and *Z. granulata*). For both the interspecies and interpopulation groups, patterns of genetic distance were congruent between F_{ST} and Cavalli-Sforza and Edwards' chord distance (Supplementary File 3 in the Supporting Information).

Summary comparisons of genetic diversity in wild versus *ex situ Z. formosa*, *Z. parrisiae*, and *Z. buxijugum* using genpofad distances and PCAs (Fig. 4) indicated that in *Z. buxijugum* overall genetic diversity is reasonably well represented by *ex situ* collections. In *Z. formosa* and *Z. parrisiae*, the genetic diversity maintained in *ex situ* collections provides a less comprehensive representation of the diversity in wild populations.

Genetic structure

STRUCTURE analyses

After filtering, the ipyrad-analysis toolkit retained 8215 unlinked SNPs from the close ingroups dataset that were used for STRUCTURE analysis. Based on deltaK, the best number of genetic groups (K), as identified by Structure Harvester, was $K = 2$ ($\Delta k = 46.185$). After filtering out unconverged runs, mean ln P(K) values were highest for $K = 5$ (−221 026.91) and deltaK was highest for $K = 2$ (46.185) (see Supplementary Files 4, 5 in the Supporting Information for further information on K selection for the close ingroups dataset). Under the $K = 2$ scheme (Fig. 5A) for the close ingroups dataset, species were grouped according to the deepest phylogenetic split in the sample set (Fig. 3), namely the split of *Z. granulata*/*Z. aff. tuberculata* from *Z. formosa*/*Z. buxijugum*/*Z. parrisiae*. Subsequent values for K (3 and 4) retrieved two additional genetic groups corresponding to *Z. formosa* and *Z. aff. tuberculata*, however, K values 5–7 failed to distinguish *Z. buxijugum* and *Z. parrisiae* (Fig. 5A).

For the target species dataset, the ipyrad-analysis toolkit retained 7563 unlinked SNPs that were used for STRUCTURE analysis. $K = 2$ received the highest mean deltaK and ln P(K) scores from Structure Harvester ($\Delta k = 2939.204$, ln P(K) −189 826.710). After filtering out unconverged runs, mean ln P(K) values were highest for $K = 3$ (−176 619.0) and deltaK was highest for $K = 4$ (80.621) (see Supplementary Files S6, S7 in the Supporting Information for further information on K selection for the target species dataset). Under $K = 2$ for the target species dataset, *Z. formosa* was clearly separated from *Z. buxijugum* and *Z. parrisiae* (Fig. 5B). Under the $K = 3$ scheme, *Z. buxijugum* and *Z. parrisiae* formed clearly identifiable clusters, with *Z. formosa* remaining distinct from both clusters. For K values of 4 and 5, no additional discernible clusters were apparent.

are labelled according to population and plant number, and samples collected from only one wild population (i.e. *Z. formosa*, *Z. parrisiae*, and *Z. buxijugum*) are labelled by collector number. Scale bar corresponds to the number of substitutions per site. B, Summary cladogram of SVDquartets bootstrap consensus tree generated from the alignment of 8591 unlinked SNPs. For the analysis, samples were not assigned to species groups (i.e. each sample was treated as a terminal); following high support for the monophyly of all species, tips were subsequently grouped into species post-analysis to aid visualization in this cladogram. Support for nodes is quantified by bootstrap values.

Table 3. Species mean genetic diversity (expected and observed heterozygosity, H_e and H_o) and inbreeding (F_{IS}) measures, with interspecies pairwise F_{ST} estimates. F_{ST} values below the diagonal are mean values and values above the diagonal are 95% confidence intervals based on 100 bootstrap replicates. Cells with mean pairwise F_{ST} values are coloured according to the dataset mean (0.484), where darker red indicates values above the mean and darker blue indicates values below the mean.

						Interspecies pairwise F_{ST}						
Species	**Autosomal H_e**	**Autosomal H_o**	**SNP H_e**	**SNP H_o**	**F_{IS}**	***Z. aff. tuberculata***	***Z. buxijugum***	***Z. formosa***	***Z. granulata***	***Z. littoralis***	***Z. parrisiae***	***Z. tuberculata***
Z. aff. tuberculata	0.0021	0.0026	0.070	0.085	-0.069		0.488–0.532	0.496–0.545	0.239–0.294	0.536–0.579	0.495–0.542	0.567–0.617
Z. buxijugum	0.0023	0.0024	0.081	0.081	0.038	0.515		0.31–0.358	0.43–0.477	0.512–0.554	0.146–0.183	0.544–0.585
Z. formosa	0.0024	0.0025	0.079	0.082	0.010	0.521	0.336		0.441–0.489	0.498–0.543	0.311–0.359	0.56–0.601
Z. granulata	0.0028	0.0026	0.096	0.089	0.109	0.284	0.461	0.467		0.511–0.55	0.439–0.477	0.524–0.56
Z. littoralis	0.0028	0.0017	0.088	0.053	0.488	0.563	0.519	0.507	0.528		0.538–0.578	0.577–0.617
Z. parrisiae	0.0024	0.0024	0.078	0.080	0.014	0.519	0.166	0.334	0.461	0.544		0.558–0.596
Z. tuberculata	0.0023	0.0022	0.074	0.070	0.088	0.591	0.567	0.578	0.543	0.576	0.580	

TESS3 analysis

After removing cultivated specimens and SNPs with > 75% missing data, a total of 7823 unlinked SNPs were analysed using TESS3. The cross-validation plot identified $K = 3$ as the best choice for K (Supplementary File S8 in the Supporting Information). For this value of *K*, each of *Z. formosa*, *Z. parrisiae*, and *Z. buxijugum* formed clear clusters (Fig. 6A). The ancestry coefficient map output by TESS3 is presented, superimposed onto topographic and hydrological features, as Figure 6B.

DISCUSSION

Our analyses of ddRADseq data support the continued taxonomic recognition and separate conservation management of the three Critically Endangered, closely related, and geographically proximal *Zieria* species (Fig. 1), and suggest that *Z. aff. tuberculata* is a species new to science. In particular, our analyses indicate that *Z. buxijugum*, *Z. formosa* and *Z. parrisiae* are monophyletic and are distinguished from each other by levels of differentiation in line with other closely related species, with no reticulation and negligible admixture between species.

Resolution of phylogenetic relationships

Our phylogenetic analyses have provided further insights into relationships among the sampled *Zieria* species. In line with results from previous genetic studies (Barrett *et al.* 2014, 2018), we confirm a close relationship between *Z. formosa*, *Z. parrisiae*, and *Z. buxijugum*, that together form a clade. In addition, our results have clarified the order of relationships between the species, with *Z. formosa* found to be sister to *Z. parrisiae* and *Z. buxijugum*. More broadly, the recovery of a sister relationship between the clade of these three species and the clade of *Z. granulata* and *Z. aff. tuberculata* improves on the resolution of Barrett *et al.* (2018) and clarifies the nearest relations to *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae*. In our analyses, the distant position of *Z. tuberculata* to the *Z. formosa*–*Z. granulata* clade contrasts with Barrett *et al.* (2018), who resolved that species firmly in a clade with *Z. buxijugum*, *Z. formosa*, *Z. parrisiae*, and *Z. granulata*. However, our result is consistent with the morphology-based phylogenetic tree of Armstrong (2002), in which *Z. tuberculata* was placed outside the *Z. formosa*–*Z. granulata* clade. By including samples of *Z. aff. tuberculata* in our analyses, we have been able to provide the first phylogenetic placement for that putative taxon. The position of *Z. aff. tuberculata*, sister to *Z. granulata*, indicates that the previously hypothesized morphological affinity of that taxon to *Z. tuberculata* does not reflect its true relationships.

Species delimitation

When making decisions about species delimitation it is important to consider multiple lines of evidence (Schlick-Steiner *et al.* 2010, Carstens *et al.* 2013, Coates *et al.* 2018, Stanton *et al.* 2019). Up to this point, evidence for the taxonomic recognition of *Zieria buxijugum*, *Z. formosa*, and *Z. parrisiae* has come entirely from morphology (Armstrong 2002). Our analyses provide additional lines of evidence that confirm that, while the species are closely related, each is a monophyletic group with no evidence of ongoing reticulate evolution between terminal groups in the phylogenetic network, and negligible admixture in STRUCTURE.

Table 4. Interpopulation pairwise F_{ST} estimates. F_{ST} values below the diagonal are mean values and values above the diagonal are 95% confidence intervals based on 100 bootstrap replicates. Cells with mean pairwise F_{ST} values are coloured according to the dataset mean (0.461), where darker red indicates values above the mean and darker blue indicates values below the mean.

