Conservation genomics of *Zieria covenyi* in support of management and translocation activities.

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FINAL REPORT

Research Centre for Ecosystem Resilience

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Acknowledgements

We acknowledge the Traditional Custodians of the land on which the plant species in this study are found on, and pay respects to Elders past and present. We acknowledge all collectors who conducted the field sampling.

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Cover image

Photograph is provided by Sam Yap.

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EXECUTIVE SUMMARY

The genetic diversity of *Zieria covenyi*, a species suspected to be impacted by hybridisation, was measured using high quality genome scans. We provide empirical evidence to show that it is genetically distinct, not prone to hybridisation, and should be recognised as a species. Infraspecific variation is present within the species, with high diversity present at the recently discovered Breakfast Creek site rather than at Narrow Neck which is characterised by extensive clonality, and high genetic disparity reported between the two sites, presumably through historical isolation. Given that the species is currently deemed as pollen sterile based on information obtained from Narrow Neck plants, it is important to update its life history status across the entire distribution of the species (i.e. learn more about the Breakfast Creek site) so that conservation actions can better assist in its recovery. The Breakfast Creek population can be regarded as 'genetically healthy', but continued conservation efforts need to carefully select individuals to preserve the distinct entities within *Z. covenyi*. Assuring maximum levels of genetic diversity in a translocated population increases fitness by reducing the risk of inbreeding and increasing the adaptive potential to environmental change and other pressures. There is currently a paucity of genetic diversity represented in the *ex situ* collections, with only eight genets derived from the Breakfast Creek site and some genets from Narrow Neck. We estimated the necessary combinations of propagules to ensure the establishment of suitably evolutionary resilient translocated populations of various sizes.

1. INTRODUCTION

1.1 Background

Zieria covenyi J.A.Armstr. (Rutaceae), commonly known as Coveny's Zieria is a species listed as Endangered on the NSW Biodiversity Conservation Act 2016 (BC) and the Environment Protection and Biodiversity Conservation Act 1999 (EPBC). It is an aromatic shrub, up to 2 m high, proliferates from root suckers, and known only from the Narrow Neck Peninsula and the Breakfast Creek area, south-west of Katoomba in the Central Blue Mountains. Two populations are known, a small population on Narrow Neck Plateau (between 100-200 plants; this study defines the "Narrow Neck" population as all individuals identified on the Narrow Neck Plataeu and the "Farside" subpopulation which is on the west side of Narrow Neck Plateau) and a larger population of more than 2,000 plants in the upper Breakfast Creek area, approximately 4 km from Narrow Neck (hereon referred to as the "Breakfast Creek" population; *Zieria covenyi* Translocation Plan 2017). All biological / ecological details describing the species are derived from the Narrow Neck population (e.g. Harden 1991, Armstrong 2002, NSW National Parks and Wildlife Service 2002, Threatened Species Scientific Committee 2008) because the Breakfast Creek population was only discovered recently. Morphologically, the Breakfast Creek plants have flowers with a pinkish hue and longer and narrower leaves than those at Narrow Neck (M. Jones pers. obs), and without the necessary population-level genetic investigations it is unclear whether the plants from the Narrow Neck and Breakfast Creek sites are separate populations of *Z. covenyi* or different species entirely.

Recently, the classification and understanding of the genus *Zieria* has been significantly challenged. Poor resolution was observed within multiple clades of *Zieria* based on chloroplast and nuclear DNA (Morton 2015, Barrett et al. 2018), and morphological characters provided little support for species distinction (Armstrong 2002). Given the taxonomic uncertainty within *Zieria* and the lack of sampling at population level, it is impossible to address whether *Z. covenyi* is a distinct species or a morphologically distinct population of the similar-looking *Z. compacta, Z. cytisoides*, and *Z. murphyi* (or *Z. caducibracteata*, M. Duretto pers. comm). A better understanding of the taxonomic status of *Z. covenyi* and its phylogenetic position, necessitates an in-dept analysis of molecular and morphological evidence, providing validation for conservation decisions.

Hybridisation is a relatively common process associated with the evolution of new lineages or species of plants. This process tends to be more pronounced in circumstances where a small population of one species is found in close proximity to a larger population of a closely related species with a shared flowering time. If postzygotic barriers are relaxed, which is common within some *Zieria* species (Barrett et al. 2018), disproportionately large pollen loads received from larger populations can result in genetic swamping of smaller ones. In outcrossing species, the underlying preference of the smaller population to receive outcrossed pollen can maximise the uptake of inter-specific pollen and augment the production of hybrid propagules. If *Z. covenyi* is not a hybrid swarm itself, its small size and restricted distribution are the concerning hallmarks of potential genetic swamping. Surrounding the distribution of *Z. covenyi* a number of species that could potentially introgress into its populations are: *Z. compacta, Z. cytisoides*, *Z. murphyi, Z. arborescens*, *Z. laevigata*, *Z. caducibracteata*, *Z. pilosa*, *Z. involucrata*, *Z. robusta* and *Z. smithii*. Hybridisation can also result in polyploidy, and the high chromosome number (2n=54) of *Z. covenyi* at Narrow Neck suggests it could have arisen from a hybridisation event between diploid and tetraploid parents, such as *Z. caducibracteata* (2n=18) and *Z. cytisoides* (2n=36) (Armstrong 2002). Infertility, which is suspected in *Z. covenyi* given its lack of viable pollen, and dependence on asexual reproduction through prolific root suckering could also be attributable to polyploidy (Herben et al. 2017). Any impact of hybridisation, as well as confirming *Z. covenyi* as a *bona fide* species, will be critical to conservation and management.

Loss of genetic diversity can reduce the health of a population, and consequently increase the risk of local extinction. Genetic health is the concept used to combine current population fitness, and long-term adaptive potential to climatic and environmental changes. The species existence has recently come under further threat as its habitat was impacted by fire that took place in December 2019. The fire burnt all the species' habitat, with only some plants at Narrow Neck left unburnt. Recovery is variable, with most individuals resprouting at Narrow Neck, but not the larger population at Breakfast Creek where surveying still is underway. The populations of *Z. covenyi* have likely suffered loss of genetic diversity and gene flow disruptions. The resprouting habit of *Z. covenyi* compromises our ability to estimate an individual's age and the physical extent of single genets, therefore preventing an accurate assessment of recruitment and demography, let alone estimate how much diversity was lost. Genetic tests of relationships at the population scale are a tractable approach for measuring diversity and identifying demography and relationships within populations of *Z. covenyi*.

Under the premise that *Zieria covenyi* is an endangered species, a conservation and management plan was developed under the Saving Our Species initiative by the New South Wales Office of Environment & Heritage (OEH, now part of the Department of Planning, Industry and Environment (DPIE)). This plan identified site-based management as a priority conservation action, principally because of an inexplicably progressive decline of *Z. covenyi*. In recent years, a translocation plan was proposed for the species to create *ex situ* insurance populations, and there is interest to determine the genetic health of the translocation stock at the Australian Botanic Garden Mt Annan. The Royal Botanic Gardens & Domain Trust (RBG&DT) was contracted by the OEH to conduct a conservation genomics study on *Z. covenyi* to provide foundational knowledge essential for developing effective conservation strategies and help guide management strategies. The benefits of a single genetic study in the framework of conservation work are manifold as outputs can be used to rigorously test species status, quantitatively assess genetic diversity, determine genetic health, identify hybrids and provide practical solutions to long-term management strategies.

1.2 Aims and objective of the conservation genomics study of *Zieria covenyi*

In order to support the long-term management and conservation of *Zieria covenyi*, the conservation genomics study had the following aims:

1. Test the species concept of *Zieria covenyi* by examining it within the current phylogenetic framework.
2. Assess the presence and extent of clonality, level of kinship and remaining genetic diversity within and between populations.
3. Assess diversity and genetic provenance of the *ex situ Z. covenyi* collection at the Australian Botanic Garden Mt Annan (ABGMA).
4. Determine an optimal selection of individuals to be used in a range of translocation scenarios.