Population	Interpopulation pairwise F_{ST}											
	Z. aff. tuberculata	*Z. buxijugum*	*Z. formosa*	*Z. granulata* Jerrara Dam	*Z. granulata* Killalea	*Z. granulata* ANBG	*Z. littoralis*	*Z. parrisiae*	*Z. tuberculata* Bellbrook	*Z. tuberculata* Gulaga	*Z. tuberculata* Tilba	*Z. tuberculata* ANBG
Z. aff. tuberculata		0.484–0.535	0.492–0.542	0.297–0.344	0.269–0.312	0.269–0.315	0.535–0.585	0.498–0.541	0.578–0.620	0.592–0.634	0.568–0.616	0.579–0.629
Z. buxijugum	0.515		0.314–0.356	0.470–0.515	0.454–0.497	0.460–0.508	0.513–0.555	0.146–0.185	0.541–0.593	0.550–0.595	0.532–0.582	0.545–0.594
Z. formosa	0.521	0.336		0.472–0.525	0.451–0.504	0.462–0.516	0.501–0.550	0.309–0.354	0.552–0.603	0.558–0.612	0.540–0.595	0.550–0.605
Z. granulata Jerrara Dam	0.326	0.487	0.495		0.084–0.107	0.012–0.043	0.528–0.572	0.465–0.514	0.537–0.587	0.550–0.600	0.531–0.580	0.537–0.590
Z. granulata Killalea	0.301	0.469	0.475	0.095		0.044–0.075	0.515–0.558	0.453–0.498	0.521–0.568	0.538–0.586	0.512–0.561	0.525–0.574
Z. granulata ANBG	0.289	0.469	0.476	0.024	0.058		0.487–0.541	0.456–0.510	0.529–0.582	0.552–0.602	0.520–0.576	0.532–0.586
Z. littoralis	0.563	0.519	0.507	0.544	0.535	0.518		0.538–0.587	0.547–0.589	0.563–0.606	0.538–0.585	0.548–0.590
Z. parrisiae	0.519	0.166	0.334	0.487	0.469	0.471	0.544		0.560–0.608	0.566–0.610	0.549–0.598	0.561–0.611
Z. tuberculata Bellbrook	0.599	0.573	0.585	0.568	0.557	0.551	0.574	0.587		0.055–0.092	0.012–0.055	0.033–0.073
Z. tuberculata Gulaga	0.614	0.587	0.599	0.584	0.573	0.571	0.588	0.601	0.073		0.072–0.117	0.075–0.116
Z. tuberculata Tilba	0.593	0.564	0.577	0.563	0.549	0.546	0.569	0.578	0.032	0.094		0.041–0.081
Z. tuberculata ANBG	0.603	0.578	0.590	0.574	0.562	0.557	0.576	0.592	0.050	0.094	0.062	

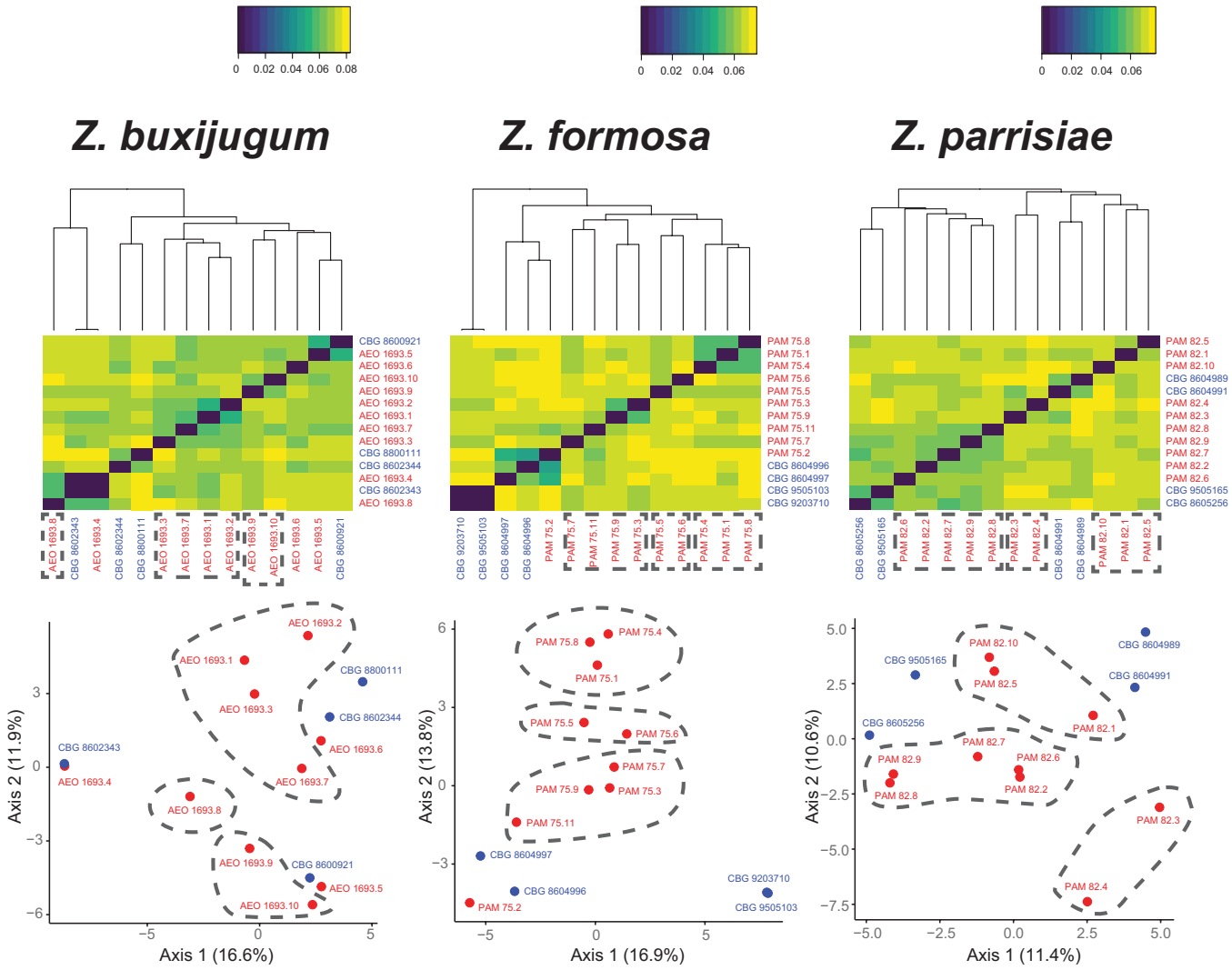

Figure 4. Heatmaps with dendrograms and PCAs for individual samples of *Zieria buxijugum*, *Z. formosa*, and *Z. parrisiae,* based on pairwise genpofad distances produced from the 2409 SNP dataset. Colour scales denote genetic distance, where lower values indicate lower pairwise distances. Dendrograms reflect relationships based on hierarchical cluster analysis of pairwise distances (R; 'stats::hclust', with default settings). Individuals in red are from wild populations, while individuals in blue are from *ex situ* collections at the Australian National Botanic Garden. Wild genotypes suggested for future sampling for *ex situ* cultivation are indicated by dashed grey polygons.

Our data appear to be hierarchically structured, with the identification of $K = 2$ as the optimal number of clusters by Structure Harvester for both STRUCTURE analyses simply reflecting the deepest phylogenetic split in each group. The tendency of deltaK to preferentially identify the uppermost level of genetic structure, particularly for hierarchically structured data, is a well-documented shortcoming of this method that has led to the suggestion that it should not be relied on too heavily (Evanno *et al.* 2005, Janes *et al.* 2017, Cullingham *et al.* 2020). Our investigation of support for various K values under different filtering thresholds highlights the sensitivity of deltaK under hierarchical structuring; for both STRUCTURE analyses we found that the optimal K-value identified by ln P(K) was congruent with the a priori number of taxa included in the analysis. For the target species dataset, the three clusters identified by STRUCTURE under $K = 3$ (Fig. 5B) perfectly align with the current delimitation of *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae*, and are probably the most accurate representation of genetic structure in these three species. Failure to detect any additional meaningful structure under $K = 4$ and $K = 5$ (Fig. 5B), along with identification of $K = 3$ as the optimal cluster number for *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae* in our TESS3 analysis (Fig. 6), provides further support for $K = 3$ as the best fit for the target species dataset. This result also indicates that TESS3, which incorporates geographic information on the species' discrete distributions, is potentially better able to detect nested clusters in situations where hierarchical structure would lead non-spatially explicit approaches to suggest $K = 2$. Previously, TESS has been shown to outperform STRUCTURE under lower levels of ancestral differentiation between populations (François and Durand 2010), and recent studies have found underestimation of K by STRUCTURE compared to TESS in hierarchically structured data (e.g. Thompson *et al.* 2019, Henson *et al.* 2022).