2. METHODS:

2.1 Sampling

Sampling of *Zieria covenyi* was mostly undertaken by the conservation officers from Department of Planning, Industry and Environment (DPIE), with supplementary sampling by the Research Centre for Ecosystem Resilience (ReCER) team at RBG&DT. A total 232 specimens of *Z. covenyi* and other *Zieria* species (Table 1) were obtained to test the *Z. covenyi* species concept, determine species relationships, identify levels of hybridisation, measure diversity and connectivity, and verify the identity and kinship of *ex situ* ramets and genets.

Sampled *Z. covenyi* consisted of 182 plants, from three locations: Breakfast Creek is the largest site, with 103 individuals sampled, while Narrow Neck and Farside are smaller with 27 and 40 individuals sampled. Sampling was undertaken across multiple populations and aimed to examine genetic diversity by collecting any *Z. covenyi* visually identified as a distinct individual (i.e. sampling only once from a multi-stemmed individual). Additionally, 12 *Z. covenyi* individuals sourced from the *ex situ* collection were included in the study to determine if the collection represent *in situ* genetic diversity.

Z. covenyi is speculated to be of hybrid origin due to the morphological similarity between the its plants from Narrow Neck and some of the *Zieria* species (*Z. caducibracteata* and *Z. cytisoides*), and unusual chromosome number of *Z. covenyi*, which was also observed from an individual from Narrow Neck (Armstrong 2002). To test this, specimens of multiple target species from different areas were included via consultation from relevant curator at the National Herbarium of New South Wales (Dr. M. Duretto): *Z. caducibracteata* (Budawangs, Newhaven Creek and Wog Wog) and *Z. cytisoides* (Minto Heights, Glenbrook Gorge, Oxley Wild Rivers National Park, Rysltone, Glen Davis, Canyonleigh).

Species in sympatry with *Z. covenyi* were also sampled: *Z. laevigata* at Narrow Neck and *Z. pilosa* at Narrow Neck and Wentworth Falls. Non sympatric individuals of *Z. laevigata* at Beacon Hill and *Z. pilosa* at Royal National Park were also included to investigate potential gene flow with *Z. covenyi*. Other species occurring in the same region as *Z. covenyi* in the Blue Mountains were included, but depending on availability of herbarium specimens / living

collection, sampling of each species may not necessary have been conducted locally: *Z. compacta* (Nepean State Conservation Area), *Z. involucrata (Glenorie and Maroota State Forest), Z. murphyi* (Mt Tomah), *Z. arborescens* (subsp. *arborescens* Laurieton, Kulnura; subsp. *decurrens* unsure origin, sourced from the Australian Botanic Garden Mt Annan).

2.2 DNA extraction and sequencing

All samples were sent to Diversity Arrays Technology (DArT) Pty Ltd in Canberra for DNA extraction and genotype-by-sequencing analysis (referred to as DArTseq analysis) using the documented in-house procedure. DNA was extracted from each sample using the Plant DNA Extraction Protocol for DArT.

2.3 Data analysis

2.3a Quality screening and control of Single Nucleotide Polymorphism data:

Single nucleotide polymorphisms (SNPs) data was quality checked using the filtering scripts implemented by an in-house designed package called RRtools package v1.0 (as described in Rossetto *et al.* 2019) in the open source program, R (version 3.3.0, R Core Development Team 2013). Loci that did not pass standardised quality thresholds were removed from the data and were not used in downstream analysis. To ensure that only the higher quality DArTseq markers were used for analyses, all SNPs with a reproducibility (proportion of replicate assay pairs for which the marker score is consistent) of less than 96% and which had more than 30% missing data were excluded from the dataset.

2.3b Genetic relationships considering reticulate evolution and hybridisation

Splitstree Program ver. 4.14.6 (Huson et al. 2008) with default software settings was used to generate a network from the quality SNP matrix to provide a preliminary estimation of genetic relationships across the entire dataset. The network can represent evolutionary histories with substantial reticulation that arise from incomplete lineage sorting and hybridisation, which in the network are indicated by the extent of “webbing” associated between branches of the network.

2.3c Phylogenetic analyses

A phylogenetic tree attempts to explain relationships from an evolutionary history (i.e. based on inference of a common ancestor) rather than by observed genetic or phenetic similarities. Since taxonomy is integrated with an understanding of evolutionary history and lineage diversification, examining the evolutionary history of *Z. covenyi* is essential for making taxonomic decisions and identifying potential hybridisation events. The coalescent-based phylogenetic tool SVDquartets package ver. 1 (Chifman and Kubatko 2014) implemented in the PAUP software v4.0a (Swofford 2002) was used to evaluate the position of *Z. covenyi* within the *Zieria* phylogeny. The multispecies coalescent model was set up with the following parameters: 100,000 quartets and 1000 bootstrap replicates (as a measure of branch support). This program is designed to accept SNP data and can accept a large number of specimens and data while still producing relatively robust phylogenetic results (see Chou et al. 2015 for a critical review of this program). We examined results of all analyses using at least three independent runs for multi-species coalescent analysis by allocating samples within their respective populations.

2.3d Principal coordinate analyses

Adegenet 2.1.1 package in R (version 3.3.0, R Core Development Team) was used to perform a Principal Component Analysis (PCA) to better understand relationships between individuals and populations. This method of PCA derives an ordination based on Euclidean transformed dissimilarity matrix of the data.

2.3e Population genetic diversity measures

In order to evaluate F-statistics and population-level measures of diversity, populations consisting of five or more individuals were used. The RRtools package v1.0 was used for generating a matrix of spatial distances between the populations and calculating expected (H_e) and observed heterozygosity (H_o) and inbreeding coefficient (F_{is}) across each population.

2.3f Kinship

Genetic similarity between individuals located at the same site and corresponding cultivated material was estimated using the unweighted pair group method with arithmetic mean (UPGMA) hierarchical clustering method as implemented in the phanghorn package v2.4.0 in

R. Kinship (relatedness) measurements were used in assessing the degree of clonality across the *in situ* plants of *Z. covenyi* and identify genets and ramets of *Z. covenyi* in cultivation. Pairwise kinship coefficient was estimated from the genotype data using an Identity-by-descent (IBD) analysis in SNPrelate package v1.17.1 in R. Distance matrices of pairwise kinship were generated for each *Z. covenyi* site based on observation from preliminary results from principal component and network analyses that clonality occur within each site. The matrices were combined to generate a supermatrix that was drawn using the heatmap function from the Phytools package v0.6-60.

2.3g Optimal genetic diversity for translocation

If maximal genetic diversity (and hence greater expected fitness) is desired in a population created by future translocation efforts, an explicit proportion of genetically distinct individuals (i.e. genets determined from the kinship results) will be required to minimise the risk of inbreeding depression and maximise diversity in translocated populations. Given that *Z. covenyi* can be propagated from cuttings, we explored what could be used from *in situ* to create optimal genetic diversity and population size for future translocation work. Optimisation of mixtures analyses were implemented on the package OptGenMix developed at the RBG&DT, Sydney (Bragg et al. 2020). This package sought the optimal proportion of genets by evaluating the highest proportion of shared alleles among individuals for all combinations. It should be noted that our estimate of genetic diversity is not always comprehensive, and therefore genetically similar individuals might represent important variation at any given allele, and survival of translocated individuals is not guaranteed.

3. RESULTS AND INTERPRETATION

3.1 Summary

We report results based on genomic analyses of 232 *Zieria* samples to assess the species concept and genetic history of the endangered *Z. covenyi,* and provide an understanding of remaining genetic diversity to assist with future conservation and management. High-quality genome scans from DArTseq enabled differentiation between *Zieria* species, quantification of genetic diversity and relatedness between and within populations, assessment of kinship and admixture, and an estimation of associative patterns between genetic and geographic structure.