Pairwise F_{ST} comparisons between species (Table 3) suggested that, of all species considered, *Z. buxijugum* and *Z. parrisiae* are the most genetically similar (mean pairwise $F_{ST} = 0.166$). That

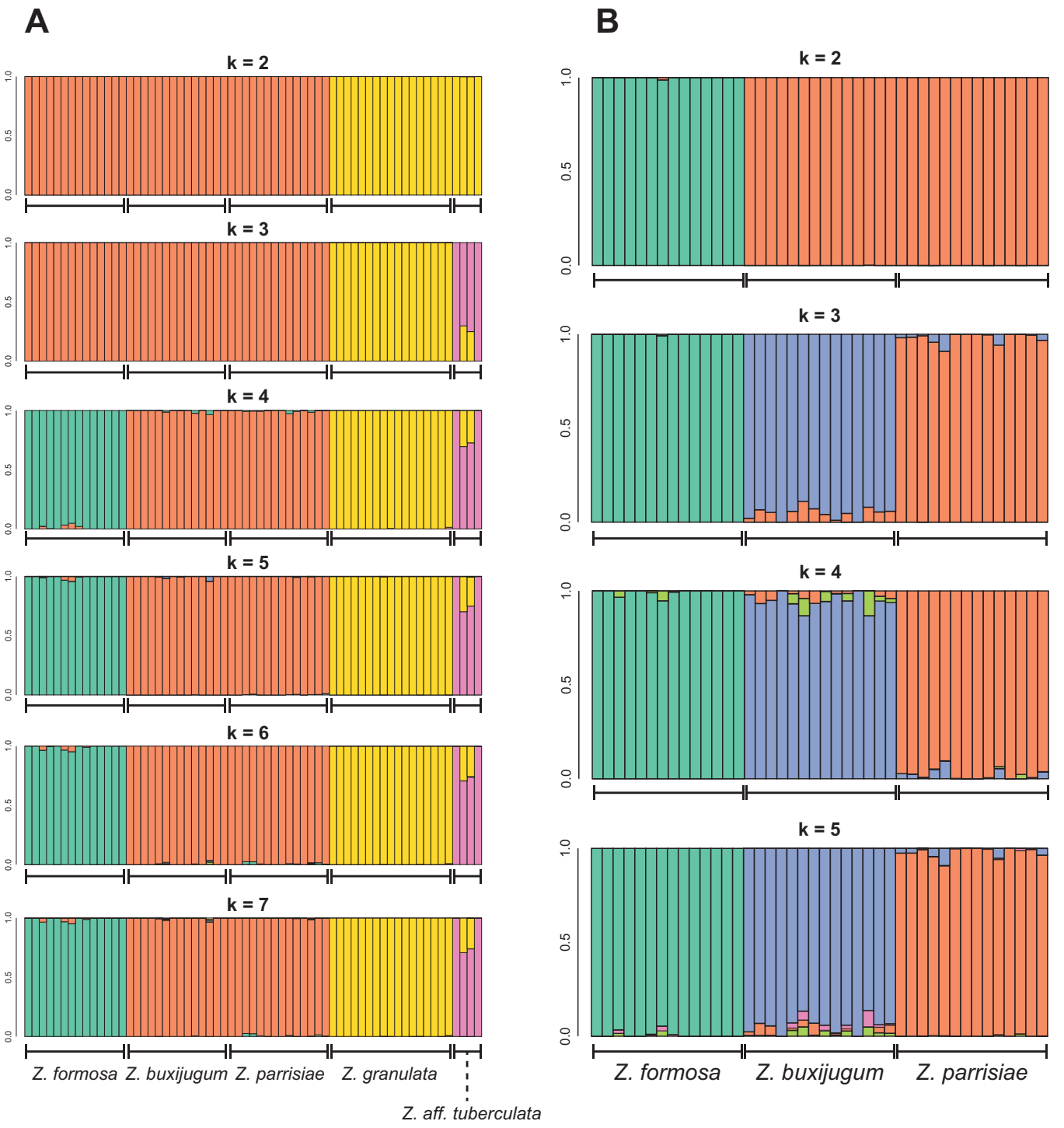

Figure 5. Genetic structure of *Zieria* species based on STRUCTURE analyses of: A, the close ingroups dataset (comprising 8215 unlinked SNPs from five species: *Z. formosa*, *Z. buxijugum*, *Z. parrisiae*, *Z. granulata*, and *Z. aff. tuberculata*) for values of *K* of 2–7, and B, the target species dataset (comprising 7563 unlinked SNPs from three species: *Z. formosa*, *Z. buxijugum*, and *Z. parrisiae*) for values of *K* of 2–5.

being said, interpopulation F_{ST} comparisons found the pairwise difference between *Z. buxijugum* and *Z. parrisiae* to be much higher than the highest differences between populations of *Z. granulata* (Table 4; e.g. Killalea × Jerrara Dam, ~10 km apart, mean pairwise F_{ST} = 0.095) and *Z. tuberculata* (Table 4; e.g. Gulaga × Tilba, ~4 km apart, mean pairwise F_{ST} = 0.094). Given that the genetic difference between *Z. buxijugum* and *Z. parrisiae* is clearly greater than that between populations of closely related species, and that they are well differentiated in the STRUCTURE analysis, the current species rank ascribed to those two taxa seems appropriate.

With respect to *Z. aff. tuberculata*, STRUCTURE analyses indicated that it is genetically more distinct than other recognized species in the analysis. Delineation of *Z. aff. tuberculata* rather than *Z. formosa* at a lower *K*-value (Fig. 5A; *K* = 3 versus *K* = 4), along with the failure to distinguish *Z. buxijugum* and

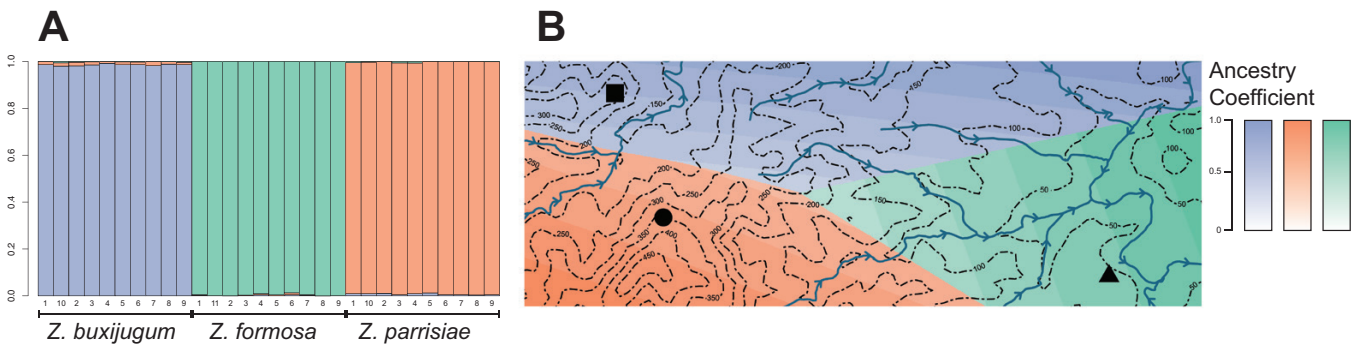

Figure 6. Results from TESS3 analysis of the target species dataset (comprising 7823 unlinked SNPs from three species: *Z. formosa*, *Z. buxijugum*, *Z. parrisiae*). A, Barplot of ancestry coefficients for the best-fit value of *K*, *K* = 3. Numbers under individual bars correspond to the sampled individual (see Appendix 1). B, Spatial map of ancestry coefficients with basic regional topography (from Jarvis *et al.* 2008) and hydrology (from Crossman and Li 2015) superimposed; the square denotes *Z. buxijugum*, the circle denotes *Z. parrisiae*, and the triangle is *Z. formosa*.

Z. parrisiae from each other in the analysis of the close ingroups dataset (Fig. 5A), suggests that *Z. aff. tuberculata* is genetically more distinct than each of the three Critically Endangered species. Interspecies pairwise F_{ST} comparisons (Table 3) found *Z. aff. tuberculata* to be highly differentiated from its sister, *Z. granulata* (mean pairwise F_{ST} = 0.284). Comparatively, the difference between *Z. aff. tuberculata* and *Z. granulata* is greater than that between *Z. buxijugum* and *Z. parrisiae* (mean pairwise F_{ST} = 0.166), and slightly less than that between *Z. formosa* and *Z. buxijugum* (mean pairwise F_{ST} = 0.336) or *Z. formosa* and *Z. parrisiae* (mean pairwise F_{ST} = 0.334). Consequently, the level of genetic differentiation between *Z. aff. tuberculata* and *Z. granulata* according to F_{ST} provides further support for taxonomic recognition of *Z. aff. tuberculata* at the rank of species.