The significant findings are:

- *Zieria covenyi* is a distinct species;
- *Z. covenyi* sampled around Narrow Neck is characterised by extensive clonality: sampled individuals formed three genetic clusters, each cluster being extremely clonal and consisting of a single genet;
- The highest diversity for *Z. covenyi* was detected at Breakfast Creek where clonality and inbreeding was minimal;
- *Ex situ* collection of *Z. covenyi* does not represent *in situ* genetic diversity. Only eight genets from the collection are derived from Breakfast Creek although the collection also contains some plants sourced from the Narrow Neck area;
- Translocation scenarios based on the empirical evolutionary information on *Z. covenyi* are provided.

3.2 *Zieria covenyi* is a distinct species.

Our genetic analysis of 2,633 genome-wide markers (SNPs) shows that 182 specimens identified as *Zieria covenyi* are distinct from 50 specimens representing eight *Zieria* species. As the species is locally highly clonal, only a representative from each genet was used in the phylogenetic analysis (Fig. 2, 3). The network shows that the main population of *Z. covenyi* in Breakfast Creek forms a tight cluster, with the three genets from Narrow Neck or Farside appearing slightly divergent from the Breakfast Creek individuals (Fig. 2). This cluster sits at the tip of a main branch separate from *Z. caducibracteata*, *Z. murphyi*, *Z. arborescens* and *Z. involucrata*, despite the fact that some of these species are considered morphologically similar to *Z. covenyi*. Even more genetically dissimilar to *Z. covenyi* are *Z. laevigata*, *Z. pilosa*,

Z. cytisoides and *Z. compacta*, some of which occur sympatrically, which suggest that there is no genetic exchange between core *Z. covenyi* and unrelated species.

A recent phylogenetic study could not resolve relationships among *Zieria* including *Z. covenyi* (Barrett et al. 2018). Our phylogenetic analyses identified well-resolved species boundaries representing two strongly differentiated clades of species (Fig. 3). A larger clade which consists of *Z. covenyi*, *Z. caducibracteata*, *Z. murphyi*, *Z. arborescens* and *Z. involucrata* (similar to the network output) and also supporting the soundness of *Z. covenyi* species concept. A distinct sister clade consists of *Z. murphyi* and *Z. caducibracteata* (Fig. 3), but a more comprehensively sampled dataset that includes more individuals of *Z. murphyi* and *Z. caducibracteata* will be required to pinpoint the closest relative of *Z. covenyi* (and test the species concept of *Z. murphyi* as the phylogenetic analysis suggests it is not monophyletic with respect to *Z. caducibracteata;* Fig. 3).

3.4 Genetic health across *Zieria covenyi*

Zieria covenyi is described as pollen sterile and only reproduces vegetatively through root suckering, based on plants sourced from Narrow Neck (Armstrong 2002). A comprehensive understanding of standing genetic diversity was required to better diagnose the genetic health and prepare well-informed long-term management strategies for *Z. covenyi.* Evolutionary resilience was quantified by measuring the extent of clonality, kinship and genetic diversity at population scale. Our genomic data from 182 samples representing all *Z. covenyi* sites showed that genetic variability is present within some sites and between sites.

Given previously described reliance of *Z. covenyi* on asexual reproduction (Armstrong 2002), our results unsurprisingly show that the individuals at Narrow Neck and Farside are clonal (Fig. 4). Clonality at those sites was extensive, and similar to that measured for the endangered *Z. bauerlenii* which is also pollen sterile and reliant on asexual reproduction (Sharma 2001). For *Z. covenyi* only two genets with multiple ramets were detected at Narrow Neck, with one genet, NN genet 1, consisting of 20 ramets, and the other, NN genet 2, of 7 ramets. More extensive clonality was detected at Farside, where only a genet with 40 ramets was observed (FarNN genet 1). The ramets from NN genet 1 are up to 80 m apart (Fig. 5), suggesting they originated and persisted for a relatively long time. As the extent and speed of the rhizomatous growth is unknown it is difficult to exactly estimate how long these individuals have been at the site.

Less clonality was observed at Breakfast Creek where, out of 112 samples, we identified 17 genets with multiple ramets, as well as 60 genets consisting of a single ramet (for a total of 77 genetically distinct individuals; Table 2). Less clonality, coupled with the presence of genets of single ramet and genets that are related but not clonal (i.e. siblings) suggest *Z. covenyi* is capable of sexual reproduction, contradicting the description that the species only reproduces vegetatively through root suckering (Armstrong 2002). This is because species' description preceded the recent discovery of the Breakfast Creek site in 2015, which means past literature describing the species' characteristics and life history traits was based on Narrow Neck alone (literature sources: Harden 1991, Armstrong 2002, NSW National Parks and Wildlife Service 2002, Threatened Species Scientific Committee 2008). This would be adequate for a species with admixed populations and consistent morphology, but this is not the case for *Z. covenyi*. There are differences in floral and leaf morphology at Breakfast Creek and Narrow Neck (as explained in the Introduction), and in our PCA ordinations the *Z. covenyi* genets from Breakfast Creek form a separate cluster from the Narrow Neck and Farside genets (Fig. 6). Additionally, the considerably larger Breakfast Creek population was characterised by higher levels of heterozygosity than Narrow Neck and Farside, as well as low biparental inbreeding (Table 3) indicating the importance of the Breakfast Creek site as the source of diversity for *Z. covenyi*.

Our results show the Breakfast Creek and Narrow Neck are genetically differentiated sites, presumably due to historical isolation, and therefore the population at the Breakfast Creek site needs to be surveyed in order to get a better description of *Z. covenyi*. Given that the species is currently deemed as pollen sterile, it is important to update its life history status across the entire distribution of the species (i.e. learn more about the Breakfast Creek site) so that conservation actions can better assist in its recovery. This is because our genetic findings suggest that is possible that the Narrow Neck population might not be representative of the species (hence the sterility, high levels of clonality, high ploidy).

Ex situ collection of Zieria covenyi

The *ex situ* collection of *Z. covenyi* from ABGMA was included in this study to assess if it is representative of *in situ* genetic diversity. Kinship results identified three plants derived from Narrow Neck, with two plants belonging to the NN genet 1, and one of the plants belonging to the NN genet 2. Nine plants from the ABGMA collection originated from Breakfast Creek, with four matching to genotyped individuals sampled *in situ* (Table 2, Fig. 5). Five other plants sourced from Breakfast Creek did not match any genotyped individuals. This highlights the

importance of including *ex situ* material within conservation genetic studies, as it might identify genets that are no longer alive *in situ* but might still be able to contribute to the species recovery.

3.5 Projected estimates for translocation of *Zieria covenyi*.

After field collection of *Zieria covenyi* for this study, it was reported that its entire population (on Narrow Neck Plateau and Breakfast Creek) was affected by fire in December 2019. The species is expected to recover from a low intensity fire by resprouting based on observations in July that among the 89 plants at the Narrow Neck site that were burnt, about 13 re-sprouted (M. Jones pers. obs). However, high intensity fires may have impacted Breakfast Creek site where metal tags on the *Z. covenyi* melted due to the hot fires. Post-fire surveys are currently underway to determine the extent of loss.

To aid in post-fire recovery, we analysed the genomic data to estimate the combinations of propagules needed to create evolutionary resilient translocation populations of various sizes. As an evolutionary resilient population can only be viable if propagules are sexually reproducing, our main focused was to estimate translocation populations using only genets from Breakfast Creek as potential propagules (Fig. 7, 8). We designed our translocation populations using cuttings due to the ease of this mode of propagation (as opposed to seed propagation which would be difficult in *Z. covenyi*). We provide results that include all *Z. covenyi* genets just in case, fertility is restored at Narrow Neck (Fig. 9). It is worth noticing, that as identified in the genetic study, the potential loss of the Breakfast Creek individuals could have significant consequences, as most of the genetic diversity is found within those individuals. Hence recovery and translocation efforts should be focused on those individuals (if possible).