Speciation of *Zieria* at local geographic scales

The biological characteristics of *Zieria* (outlined in the 'Study System' section) mean that pollen transfer and seed dispersal are limited to short distances from parent plants, suggesting that gene flow in *Zieria* has historically been affected more strongly by gradual connectivity and fragmentation of vegetation than by long distance cross-pollination and dispersal (Neal *et al.* 2019), and that the geographic distances required for reproductive isolation between populations are not large. While *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae* are geographically close, there are several local environmental features that may have played a role in their geographic fragmentation and affected gene flow between them. Both *Z. buxijugum* and *Z. parrisiae* occur on elevated ignimbrite outcrops on either side of a gully through which runs one main ephemeral creek (Fig. 6B). *Zieria formosa* is geographically closest to *Z. parrisiae*, occurring ~4.5 km east of *Z. parrisiae* on a rhyolite outcrop at lower elevation near the Pambula River (Fig. 6B), with a ridgeline and spur, and multiple creeks separating these two species. Fine-scale environmental heterogeneity is an important driver of speciation in sister plant species (Anaker and Strauss 2014). Preference for certain habitats or soils may drive geographic isolation either through ecological differentiation (if species prefer different environments) or by restricting potential for range expansion and re-connection (if species prefer the same geographically isolated and limited environment) (Sobel *et al.* 2010). In the case of *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae* a combination of fine-scale environmental preferences, limited pollination distances and local vicariant barriers have probably resulted in their allopatry and speciation over a such a small geographic scale.

Genetic diversity, breeding systems, and seed viability in endangered *Zieria* species

Genetic diversity in *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae* is comparable with that of the less threatened and more widely distributed species *Z. granulata* and *Z. tuberculata* (Table 3). In particular, mean H_e estimates for *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae* were all slightly higher than *Z. tuberculata* and slightly lower than *Z. granulata*.

Although it is not known if plants of *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae* are self-compatible, the patterns of genetic diversity we found, including comparisons with related species, together with data on seed viability, provide some insight into their mating systems. Among the taxa included in the present study, *Z. littoralis*, *Z. tuberculata*, and *Z. granulata* have mixed mating systems and are capable of self-compatibility, although fruit set in *Z. tuberculata* and *Z. granulata* under such circumstances is low (Armstrong 2002, Lopresti *et al.* 2023). Our estimates of high F_{IS} and much lower H_o than H_e in *Z. littoralis* are consistent with previous findings by Armstrong (2002) of frequent selfing in that species. Moderately low F_{IS} and only slightly lower H_o than H_e in *Z. tuberculata* and *Z. granulata* suggest that they are potentially affected by low levels of inbreeding, however not to the extent of *Z. littoralis*, implying that self-compatibility is probably not the primary breeding system in *Z. tuberculata* and *Z. granulata*. This result is consistent with Lopresti *et al.* (2023), who found fruit and seed set from both self and outcrossing pollination events in *Z. granulata*, although no viable seed was produced from selfed flowers. In *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae* low F_{IS} values and estimates of H_o that are slightly higher than H_e indicate no inbreeding in each of these species. The high fruit set output by these species (NPWS 2002), along with low seed fill and seed viability (Martyn *et al.* 2009), could be the product of these species possessing a superficially mixed mating system (similar to their sister clade species, *Z. granulata*) that permits fruit and seed set but results in unviable seed when flowers are self-pollinated. In other threatened plant species, self-incompatibility and small

population sizes have been found to limit mate availability and increase reproductive failure (Scobie and Wilcock 2009, Young and Pickup 2010). Given the small populations of *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae* it is probable that population viability has been heavily reduced due to a lack of available unrelated mates.

While the process responsible for low seed viability (including seed fill; Martyn *et al.* 2009) remains unclear, the fact that we have found no evidence for historical inbreeding in any of *Z. buxijugum*, *Z. formosa*, or *Z. parrisiae* could suggest that it is not an expression of inbreeding depression, but rather a late-acting self-incompatibility mechanism (Seavey and Bawa 1986, Ghazoul and Satake 2009). It is worth noting that seed predation in *Zieria* is quite high (Armstrong 2002, Lopresti *et al.* 2023), and that the high-density production of unviable seed may provide a selective advantage by satiating seed predators (Ghazoul and Satake 2009). Seed viability may also be affected by the plants' pollinators; under suboptimal pollination regimes, plant species with multi-seeded fruits may gain an adaptive advantage by developing fruits despite low levels of embryo formation to ensure that at least a few viable seeds are still produced (Picarella and Mazzucato 2019). Future research on seed and embryo development in *Zieria* species with mixed mating systems would prove useful in unravelling these dynamics. Along with this, pollination studies of *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae* might yield insights into biotic factors affecting cross-pollination that have potentially led to the genetic divergence of the species.

Implications for conservation management

Taken together and considering all potential alternative taxonomic treatments for these species, our results are most consistent with the current delimitation of *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae* as distinct species. We therefore recommend the continued recognition and conservation management of these entities as discrete species.

Our insights into the genetic diversity of *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae*, along with previous work on seed viability and germination in *Zieria* (Armstrong 2002, Martyn *et al.* 2009, Lopresti *et al.* 2023), have highlighted that the species may face demographic and extinction risks due to the low viability of seed, which could result from a preponderance of self-pollination, assuming similar biology to *Z. granulata* as shown by Lopresti *et al.* (2023). Martyn *et al.* (2009) highlighted that, among the Australian Rutaceae, threatened species generally display lower seed fill than common species. Given the low seed fill and viability (< 30%) in *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae* from wild-collected seed (Martyn *et al.* 2009), efforts should be made to boost the cohort of unrelated plants growing *ex situ*. This would have two beneficial effects for conservation of the species, namely: (i) increasing the overall *ex situ* genetic diversity of each species, and (ii) potentially increasing the chances of producing viable seed from the manual outcrossing of *ex situ* plants. On the basis of our sampling of the three species, current *ex situ* genetic diversity in living collections is best representative of wild diversity in *Z. buxijugum*, although this could still be improved through the inclusion of the genotypes of AEO 1693.8, AEO 1693.1/2/3/7, and AEO 1693.9/10 (indicated on Fig. 4). The genetic diversity of *Z. formosa* is poorly represented by the *ex situ* living collections, although an important lineage of two *ex situ* plants (probably the same genotype) was identified sister to all other samples (Fig. 3). *Ex situ* genetic diversity of *Z. formosa* would be improved by the inclusion of the genotypes of PAM 75.1/4/8, PAM 75.5/6, and PAM 75.3/7/9/11 (Fig. 4). In *Z. parrisiae*, *ex situ* genetic diversity would be increased by the inclusion of the genotypes of PAM 82.1/5/10, PAM 82.3/4, and PAM 82.2/6/7/8/9 (Fig. 4). Seed collections held at the National Seed Bank at ANBG (*Z. buxijugum*: 15 maternal lines, *Z. formosa*: 13 maternal lines, and *Z. parrisiae*: 14 maternal lines; information available from https://www.anbg.gov.au/gardens/living/seedbank/seeddata.html) probably contribute to the genetic diversity represented *ex situ*, however, were not sequenced in this study. Further research is required to determine the fill, viability, dormancy, and germination of the seeds to enable their propagation, and understand the contribution of seed collections to the genetic diversity secured *ex situ*.

All genetic analyses conducted as part of this study suggest that *Z. aff. tuberculata* is worthy of taxonomic recognition at the rank of species. As the samples of *Z. aff. tuberculata* that were included in this study were sourced solely from *ex situ* living collections, it is possible that unsampled wild genetic diversity exists. However, field surveys and collections of *Z. aff. tuberculata* from the wild that were undertaken concurrently with this study have confirmed that the putative species is restricted to an allopatric population of only a few hundred mature individuals occurring over a very small area on a single mountaintop in NSW; making it more likely that our sampling covers a significant proportion of the wild diversity. Examination of field collected material after this genetic study has identified obvious distinguishing morphological features that confirm *Z. aff. tuberculata* should be recognized as a distinct species (Orel *et al.* 2023).

CONCLUSION

The current study has provided support for the current classification of *Zieria buxijugum*, *Z. formosa*, and *Z. parrisiae*, reinforcing their status as three distinct species. Species-level relationships among a closely related group have been further clarified, with genetic evidence suggesting that *Z. aff. tuberculata* is distinct enough to be recognized as a new species. The genetic diversity of each of *Z. buxijugum*, *Z. formosa*, and *Z. parrisiae* was found to be similar to that of more widespread, closely related species. We detected no evidence of inbreeding within *Z. buxijugum*, *Z. formosa*, or *Z. parrisiae*; a finding that is likely to be consequential for their future management in *ex situ* gardens and seed banks. Finally, we have provided an assessment of the genetic diversity of plants of these species currently held in *ex situ* living collections, along with guidance for targeting collections from certain wild genotypes in the future to increase *ex situ* diversity.

SUPPLEMENTARY DATA

Supplementary data is available at the *Botanical Journal of the Linnean Society* online.

ACKNOWLEDGEMENTS

We thank Tamera Beath, Toby Golson, Jedda Lemmon, Andrew Orme, Seanna McCune, and David McCreery for undertaking sample

collection. We would like to acknowledge the contribution of the Genomics for Australian Plants Framework Initiative consortium (https://www.genomicsforaustralianplants.com/consortium/) in the generation of data used in this publication. The Initiative is supported by funding from Bioplatforms Australia (enabled by NCRIS), the Ian Potter Foundation, Royal Botanic Gardens Foundation (Victoria), Royal Botanic Gardens Victoria, the Royal Botanic Gardens and Domain Trust, the Council of Heads of Australasian Herbaria, CSIRO, Centre for Australian National Biodiversity Research, and the Department of Biodiversity, Conservation and Attractions, Western Australia. In particular, we thank Mabel Lum for her role liaising between the authors and the consortium. H.K.O. is supported by an Australian Government Research Training Program scholarship, and a Sophie Ducker scholarship from the University of Melbourne Botany Foundation. Collections for the study were made under permits granted by the New South Wales National Parks and Wildlife Service (SL100569 and SL101283), and an Agreement for Supply of Biological Material for Non-Commercial Use granted by the Director of National Parks (DNP ref. 2020/21-8) for plant material from ANBG. We also thank the handling editor and three anonymous reviewers for their constructive comments and suggestions that helped improve this manuscript.

DATA AVAILABILITY

Raw sequence data utilised in this study are available from the Bioplatforms Australia Data Portal (https://data.bioplatforms.com, search 'ticket:BPAOPS-1119') and Figshare (https://doi.org/10.26188/21686159). Datasets output from the ipyrad pipeline and files produced from phylogenetic, network and STRUCTURE analyses are available from Figshare (https://doi.org/10.26188/21280542). Further data are available from the authors upon reasonable request.

FUNDING

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

APPENDIX

Table A1. Details of samples used in this study.

Living Collections plant number	Herbarium voucher number	Collector number[a]	State	Locality
Zieria formosa J.D.Briggs & J.A.Armstr.				
–	NSW1101007[b]	McCune PAM 75.1	NSW	471 Mount Darragh Road, ~10 km south-west of Pambula.
–	NSW1101007[b]	McCune PAM 75.2	NSW	471 Mount Darragh Road, ~10 km south-west of Pambula.
–	NSW1101007[b]	McCune PAM 75.3	NSW	471 Mount Darragh Road, ~10 km south-west of Pambula.
–	NSW1101007[b]	McCune PAM 75.4	NSW	471 Mount Darragh Road, ~10 km south-west of Pambula.
–	NSW1101007[b]	McCune PAM 75.5	NSW	471 Mount Darragh Road, ~10 km south-west of Pambula.
–	NSW1101007[b]	McCune PAM 75.6	NSW	471 Mount Darragh Road, ~10 km south-west of Pambula.
–	NSW1101007[b]	McCune PAM 75.7	NSW	471 Mount Darragh Road, ~10 km south-west of Pambula.
–	NSW1101007[b]	McCune PAM 75.8	NSW	471 Mount Darragh Road, ~10 km south-west of Pambula.
–	NSW1101007[b]	McCune PAM 75.9	NSW	471 Mount Darragh Road, ~10 km south-west of Pambula.
–	NSW1101007	McCune PAM 75.11	NSW	471 Mount Darragh Road, ~10 km south-west of Pambula.
CBG 8604996	CBG 8604996	Parris, M. 9150a	NSW	Cultivated, ANBG ex. Bega Valley.
CBG 8604997	CBG 8604997	Parris, M. 9150b	NSW	Cultivated, ANBG ex. Bega Valley.
CBG 9505103	CBG 8604998[b]	Parris, M. 9151b	NSW	Cultivated, ANBG ex. Bega Valley.
CBG 9203710	CBG 8604998[b]	Parris, M. 9151c	NSW	Cultivated, ANBG ex. Bega Valley.
Zieria parrisiae J.D.Briggs & J.A.Armstr.				
–	NSW1101008	McCune PAM 82.1	NSW	Box Range Farm, 9 km directly west of Pambula.
–	NSW1101008[b]	McCune PAM 82.2	NSW	Box Range Farm, 9 km directly west of Pambula.
–	NSW1101008[b]	McCune PAM 82.3	NSW	Box Range Farm, 9 km directly west of Pambula.

Table A1. Continued

Living Collections plant number	Herbarium voucher number	Collector number[a]	State	Locality
–	NSW1101008[b]	McCune PAM 82.4	NSW	Box Range Farm, 9 km directly west of Pambula.
–	NSW1101008[b]	McCune PAM 82.5	NSW	Box Range Farm, 9 km directly west of Pambula.
–	NSW1101008[b]	McCune PAM 82.6	NSW	Box Range Farm, 9 km directly west of Pambula.
–	NSW1101008[b]	McCune PAM 82.7	NSW	Box Range Farm, 9 km directly west of Pambula.
–	NSW1101008[b]	McCune PAM 82.8	NSW	Box Range Farm, 9 km directly west of Pambula.
–	NSW1101008[b]	McCune PAM 82.9	NSW	Box Range Farm, 9 km directly west of Pambula.
–	NSW1101008[b]	McCune PAM 82.10	NSW	Box Range Farm, 9 km directly west of Pambula.
CBG 8605256	CBG 8604989[b]	Parris, M. 9145d	NSW	Cultivated, ANBG ex. 9 km directly W of Pambula.
CBG 8604989	CBG 8604989	Parris, M. 9145a	NSW	Cultivated, ANBG ex. Bega Valley.
CBG 8604991	CBG 8604989[b]	Parris, M. 9145c	NSW	Cultivated, ANBG ex. 9 km directly W of Pambula.
CBG 9505165	CBG 8604989[b]	Parris, M. 9145e	NSW	Cultivated, ANBG ex. Bega Valley.
Zieria buxijugum J.D.Briggs & J.A.Armstr.				
–	NSW1100980	Orme AEO 1693.1	NSW	Box Range Farm, Lochiel. ~15 km W of Pambula.
–	NSW1100980[b]	Orme AEO 1693.2	NSW	Box Range Farm, Lochiel. ~15 km W of Pambula.
–	NSW1100980[b]	Orme AEO 1693.3	NSW	Box Range Farm, Lochiel. ~15 km W of Pambula.
–	NSW1100980[b]	Orme AEO 1693.4	NSW	Box Range Farm, Lochiel. ~15 km W of Pambula.
–	NSW1100980[b]	Orme AEO 1693.5	NSW	Box Range Farm, Lochiel. ~15 km W of Pambula.
–	NSW1100980[b]	Orme AEO 1693.6	NSW	Box Range Farm, Lochiel. ~15 km W of Pambula.
–	NSW1100980[b]	Orme AEO 1693.7	NSW	Box Range Farm, Lochiel. ~15 km W of Pambula.
–	NSW1100980[b]	Orme AEO 1693.8	NSW	Box Range Farm, Lochiel. ~15 km W of Pambula.
–	NSW1100980[b]	Orme AEO 1693.9	NSW	Box Range Farm, Lochiel. ~15 km W of Pambula.
–	NSW1100980[b]	Orme AEO 1693.10	NSW	Box Range Farm, Lochiel. ~15 km W of Pambula.
CBG 8600921	CBG 8600921	Parris, M. 8971	NSW	Cultivated, ANBG ex. Bega Valley.
CBG 8602343	CBG 8602343	Parris, M. 9018a	NSW	Cultivated, ANBG ex. Bega Valley.
CBG 8800111	CBG 8602343[b]	Parris, M. 9018e	NSW	Cultivated, ANBG ex. Bega Valley.
CBG 8602344	CBG 8602343[b]	Parris, M. 9018b	NSW	Cultivated, ANBG ex. Bega Valley.
Zieria granulata C.Moore ex Benth.				
–	NSW1101995[b]	J. Lemmon Killalea 1	NSW	Killalea Reserve, northern end, north-west of Killalea lagoon. Shellharbour LGA.
–	NSW1101995[b]	J. Lemmon Killalea 2	NSW	Killalea Reserve, northern end, north-west of Killalea lagoon. Shellharbour LGA.
–	NSW1101995[b]	J. Lemmon Killalea 3	NSW	Killalea Reserve, northern end, north-west of Killalea lagoon. Shellharbour LGA.