We tested the diversity of the translocation populations by examining the proportion of common SNPs *in situ* were polymorphic (i.e. captured) in the propagation populations. All simulations showed that translocated individuals selected by optimisation based on available genomic data or by using a random sampling approach (once the understanding of clonality is taken into account) can both be effective in capturing high genetic diversity (Fig. 7, 8). In the case where only Breakfast Creek genets were used, at least 30 propagules each from a genetically different individual that is spaced at least 10 m apart will be required (Fig. 8). Having propagules from at least 30 genetically distinct individuals ensures 98% or more of the

diversity represented in the dataset is captured (Fig. 7). If Narrow Neck genets are to be included at least 20 propagules will be required (Fig. 9). The number needed is lower because the Narrow Neck population is more differentiated from the Breakfast Creek individuals, however based on the current uncertainty of the highly clonal and apparently sterile individuals at Narrow Neck actually represent, a cautionary approach would be to leave them out from a translocation plan.

It should be noted that if additional individuals of *Z. covenyi* were to be genotyped, it will be possible to re-calculate the optimal selection scenarios accordingly (although given the amount of diversity captured and its distribution, we would not expect a significantly different outcome). Furthermore, we will be happy to re-calculate the translocation scenarios when the individuals that perished during the fire have been determined, to estimate the necessary combinations of propagules to ensure the establishment of suitably evolutionary resilient translocated populations of various sizes.

Accounting the loss of genetic diversity as a result of this event will also be important because genetic diversity promotes fitness and reduces the risk of inbreeding and adaptation to selective environmental pressures. An approach to estimate the amount of evolutionary potential lost has been developed by the ReCER team at RBG&DT, and was used in other SoS projects. We will be happy to assess the loss through the genomic data if requested.

4. CONCLUSIONS AND IMPLICATIONS

This project highlights the following:

- *Zieria covenyi* is a distinct species;
- The considerably larger and more diverse Breakfast Creek is genetically distinct from the Narrow Neck population, and consequently the original species descriptions that only characterised plants from Narrow Neck needs updating;
- *Z. covenyi* at Narrow Neck and Farside are characterised by extensive clonality;
- Most genetic diversity of *Z. covenyi* is at Breakfast Creek where low clonality was detected;
- The *ex situ* collection for *Z. covenyi* consists of a low amount of genetic diversity, consisting of 11 genets, three from Narrow Neck area and eight from Breakfast Creek area (six of which represent identified *in situ* genets of *Z. covenyi*.

Translocation scenarios for *Zieria covenyi* is proposed. To maximise genetic diversity in translocated populations, we recommend:

- A part targeted, part random approach, involving the selection of at least 30 individuals, that is spaced at least 10 m apart in the source population to avoid selecting ramets of a genet.
- A targeted approach, relying on genetically selected individuals based on a range of propagation targets. This is currently not achievable, as the tags on individuals are melted.

5. FIGURES AND TABLES

Figure 1: Location of *Zieria covenyi* individuals in the Narrow Neck Peninsula and Breakfast Creek area at the Blue Mountains National Park.

Each dot on the map represents an individual of *Z. covenyi* with genotype information.

The location of genets of multiple ramets identified at the Narrow Neck site is labelled in the map. The location of genets of multiple ramets at Breakfast Creek is labelled in Fig. 6.

Table 1: *Zieria covenyi* study sampling details. Asterisk means sample was obtained from voucher specimen from the National Herbarium of New South Wales.

Species	Site	N samples
Zieria covenyi	Breakfast Creek (103), Narrow Neck (27), Farside (40) ABGMA (9 sourced Breakfast Creek, 3 sourced from Narrow Neck),	182
Zieria arborescens	subspecies arborescens (Kulnurra*, Laurieton*) subspecies decurrens (unknown source, cultivated at Mt Annan)	3
Zieria caducibracteata	Budawangs*, Morton National Park*, Newhaven Creek*, Wog Wog*	5
Zieria involucrata	Glenorie*, Maroota State Forest*	2
Zieria murphyi	Mt Tomah*	1
Zieria laevigata	Narrow Neck (5), Beacon Hill (6)	11
Zieria pilosa	Narrow Neck (1), WentWorth Falls (6), Lake Parramatta (2), Karloo Pools (6),	15
Zieria cytisoides	Canyonleigh*, Glen Davis*, Glenbrook Gorge*, Nortons Basin Reserve*, Oxley Wild Rivers National Park*, Rylstone* (1 each) Minto Heights (6)	12
Zieria compacta	Nepean State Conservation Area*	1
	Total	232

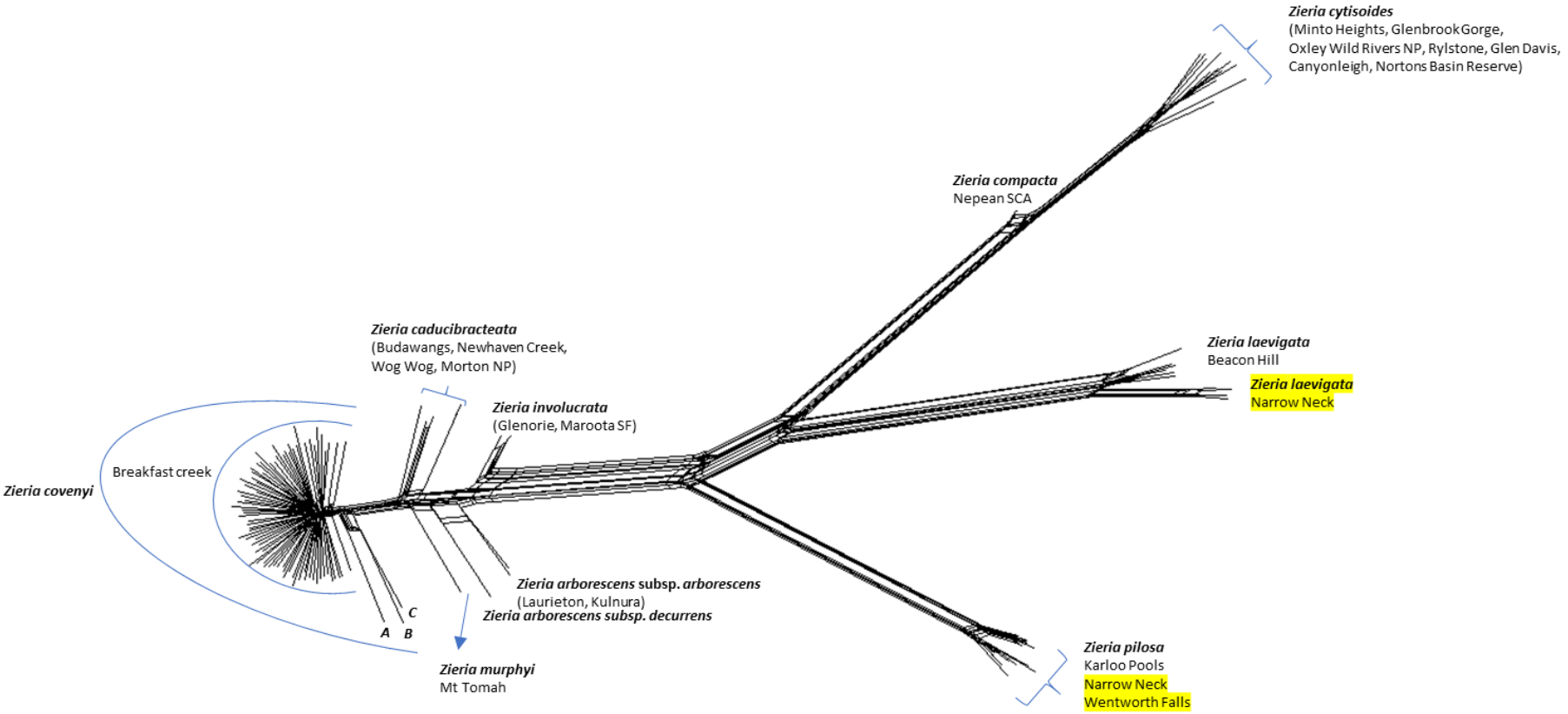

Figure 2: Splitstree network analysis of Single Nucleotide Polymorphism (SNP) data from individuals from 130 specimens representing genets of *Z. covenyi* and its associated putative hybrids, and specimens of eight *Zieria* taxa.

Clones were removed before running this analysis. Individuals from Narrow Neck belong to genets of multiple ramet: A: Farside genet 1, B: NN genet 1, C: NN genet 2.

Species that are sympatric with *Z. covenyi* are highlighted in yellow. Note that *Zieria pilosa* collected from Wentworth falls occurs about 10.5 km from the Narrow Neck sites.

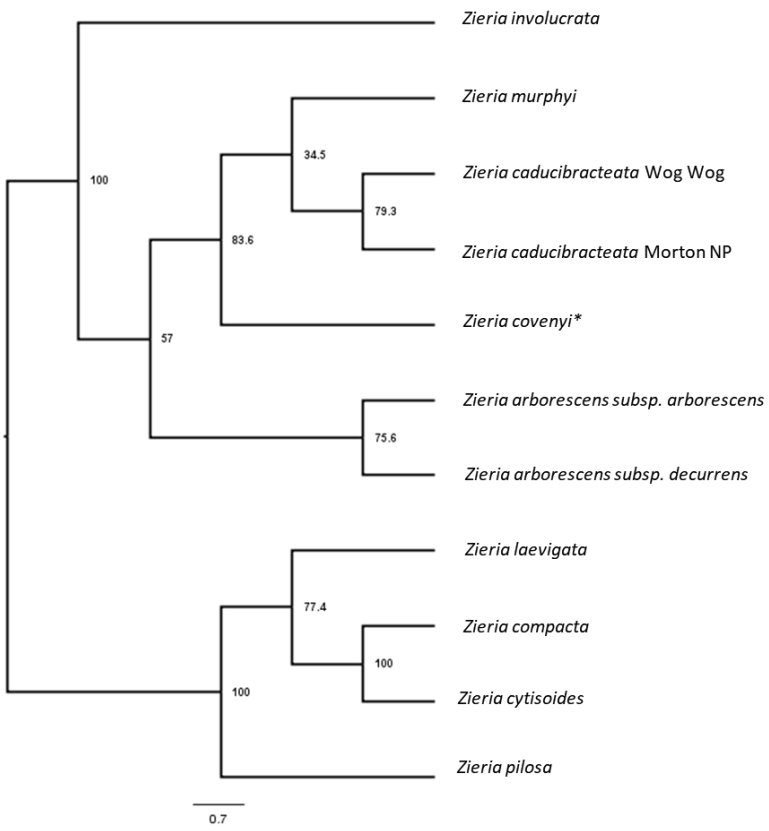

Figure 3: Phylogenetic coalescent tree produced from SVDquartets analysis of SNP data generated from multiple representatives of multiple representatives of *Zieria* and *Zieria covenyi*.

The asterisk next to *Z. covenyi* is to indicate both Breakfast Creek genets were included in this analysis.

Representatives of *Zieria* used here includes eight *Zieria* species, two subspecies of a species and two potentially different lineages of another species. Our results indicate *Z. covenyi* is bona fide species, sister a clade of *Z. caducibracteata* and *Z. murphyi*, and there is strong support that these three species are highly distinct from the clade consisting of *Z. laevigata*, *Z. compacta*, *Z. cytisoides* and *Z. pilosa*.

Bootstrap support values above 50% are placed above branches, and higher than 80% are considered strong support. The total weight of compatible quartets = 84.52 % was relatively higher than total weight of incompatible quartets = 15.48 %, which means probability of lineage sorting is relatively low, supporting *Z. covenyi* as a distinct species.

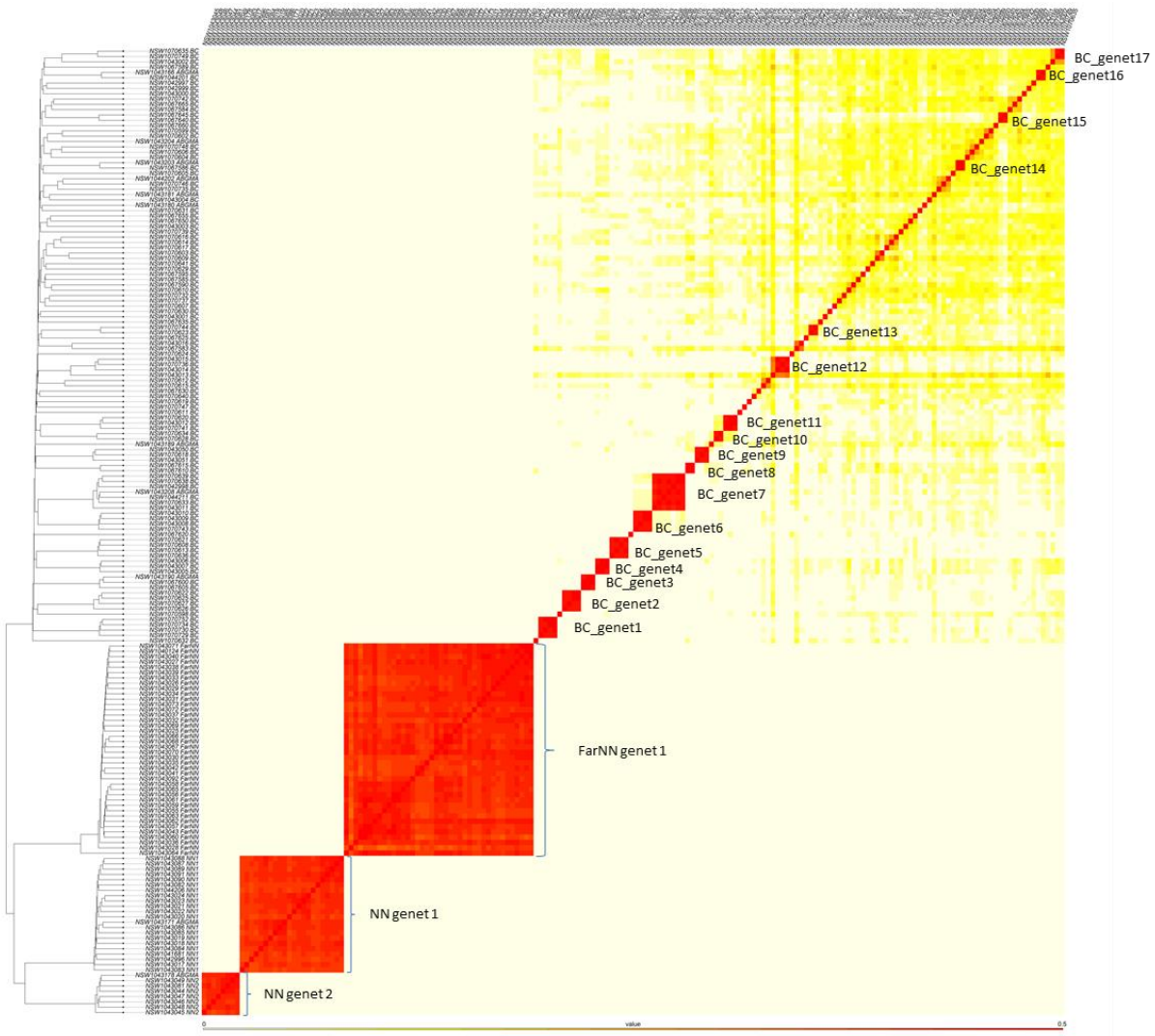

Figure 4: Composite UPGMA tree/Kinship heatmap analysed from Single Nucleotide Polymorphism loci for 182 specimens of *Zieria covenyi* from Breakfast Creek, Narrow Neck and Farside.