Table A1. Continued

Living Collections plant number	Herbarium voucher number	Collector number[a]	State	Locality
–	NSW1101995[b]	J. Lemmon Killalea 4	NSW	Killalea Reserve, northern end, north-west of Killalea lagoon. Shellharbour LGA.
–	NSW1101995[b]	J. Lemmon Killalea 5	NSW	Killalea Reserve, northern end, north-west of Killalea lagoon. Shellharbour LGA.
–	NSW1101995[b]	J. Lemmon Killalea 6	NSW	Killalea Reserve, northern end, north-west of Killalea lagoon. Shellharbour LGA.
–	NSW1101995[b]	J. Lemmon Killalea 7	NSW	Killalea Reserve, northern end, north-west of Killalea lagoon. Shellharbour LGA.
–	NSW1101996[b]	J. Lemmon Jerrara Dam 1	NSW	Jerrara Dam, Kiama, on the west facing slopes south-west of the dam.
–	NSW1101996[b]	J. Lemmon Jerrara Dam 2	NSW	Jerrara Dam, Kiama, on the west facing slopes south-west of the dam.
–	NSW1101996[b]	J. Lemmon Jerrara Dam 3	NSW	Jerrara Dam, Kiama, on the west facing slopes south-west of the dam.
–	NSW1101996[b]	J. Lemmon Jerrara Dam 4	NSW	Jerrara Dam, Kiama, on the west facing slopes south-west of the dam.
–	NSW1101996[b]	J. Lemmon Jerrara Dam 5	NSW	Jerrara Dam, Kiama, on the west facing slopes south-west of the dam.
–	NSW1101996[b]	J. Lemmon Jerrara Dam 7	NSW	Jerrara Dam, Kiama, on the west facing slopes south-west of the dam.
–	NSW1101996[b]	J. Lemmon Jerrara Dam 8	NSW	Jerrara Dam, Kiama, on the west facing slopes south-west of the dam.
CBG 8501504	CBG 8501504	Mills, K. 1A	NSW	Cultivated, ANBG ex. Jamberoo Valley.
CBG 9505089	CBG 8208554[c]	Armstrong, J.A. 757	NSW	Cultivated, ANBG ex. John Cleary Look-out.
CBG 8404676	CANB 653314	Zich, F.A. 434	NSW	Cultivated, ANBG ex. Minnamurra Head-land.
Zieria tuberculata J.A.Armstr.				
–	NSW1102000[b]	D. McCreery Gulaga 1	NSW	Gulaga FT, 10 m north of Gulaga FT at topside of N facing rocky outcrop.
–	NSW1102000[b]	D. McCreery Gulaga 2	NSW	Gulaga FT, 10 m north of Gulaga FT at topside of N facing rocky outcrop.
–	NSW1102000[b]	D. McCreery Gulaga 3	NSW	Gulaga FT, 10 m north of Gulaga FT at topside of N facing rocky outcrop.
–	NSW1102000[b]	D. McCreery Gulaga 4	NSW	Gulaga FT, 10 m north of Gulaga FT at topside of N facing rocky outcrop.
–	NSW1102000[b]	D. McCreery Gulaga 5	NSW	Gulaga FT, 10 m north of Gulaga FT at topside of N facing rocky outcrop.
–	NSW1102001[b]	D. McCreery Tilba 1	NSW	Tilba Reservoir, 10 m west of water tower N of Bellbrook loop track.
–	NSW1102001[b]	D. McCreery Tilba 2	NSW	Tilba Reservoir, 10 m west of water tower N of Bellbrook loop track.
–	NSW1102001[b]	D. McCreery Tilba 3	NSW	Tilba Reservoir, 10 m west of water tower N of Bellbrook loop track.
–	NSW1102001[b]	D. McCreery Tilba 4	NSW	Tilba Reservoir, 10 m west of water tower N of Bellbrook loop track.
–	NSW1102002[b]	D. McCreery Bellbrook 1	NSW	Bellbrook Farm, 100 m above track at edge of paddock.
–	NSW1102002[b]	D. McCreery Bellbrook 2	NSW	Bellbrook Farm, 100 m above track at edge of paddock.
–	NSW1102002[b]	D. McCreery Bellbrook 3	NSW	Bellbrook Farm, 100 m above track at edge of paddock.
–	NSW1102002[b]	D. McCreery Bellbrook 4	NSW	Bellbrook Farm, 100 m above track at edge of paddock.

Table A1. Continued

Living Collections plant number	Herbarium voucher number	Collector number[a]	State	Locality
CBG 8806089	CANB 387032[b]	Briggs, J.D. 2344e	NSW	Cultivated, ANBG ex. Dromedary Flora Reserve.
CBG 8806087	CANB 387032[b]	Briggs, J.D. 2344c	NSW	Cultivated, ANBG ex. Dromedary Flora Reserve.
CBG 8806088	CANB 387032[b]	Briggs, J.D. 2344d	NSW	Cultivated, ANBG ex. Dromedary Flora Reserve.
CBG 9203763	CANB 387032[c]	Briggs, J.D. 2344a	NSW	Cultivated, ANBG ex. Dromedary Flora Reserve.
Zieria aff. tuberculata				
CANB 773056	CANB 912870	Purdie, R.W. 11 863	NSW	Cultivated, ANBG ex. Cambewarra Mountain.
CANB 627225	CANB 889459	Briggs, J.D. 2858c	NSW	Cultivated, ANBG ex. Cambewarra Mountain.
CANB 627226	CANB 627223[b]	Briggs, J.D. 2858d	NSW	Cultivated, ANBG ex. Cambewarra Mountain.
CANB 627227	CANB 627223[b]	Briggs, J.D. 2858e	NSW	Cultivated, ANBG ex. Cambewarra Mountain.
Zieria littoralis J.A.Armstr.				
CANB 662814	CANB 662814	Pedersen, S. 743	NSW	Cultivated, ANBG ex. Nadgee Nature Reserve.
CBG 9603015	CBG 8603729[c]	Burns, R. ANBG 1062	TAS	Cultivated, ANBG ex. Bicheno.
CBG 9505088	CBG 8703977[c]	Parris, M. 9240	NSW	Cultivated, ANBG ex. Bournda National Park.
ANBG 612929	CANB 643988	Zich, F.A. 384a	NSW	Cultivated, ANBG ex. Eden Lookout.
CBG 8413677	CBG 8413677	Parris, M. 8849	NSW	Cultivated, ANBG ex. Nadgee State Forest.
Zieria collina C.T.White				
CBG 8413674	CBG 8413674	Parris, M. 8846	QLD	Cultivated, ANBG ex. Mount Tamborine.
CBG 8604923	CBG 8604923	Parris, M. 9085	QLD	Cultivated, ANBG ex. Cedar Creek National Park.
Zieria baeuerlenii J.A.Armstr.				
CBG 9516971	CBG 8704035[b]	Davies, F.E. 214e	NSW	Cultivated, ANBG ex. Bomaderry Creek.
CANB 751714	CANB 909041	S. Pedersen 1014	NSW	Cultivated, ANBG ex. Bomaderry Creek.
Zieria eungellaensis Duretto & P.I.Forst.				
CBG 9516991	CBG 9102372[c]	Telford, I.R. 11 165	QLD	Cultivated, ANBG ex. Mt Dalrymple.
Zieria verrucosa J.A.Armstr.				
CBG 8604310	CBG 8604310	Beesley, P. 970A	QLD	Cultivated, ANBG ex. ~5 km NW of Monogorilby.
CBG 9203795	CBG 8305836[b]	Hargraves, J. s.n.	QLD	Cultivated, ANBG ex. Naragen Research Station.
Zieria obcordata A.Cunn.				
CANB 741033	CBG 9103467[b]	Briggs, J.D. 2376c	NSW	Cultivated, ANBG ex. 16 km ENE of Wellington on 'Bulbudgeree' station.
CANB 782780	CANB 782780	Mulvaney, M.F. 2	NSW	Cultivated, ANBG ex. Bathurst.

[a]Collector number for the sampled individual or, if the Living Collections Plant Number is populated, collector number for the original parent of the sampled individual.
[b]Denotes vouchers that represent a population, but are from a different individual from that sampled.
[c]Denotes vouchers that represent the original parent of a descendant that was sampled.

REFERENCES

Anacker BL, Strauss SY. The geography and ecology of plant speciation: range overlap and niche divergence in sister species. *Proceedings of the Royal Society B: Biological Sciences* 2014;**281**:20132980. https://doi.org/10.1098/rspb.2013.2980

Andrews S. FastQC: a quality control tool for high throughput sequence data. https://www.bioinformatics.babraham.ac.uk/projects/fastqc/, 2010. Date accessed 15 December 2021.

Armstrong JA. *Studies on Pollination and Systematics in the Australian Rutaceae*. Australia: University of New South Wales, 1991.

Armstrong JA. The genus *Zieria* (Rutaceae): a systematic and evolutionary study. *Australian Systematic Botany* 2002;**15**:277–463. https://doi.org/10.1071/sb00040

Arnold B, Corbett-Detig RB, Hartl D *et al.* RADseq underestimates diversity and introduces genealogical biases due to nonrandom haplotype sampling. *Molecular Ecology* 2013;**22**:3179–90. https://doi.org/10.1111/mec.12276

AVH. *The Australasian Virtual Herbarium*, Council of Heads of Australasian Herbaria, 2022 https://avh.chah.org.au. Date accessed 14 June 2022.