All 182 specimens were studied in a pairwise kinship analysis, and this resulted in the heatmap above consists of pairwise kinship coefficients displayed as colours: RED colouration corresponding to the highest pairwise kinship coefficients (0.4 or greater = clone), ORANGE-YELLOW colouration corresponding to medium pairwise kinship coefficients (less than 0.4 but greater than 0.25 = sibling) and EGGSHELL WHITE colouration corresponding to the lowest pairwise kinship coefficients (0). The descending red diagonal on the graph is the result of an individual matched with itself.

To the left of the heatmap is a UPGMA tree generated from a distance-based matrix of the SNP data. The tree tip labels include each *Z. covenyi* individual's unique ID (i.e. NSW number) and site information (see Table 2 for details on genets of multiple ramets). The tree shows Z. *covenyi* individuals from Breakfast Creek are different from those at Narrow Neck and Farside.

The heatmap shows extensive clonality around Narrow neck, individuals belong to one of three unique genets of multiple ramets.

Table 2 List of samples and notes on clonality.

Under the "Genet info" column, only samples belonging to genets of multiple ramets were listed.

Sampling Locality	Sample	Genet info	Information on yellow packet
Breakfast Creek	NSW1070734	BC genet1	B. Ck-ZcBg88
	NSW1070752	BC genet1	B. Ck-ZcBg87
	NSW1070729	BC genet1	B. Ck-ZcBg86
	NSW1070730	BC genet1	B. Ck-ZcBg85
	NSW1070627	BC genet2	B. Ck-ZcBg37
	NSW1070625	BC genet2	B. Ck-ZcBg34
	NSW1070622	BC genet2	B. Ck-ZcBg35
	NSW1070626	BC genet2	B. Ck-ZcBg36
	NSW1067605	BC genet3	B. Ck-ZcBg6
	NSW1067600	BC genet3	B. Ck-ZcBg5
	NSW1043007	BC genet4	B. Ck-ZcBg44
	NSW1043006	BC genet4	B. Ck-ZcBg45
	NSW1043005	BC genet4	B. Ck-ZcBg46
	NSW1070636	BC genet5	B. Ck-ZcBg50
	NSW1070613	BC genet5	B. Ck-ZcBg31
	NSW1070608	BC genet5	B. Ck-ZcBg30
	NSW1070621	BC genet5	B. Ck-ZcBg29
	NSW1043008	BC genet6	B. Ck-ZcBg43
	NSW1070743	BC genet6	B. Ck-ZcBg84
	NSW1043009	BC genet6	B. Ck-ZcBg42
	NSW1043010	BC genet6	B. Ck-ZcBg41
	NSW1044211	BC genet7	No_data
	NSW1070633	BC genet7	B. Ck-ZcBg81
	NSW1070638	BC genet7	B. Ck-ZcBg83
	NSW1043011	BC genet7	B. Ck-ZcBg40
	NSW1070639	BC genet7	B. Ck-ZcBg82
	NSW1042998	BC genet7	B. Ck-ZcBg39
	NSW1067610	BC genet8	B. Ck-ZcBg7
	NSW1067615	BC genet8	B. Ck-ZcBg08
	NSW1070618	BC genet9	B. Ck-ZcBg38
	NSW1043050	BC genet9	B. Ck-ZcBg48
	NSW1043051	BC genet9	B. Ck-ZcBg47
	NSW1070628	BC genet10	B. Ck-ZcBg80
	NSW1070634	BC genet10	B. Ck-ZcBg79
	NSW1070741	BC genet11	B. Ck-ZcBg26
	NSW1043012	BC genet11	B. Ck-ZcBg27
	NSW1070620	BC genet11	B. Ck-ZcBg28
	NSW1043014	BC genet12	B. Ck-ZcBg24
	NSW1070736	BC genet12	B. Ck-ZcBg61

	NSW1043015	BC genet12	B. Ck-ZcBg23
	NSW1070623	BC genet13	B. Ck-ZcBg91
	NSW1070744	BC genet13	B. Ck-ZcBg90
	NSW1067586	BC genet14	B. Ck-ZcBg1
	NSW1067640	BC genet15	B. Ck-ZcBg13
	NSW1067645	BC genet15	B. Ck-ZcBg14
	NSW1044201	BC genet16	No_data
	NSW1070749	BC genet17	B. Ck-ZcBg53
	NSW1070635	BC genet17	B. Ck-ZcBg52
	NSW1067585		B. Ck-ZcBg2
	NSW1070629		B. Ck-ZcBg78
	NSW1070641		B. Ck-ZcBg77
	NSW1070614		B. Ck-ZcBg73
	NSW1070640		B. Ck-ZcBg76
	NSW1067595		B. Ck-ZcBg4
	NSW1070616		B. Ck-ZcBg71
	NSW1070609		B. Ck-ZcBg74
	NSW1070603		B. Ck-ZcBg75
	NSW1070615		B. Ck-ZcBg70
	NSW1070617		B. Ck-ZcBg72
	NSW1070611		B. Ck-ZcBg68
	NSW1070737		B. Ck-ZcBg65
	NSW1070607		B. Ck-ZcBg66
	NSW1070612		B. Ck-ZcBg69
	NSW1070610		B. Ck-ZcBg67
	NSW1070732		B. Ck-ZcBg64
	NSW1070747		B. Ck-ZcBg63
	NSW1067590		B. Ck-ZcBg3
	NSW1067620		B. Ck-ZcBg9
	NSW1067650		B. Ck-ZcBg15
	NSW1067625		B. Ck-ZcBg10
	NSW1067630		B. Ck-ZcBg11
	NSW1067635		B. Ck-ZcBg12
	NSW1067655		B. Ck-ZcBg16
	NSW1067660		B. Ck-ZcBg17
	NSW1067665		B. Ck-ZcBg 18
	NSW1067584		B. Ck-ZcBg19
	NSW1043001		B. Ck-ZcBg97
	NSW1067589		B. Ck-ZcBg20
	NSW1067583		B. Ck-ZcBg21
	NSW1043000		B. Ck-ZcBg98
	NSW1043002		B. Ck-ZcBg96
	NSW1043013		B. Ck-ZcBg25
	NSW1043016		B. Ck-ZcBg22
	NSW1043003		B. Ck-ZcBg95
	NSW1070742		B. Ck-ZcBg62

	NSW1042999		B. Ck-ZcBg99
	NSW1070606		B. Ck-ZcBg60
	NSW1043004		B. Ck-ZcBg94
	NSW1070602		B. Ck-ZcBg56
	NSW1070604		B. Ck-ZcBg59
	NSW1070748		B. Ck-ZcBg57
	NSW1042997		B. Ck-ZcBg100
	NSW1070605		B. Ck-ZcBg58
	NSW1070599		B. Ck-ZcBg55
	NSW1070735		B. Ck-ZcBg93
	NSW1070746		B. Ck-ZcBg92
	NSW1070632		B. Ck-ZcBg101
	NSW1070739		B. Ck-ZcBg89
	NSW1070598		B. Ck-ZcBg54
	NSW1070624		B. Ck-ZcBg33
	NSW1070630		B. Ck-ZcBg51
	NSW1070631		B. Ck-ZcBg49
	NSW1070619		B. Ck-ZcBg32
Farside	NSW1043032	FarNN genet 1	Farside33
	NSW1043035	FarNN genet 1	Farside24
	NSW1043036	FarNN genet 1	Farside28
	NSW1043033	FarNN genet 1	Farside32
	NSW1043034	FarNN genet 1	Farside30
	NSW1040124	FarNN genet 1	Farside31
	NSW1043030	FarNN genet 1	Farside35
	NSW1043038	FarNN genet 1	Farside26
	NSW1043027	FarNN genet 1	Farside38
	NSW1043029	FarNN genet 1	Farside36
	NSW1043031	FarNN genet 1	Farside34
	NSW1043025	FarNN genet 1	Farside40
	NSW1043026	FarNN genet 1	Farside39
	NSW1043028	FarNN genet 1	Farside37
	NSW1043037	FarNN genet 1	Farside27
	NSW1043040	FarNN genet 1	Farside24
	NSW1043072	FarNN genet 1	Farside2
	NSW1043041	FarNN genet 1	Farside23
	NSW1043073	FarNN genet 1	Farside1
	NSW1043039	FarNN genet 1	Farside25
	NSW1043056	FarNN genet 1	Farside19
	NSW1043071	FarNN genet 1	Farside3
	NSW1043068	FarNN genet 1	Farside6
	NSW1043055	FarNN genet 1	Farside20
	NSW1043067	FarNN genet 1	Farside8
	NSW1043059	FarNN genet 1	Farside17
	NSW1043063	FarNN genet 1	Farside13
	NSW1043042	FarNN genet 1	Farside22

	NSW1043060	FarNN genet 1	Farside16
	NSW1043043	FarNN genet 1	Farside21
	NSW1043092	FarNN genet 1	Farside7
	NSW1043062	FarNN genet 1	Farside14
	NSW1043066	FarNN genet 1	Farside9
	NSW1043069	FarNN genet 1	Farside5
	NSW1043061	FarNN genet 1	Farside15
	NSW1043070	FarNN genet 1	Farside4
	NSW1043065	FarNN genet 1	Farside10
	NSW1043057	FarNN genet 1	Farside18
	NSW1043058	FarNN genet 1	Farside11
	NSW1043064	FarNN genet 1	Farside12
Narrow neck	NSW1043020	NN genet 1	Narrow neck-Zcg11
	NSW1043022	NN genet 1	Narrow neck-Zcg9
	NSW1043023	NN genet 1	Narrow neck-Zcg8
	NSW1043021	NN genet 1	Narrow neck-Zcg10
	NSW1043024	NN genet 1	Narrow neck-Zcg7
	NSW1043018	NN genet 1	Narrow neck-Zcg13
	NSW1043019	NN genet 1	Narrow neck-Zcg12
	NSW1043017	NN genet 1	Narrow neck-Zcg14
	NSW1043085	NN genet 1	Narrow neck-Zcg23
	NSW1042996	NN genet 1	Narrow neck-Zcg15
	NSW1041681	NN genet 1	Narrow neck-Zcg16
	NSW1043086	NN genet 1	Narrow neck-Zcg22
	NSW1043091	NN genet 1	Narrow neck-Zcg17
	NSW1043087	NN genet 1	Narrow neck-Zcg21
	NSW1043088	NN genet 1	Narrow neck-Zcg20
	NSW1043082	NN genet 1	Narrow neck-Zcg26
	NSW1043083	NN genet 1	Narrow neck-Zcg25
	NSW1043090	NN genet 1	Narrow neck-Zcg18
	NSW1043089	NN genet 1	Narrow neck-Zcg19
	NSW1043084	NN genet 1	Narrow neck-Zcg24
	NSW1043046	NN genet 2	Narrow neck-Zcg4
	NSW1043047	NN genet 2	Narrow neck-Zcg3
	NSW1043044	NN genet 2	Narrow neck-Zcg6
	NSW1043048	NN genet 2	Narrow neck-Zcg2
	NSW1043045	NN genet 2	Narrow neck-Zcg5
	NSW1043081	NN genet 2	Narrow neck-Zcg27
	NSW1043049	NN genet 2	Narrow neck-Zcg1
ABGMA	NSW1044206	NN genet 1 (ABGMA)	Mt Annan Accession Number: A20180186111
	NSW1043171	NN genet 1 (ABGMA)	Mt Annan Accession Number: A2018-0186/1
	NSW1043178	NN genet 2 (ABGMA)	Mt Annan Accession Number: A2018-0191/2
	NSW1043190	BC genet3 (ABGMA)	Mt Annan Accession Number: AC20170467

	NSW1043208	BC genet7 (ABGMA)	Mt Annan Accession Number: A2018-0189/1
	NSW1043203	BC genet14 (ABGMA)	Mt Annan Accession Number: AC20170466
	NSW1043166	BC genet16 (ABGMA)	Mt Annan Accession Number: 2018-0190/1
	NSW1044202		Mt Annan Accession Number: A2018018811
	NSW1043189		Mt Annan Accession Number: AC20170466
	NSW1043204		Mt Annan Accession Number: AA20170471
	NSW1043181		Mt Annan Accession Number: A2018-0188/1
	NSW1043180		Mt Annan Accession Number: AA20170472


Sensitive data

Figure 5: Zoomed in maps of the Narrow Neck and Breakfast Creek area indicating the location of each *Z. covenyi* individual.

Each dot is an individual and each red dot is a genet of single ramet. Other coloured dots indicate genets with multiple ramets, with dots of the same colour showing ramets of a unique genet. More information about the genets of multiple ramets is provided in Table 2. Dot "A" is the individual (NSW1067586) labelled as BC genet 14 (a genet of single ramet), and source of *ex situ* plant, NSW1043203. Dot "B" is the individual (NSW1044201) labelled as BC genet 16 (a genet of single ramet), and source of *ex situ* plant, NSW1043166.

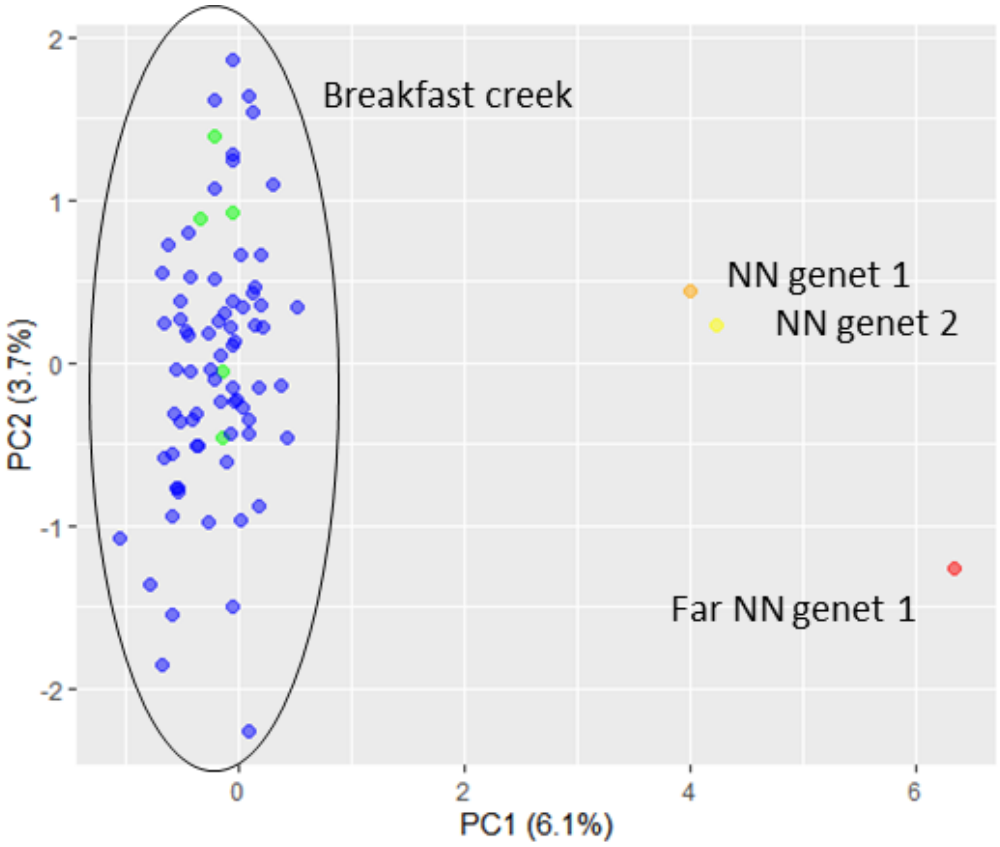

Figure 6: Principal component analysis generated from Single Nucleotide Polymorphism (SNP) data of *Zieria covenyi*.