Barrett RA, Bayly MJ, Duretto MF *et al.* A chloroplast phylogeny of *Zieria* (Rutaceae) in Australia and New Caledonia shows widespread incongruence with species-level taxonomy. *Australian Systematic Botany* 2014;**27**:427–49. https://doi.org/10.1071/sb14033

Barrett RA, Bayly MJ, Duretto MF *et al.* Phylogenetic analysis of *Zieria* (Rutaceae) in Australia and New Caledonia based on nuclear ribosomal DNA shows species polyphyly, divergent paralogues and incongruence with chloroplast DNA. *Australian Systematic Botany* 2018;**31**:16–47. https://doi.org/10.1071/sb16034

BCA. 2016. Biodiversity Conservation Act. Government of New South Wales. https://legislation.nsw.gov.au/view/html/inforce/current/act-2016-063

Binks RM, Byrne M. Species delimitation, hybridization and possible apomixis in a rapid radiation of Western Australian *Leptospermum* (Myrtaceae). *Botanical Journal of the Linnean Society* 2022;**200**:378–94. https://doi.org/10.1093/botlinnean/boac022

Booy G, Hendriks RJJ, Smulders MJM *et al.* Genetic diversity and the survival of populations. *Plant Biology* 2000;**2**:379–95. https://doi.org/10.1055/s-2000-5958

Bryant D, Moulton V. Neighbor-net: an agglomerative method for the construction of phylogenetic networks. *Molecular Biology and Evolution* 2004;**21**:255–65. https://doi.org/10.1093/molbev/msh018

Carstens BC, Pelletier TA, Reid NM *et al.* How to fail at species delimitation. *Molecular Ecology* 2013;**22**:4369–83. https://doi.org/10.1111/mec.12413

Cavalli-Sforza LL, Edwards AWF. Phylogenetic analysis: models and estimation procedures. *American Journal of Human Genetics* 1967;**19**:233–57.

Caye K, Deist TM, Martins H *et al.* TESS3: fast inference of spatial population structure and genome scans for selection. *Molecular Ecology Resources* 2016;**16**:540–8. https://doi.org/10.1111/1755-0998.12471

Caye K, Jay F, Michel O *et al.* Fast inference of individual admixture coefficients using geographic data. *Annals of Applied Statistics* 2018;**12**:586–608. https://doi.org/10.1214/17-AOAS1106

Chifman J, Kubatko L. Quartet inference from SNP data under the coalescent model. *Bioinformatics* 2014;**30**:3317–24. https://doi.org/10.1093/bioinformatics/btu530

Chifman J, Kubatko L. Identifiability of the unrooted species tree topology under the coalescent model with time-reversible substitution processes, site-specific rate variation, and invariable sites. *Journal of Theoretical Biology* 2015;**374**:35–47. https://doi.org/10.1016/j.jtbi.2015.03.006

Coates DJ, Byrne M, Moritz C. Genetic diversity and conservation units: dealing with the species-population continuum in the age of genomics. *Frontiers of Ecology and Evolution* 2018;**6**:165. https://doi.org/10.3389/fevo.2018.00165

Crossman S, Li O. *Surface Hydrology Lines (National)*. Canberra: Geoscience Australia,2015. https://pid.geoscience.gov.au/dataset/ga/83130

Crotti M, Barratt CD, Loader SP *et al.* Causes and analytical impacts of missing data in RADseq phylogenetics: insights from an African frog (*Afrixalus*). *Zoologica Scripta* 2019;**48**:157–67. https://doi.org/10.1111/zsc.12335

Cullingham C, Miller J, Peery R *et al.* Confidently identifying the correct *K* value using the Δ*K* method: When does *K* = 2? *Molecular Ecology* 2020;**29**:862–9. https://doi.org/10.1111/mec.15374

Dray S, Dufour A-B. The ade4 package: implementing the duality diagram for ecologists. *Journal of Statistical Software* 2007;**22**:1–20.

Dress AWM, Huson DH. Constructing splits graphs. *IEEE/ACM Transactions on Computational Biology and Bioinformatics* 2004;**1**:109–15. https://doi.org/10.1109/TCBB.2004.27

Duretto MF. *Zieria fordii* and *Z. wilhelminae* (Rutaceae), two new and restricted Queensland species segregated from the morphologically similar and widespread *Z. cytisoides*. *Telopea* 2019;**22**:135–40. https://doi.org/10.7751/telopea13458

Earl DA, vonHoldt BM. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources* 2012;**4**:359–61. https://doi.org/10.1007/s12686-011-9548-7

Eaton DAR, Overcast I. ipyrad: interactive assembly and analysis of RADseq datasets. *Bioinformatics* 2020;**36**:2592–4. https://doi.org/10.1093/bioinformatics/btz966

Evanno G, Regnaut S, Goudet J. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* 2005;**14**:2611–20.

Fahey PS, Fowler RM, McLay TGB *et al.* Divergent lineages in a semi-arid mallee species, *Eucalyptus behriana*, correspond to a major geographic break in southeastern Australia. *Ecology and Evolution* 2021;**11**:664–78. https://doi.org/10.1002/ece3.7099

Forster PI. *Zieria abscondita* PIForst (Rutaceae), a new and restricted species from south-east Queensland. *Austrobaileya* 2020;**10**:621–7.

François O, Durand E. Spatially explicit Bayesian clustering models in population genetics. *Molecular Ecology Resources* 2010;**10**:773–84. https://doi.org/10.1111/j.1755-0998.2010.02868.x

Frichot E, François O. LEA: an R package for landscape and ecological association studies. *Methods in Ecology and Evolution* 2015;**6**:925–9. https://doi.org/10.1111/2041-210x.12382

George AS, Duretto MF, Forster PI. *Zieria*. In: Wilson A. (Ed.), *Flora of Australia. Vol. 26: Meliaceae, Rutaceae and Zygophyllaceae*. Melbourne: Australian Biological Resources Study, Canberra and CSIRO Publishing, 2013, 282–336.

Georges A, Mijangos L, Hardip P *et al.* Distances and their visualization in studies of spatial-temporal genetic variation using single nucleotide polymorphisms (SNPs). *bioRxiv* 2023. https://doi.org/10.1101/2023.03.22.533737. Preprint: not peer reviewed.

Ghazoul J, Satake A. Nonviable seed set enhances plant fitness: the sacrificial sibling hypothesis. *Ecology* 2009;**90**:369–77. https://doi.org/10.1890/07-1436.1

Goudet J. Hierfstat, a package for R to compute and test hierarchical F-statistics. *Molecular Ecology Notes* 2005;**5**:184–6.

Guillot G, Leblois R, Coulon A *et al.* Statistical methods in spatial genetics. *Molecular Ecology* 2009;**18**:4734–56. https://doi.org/10.1111/j.1365-294X.2009.04410.x

Guindon S, Dufayard J-F, Lefort V *et al.* New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 30. *Systematic Biology* 2010;**59**:307–21. https://doi.org/10.1093/sysbio/syq010

Hamming RW. Error detecting and error correcting codes. *The Bell System Technical Journal* 1950;**29**:147–60. https://doi.org/10.1002/j.1538-7305.1950.tb00463.x

Henson LH, Service C, Stronen AV *et al.* Genetic evidence to inform management of rare genetic variants and gene flow: balancing the conservation of the rare 'Spirit bear' allele and population genetic diversity across a complex landscape. *Conservation Science and Practice* 2022;**4**:e12769. https://doi.org/10.1111/csp2.12769

Hoang DT, Chernomor O, von Haeseler A *et al.* UFBoot2: improving the ultrafast bootstrap approximation. *Molecular Biology and Evolution* 2018;**35**:518–22. https://doi.org/10.1093/molbev/msx281

Hoban S, Callicrate T, Clark J *et al.* Taxonomic similarity does not predict necessary sample size for ex situ conservation: a comparison among five genera. *Proceedings of the Royal Society B: Biological Sciences* 2020;**287**:20200102. https://doi.org/10.1098/rspb.2020.0102

Hogbin PM, Crisp MD. Evolution of the coastal neospecies *Zieria prostrata* (Rutaceae) and its relationship to the *Zieria smithii* species complex. *Australian Systematic Botany* 2003;**16**:515–25. https://doi.org/10.1071/SB02031

Huson DH, Bryant D. Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution* 2006;**23**:254–67. https://doi.org/10.1093/molbev/msj030

Jakobsson M, Rosenberg NA. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* 2007;**23**:1801–6. https://doi.org/10.1093/bioinformatics/btm233

Janes JK, Miller JM, Dupuis JR *et al.* The *K* = 2 conundrum. *Molecular Ecology* 2017;**26**:3594–602. https://doi.org/10.1111/mec.14187

Jarvis A, Reuter HI, Nelson A, Guevara E. Hole-filled seamless SRTM data V4, 2008. https://srtm.csi.cgiar.org

Joly S, Bryant D, Lockhart PJ. Flexible methods for estimating genetic distances from single nucleotide polymorphisms. *Methods in Ecology and Evolution* 2015;**6**:938–48. https://doi.org/10.1111/2041-210x.12343

Jombart T. adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics* 2008;**24**:1403–5. https://doi.org/10.1093/bioinformatics/btn129

Jombart T, Ahmed I. adegenet 13-1: new tools for the analysis of genome-wide SNP data. *Bioinformatics* 2011;**27**:3070–1. https://doi.org/10.1093/bioinformatics/btr521

Kalyaanamoorthy S, Minh BQ, Wong TKF *et al.* ModelFinder: fast model selection for accurate phylogenetic estimates. *Nature Methods* 2017;**14**:587–9. https://doi.org/10.1038/nmeth.4285

Lopresti LC, Sommerville KD, Gilpin AM *et al.* Floral biology, pollination vectors and breeding system of *Zieria granulata* (Rutaceae), an endangered shrub endemic to eastern Australia. *Australian Journal of Botany* 2023;**71**:252–68. https://doi.org/10.1071/bt22055

Martyn AJ, Seed LU, Ooi MKJ *et al.* Seed fill, viability and germination of NSW species in the family Rutaceae. *Cunninghamia* 2009;**11**:203–12.