Ramets of a genet detected in *Z. covenyi* were removed before generating these analyses.

Green dots are plants from the *ex situ* collection at ABGMA that are genets of single ramets.

Table 3: Observed heterozygosity (H_O), expected heterozygosity (H_E), inbreeding coefficient (F_{IS}) and number of unique genets (N) for the populations of *Z. covenyi* and populations of other *Zieria* species. The asterisk identifies samples that match to original *in situ* source plants.

Species/Site	N genets	N sampled originally	H_O	H_E	F_{IS}
Zieria smithii					
Newington	6	6	0.523	0.341	-0.473
LennoxBridge	6	6	0.438	0.293	-0.434
LakeParramatta	6	6	0.425	0.28	-0.461
Wollstonecraft	6	6	0.462	0.254	-0.746
Zieria pilosa					
Wentworth Falls	7	7	0.051	0.064	0.187
Karloo Pool, Royal National Park	6	6	0.061	0.175	0.587
Zieria cytisoides					
Minto Heights	6	6	0.146	0.087	-0.59
Zieria laevigata					
Beacon Hill	6	6	0.176	0.119	-0.375
Narrow Neck	5	5	0.155	0.277	0.538
Zieria covenyi					
BC	77	112 (9 from ABGMA)	0.316	0.302	-0.012
Far Narrow Neck	1	40	0.107		
Narrow Neck	3	30 (3 from ABGMA)	0.176		
ABGMA	11*	12	0.305	0.3	-0.007

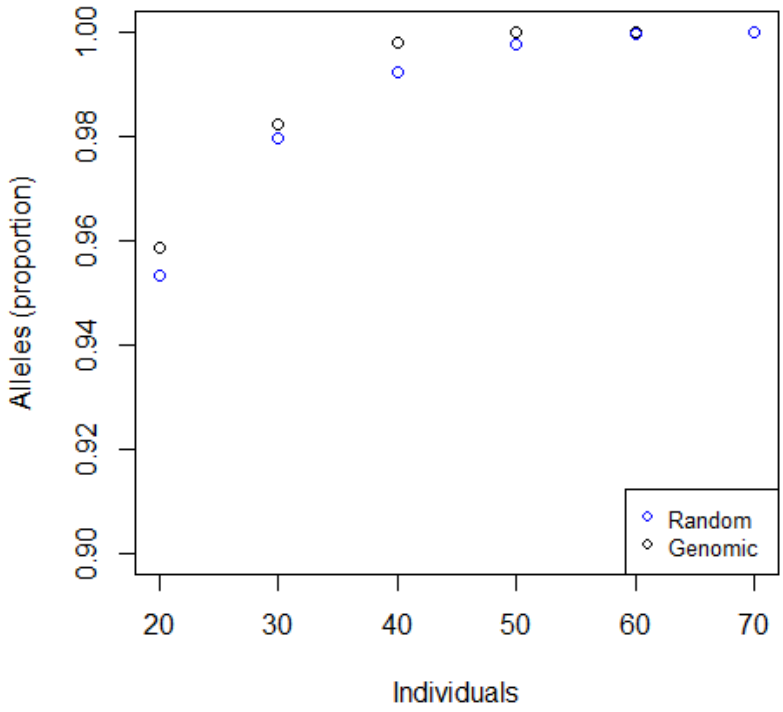

Figure 7: The proportion of loci that were polymorphic in different candidate propagation populations sourced from the genotyped Breakfast Creek population of *Zieria covenyi* generated from the optimisation of mixtures (OptGenMix) analysis.

Different numbers of individuals (horizontal axis) were selected by optimizing on the basis of gene diversity (black symbols) and by choosing at random (blue symbols, representing means of 100 replicates). Gene diversity was estimated by calculating the proportion of SNP loci that were polymorphic, i.e. where the minor allele was common (allele frequency > 3%, vertical axis).

For each analysis, using either genetic-based or random approach will enable us to maximise diversity, provided that over 30 propagules are selected (i.e. we can select just over 30 individuals to maintain over 98% of the population diversity).

Propagules should be sourced from Breakfast creek, with sampling spaced at least 10 m apart to avoid sampling clones. See Fig. 9 as an example.

Figure 8: Each map of the Breakfast Creek area where specific *Zieria covenyi* individuals to sample (red dots) for cuttings in propagation populations of different sizes (a, b).

Each set of individuals to be used in each propagation population was determined using the optimisation of mixtures (OptGenMix) analysis.

In white are individuals that were genotyped but not selected by OptGenMix as suitable candidates.

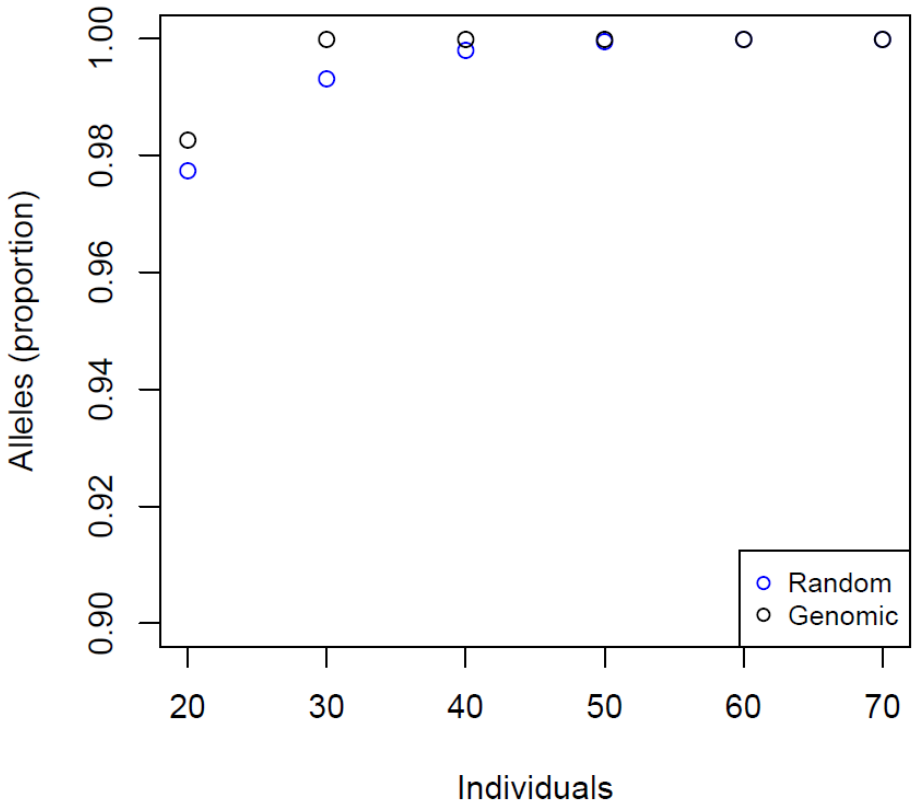

Figure 9: The proportion of loci that were polymorphic in different candidate propagation populations sourced from the genotyped Breakfast Creek and Narrow Neck (and Farside) population of *Zieria covenyi* generated from the optimisation of mixtures (OptGenMix) analysis.

Different numbers of individuals (horizontal axis) were selected by optimizing on the basis of gene diversity (black symbols) and by choosing at random (blue symbols, representing means of 100 replicates). Gene diversity was estimated by calculating the proportion of SNP loci that were polymorphic, i.e. where the minor allele was common (allele frequency > 3%, vertical axis).

For each analysis, using either genetic-based or random approach will enable us to maximise diversity, provided that over 20 propagules are selected (i.e. we can select just over 20 individuals to maintain over 98% of the population diversity). This is provided that three of the propagules include those unique genets from Narrow Neck area (NN genet 1, NN genet2 and FarNN genet 1). The rest of the propagules should be sourced from Breakfast creek, with sampling spaced at least 10m apart to avoid sampling clones.

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