McLay TGB. High quality DNA extraction protocol from recalcitrant plant tissues. In: *protocols.io*. https://doi.org/10.17504/protocols.io.i8jchun, 2017. Date accessed 16 June 2022.

Minh BQ, Nguyen MAT, von Haeseler A. Ultrafast approximation for phylogenetic bootstrap. *Molecular Biology and Evolution* 2013;**30**:1188–95. https://doi.org/10.1093/molbev/mst024

Minh BQ, Schmidt HA, Chernomor O *et al.* IQ-TREE 2: new models and efficient methods for phylogenetic inference in the genomic era. *Molecular Biology and Evolution* 2020;**37**:1530–4. https://doi.org/10.1093/molbev/msaa015

Mo YK, Lanfear R, Hahn MW *et al.* Updated site concordance factors minimize effects of homoplasy and taxon sampling. *Bioinformatics* 2022;**39**:btac741. https://doi.org/10.1093/bioinformatics/btac741

Morton CM. Phylogenetic relationships of *Zieria* (Rutaceae) inferred from chloroplast, nuclear, and morphological data. *PhytoKeys* 2015;**44**:15–38. https://doi.org/10.3897/phytokeys.44.8393

Nazareno AG, Bemmels JB, Dick CW *et al.* Minimum sample sizes for population genomics: an empirical study from an Amazonian plant species. *Molecular Ecology Resources* 2017;**17**:1136–47. https://doi.org/10.1111/1755-0998.12654

Neal WC, James EA, Bayly MJ. Phylogeography, classification and conservation of pink zieria (*Zieria veronicea*; Rutaceae): influence of changes in climate, geology and sea level in south-eastern Australia. *Plant Systematics and Evolution* 2019;**305**:503–20. https://doi.org/10.1007/s00606-019-01589-z

Nei M. *Molecular Evolutionary Genetics*. New York: Columbia University Press, 1987.

NPWS. *National Recovery plan for Zieria formosa, Zieria buxijugum and Zieria Parrisiae*. Hurstville, NSW: New South Wales National Parks and Wildlife Service, 2002.

Orel HK, Briggs JD, Duretto MF. *Zieria nubicola* (Rutaceae), A new and highly restricted species from New South Wales, Australia. *Telopea* 2023;**26**. https://doi.org/10.7751/telopea17501

Peterson BK, Weber JN, Kay EH *et al.* Double digest RADseq: an inexpensive method for de novo SNP discovery and genotyping in model and non-model species. *PLoS ONE* 2012;**7**:e37135. https://doi.org/10.1371/journal.pone.0037135

Picarella ME, Mazzucato A. The occurrence of seedlessness in higher plants; insights on roles and mechanisms of parthenocarpy. *Frontiers in Plant Science* 2019;**9**:1997. https://doi.org/10.3389/fpls.2018.01997

Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. *Genetics* 2000;**155**:945–59. https://doi.org/10.1093/genetics/155.2.945

Reaz R, Bayzid MS, Rahman MS. Accurate phylogenetic tree reconstruction from quartets: A heuristic approach. *PLoS ONE* 2014;**9**:e104008. https://doi.org/10.1371/journal.pone.0104008

Reyes-Velasco J, Adams RH, Boissinot S *et al.* Genome-wide SNPs clarify lineage diversity confused by coloration in coralsnakes of the *Micrurus diastema* species complex (Serpentes: Elapidae). *Molecular Phylogenetics and Evolution* 2020;**147**:106770. https://doi.org/10.1016/j.ympev.2020.106770

Rossetto M, Yap J-YS, Lemmon J *et al.* A conservation genomics workflow to guide practical management actions. *Global Ecology and Conservation* 2021;**26**:e01492. https://doi.org/10.1016/j.gecco.2021.e01492

Schlick-Steiner BC, Steiner FM, Seifert B *et al.* Integrative taxonomy: a multisource approach to exploring biodiversity. *Annual Review of Entomology* 2010;**55**:421–38. https://doi.org/10.1146/annurev-ento-112408-085432

Schmidt TL, Jasper M, Weeks AR *et al.* Unbiased population heterozygosity estimates from genome-wide sequence data. *Methods in Ecology and Evolution* 2021;**12**:1888–98. https://doi.org/10.1111/2041-210x.13659

Schmidt H, Trifinopoulos J, Minh B, Lanfear R. Frequently asked questions. http://www.iqtree.org/doc/Frequently-Asked-Questions#how-do-i-interpret-ultrafast-bootstrap-ufboot-support-values. 2022. Date accessed 21 April 2023.

Scobie AR, Wilcock CC. Limited mate availability decreases reproductive success of fragmented populations of *Linnaea borealis*, a rare, clonal self-incompatible plant. *Annals of Botany* 2009;**103**:835–46. https://doi.org/10.1093/aob/mcp007

Seavey SR, Bawa KS. Late-acting self-incompatibility in Angiosperms. *The Botanical Review* 1986;**52**:195–219. https://doi.org/10.1007/bf02861001

Shafer ABA, Peart CR, Tusso S *et al.* Bioinformatic processing of RAD-seq data dramatically impacts downstream population genetic inference. *Methods in Ecology and Evolution* 2017;**8**:907–17. https://doi.org/10.1111/2041-210X.12700

Sobel JM, Chen GF, Watt LR *et al.* The biology of speciation. *Evolution* 2010;**64**:295–315. https://doi.org/10.1111/j.1558-5646.2009.00877.x

Spano CA, Häussermann V, Acuña FH *et al.* Hierarchical biogeographical processes largely explain the genomic divergence pattern in a species complex of sea anemones (Metridioidea: Sagartiidae: *Anthothoe*). *Molecular Phylogenetics and Evolution* 2018;**127**:217–28. https://doi.org/10.1016/j.ympev.2018.05.022

Stace HM, Armstrong JA, James SH. Cytoevolutionary patterns in Rutaceae. *Plant Systematics and Evolution* 1993;**187**:1–28. https://doi.org/10.1007/bf00994089

Stanton DWG, Frandsen P, Waples RK *et al.* More grist for the mill? Species delimitation in the genomic era and its implications for conservation. *Conservation Genetics* 2019;**20**:101–13. https://doi.org/10.1007/s10592-019-01149-5

Stimpson ML, Bruhl JJ, Weston PH. Could this be Australia's rarest *Banksia*? *Banksia vincentia* (Proteaceae), a new species known from fourteen plants from south-eastern New South Wales, Australia. *Phytotaxa* 2014;**163**:269–86. https://doi.org/10.11646/phytotaxa.163.5.3

Swofford DL. *PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4*. Sunderland, Massachusetts: Sinauer Associates, 2003.

Takezaki N, Nei M. Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. *Genetics* 1996;**144**:389–99. https://doi.org/10.1093/genetics/144.1.389

Thompson LM, Klütsch CF, Manseau M *et al.* Spatial differences in genetic diversity and northward migration suggest genetic erosion along the boreal caribou southern range limit and continued range retraction. *Ecology and Evolution* 2019;**9**:7030–46. https://doi.org/10.1002/ece3.5269

Vähä J, Erkinaro J, Niemelä E *et al.* Life-history and habitat features influence the within-river genetic structure of Atlantic salmon. *Molecular Ecology* 2007;**16**:2638–54. https://doi.org/10.1111/j.1365-294X.2007.03329.x

Young AG, Pickup M. Low S-allele numbers limit mate availability, reduce seed set and skew fitness in small populations of a self-incompatible plant. *Journal of Applied Ecology* 2010;**47**:541–8. https://doi.org/10.1111/j.1365-2664.2010.01798.x