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Integrating genomics and evolution into the conservation and breeding management of a Threatened Australian frog

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Abstract

Conservation Breeding Programs (CBPs) are an increasingly important tool for conserving biodiversity in a rapidly changing world, with genomic approaches playing a critical role in managing genetic diversity and minimising the risks of inbreeding and outbreeding depression. The Southern Stuttering Frog (*Mixophyes australis*) is a threatened ground-dwelling amphibian native to eastern Australia, which is currently the focus of a CBP designed to secure an insurance population and reintroduce the species to their historic range. As a newly described threatened species with a recently established CBP, *M. australis* provides an ideal case study for demonstrating how genomic data can inform and direct conservation management decisions. Here, we integrate genome-wide single nucleotide polymorphism (SNP) data from founders of the CBP alongside samples from wild populations to assess population structure, genetic diversity, and relatedness among individuals used in the CBP. Using evolutionary methods, we uncover patterns of gene flow and quantify differentiation between wild populations, identifying structuring between geographically distinct northern and southern populations of *M. australis* and translating these findings into management recommendations. We compare genetic diversity metrics, illustrating how these metrics can be used to assess populations genetic health and adaptive potential. Finally, we demonstrate how genomic data can characterise founder relatedness when pedigree data is unavailable and compare widely used relatedness estimators, providing managers with an informed framework for future mate-pairings. Overall, our study provides a foundation for the long-term genetic management of a threatened frog species, and highlights the value of evolution and genomics in conservation assessments

Introduction

The Earth is now experiencing what many scientists consider the sixth mass extinction in geological history (Dirzo et al., 2022), with species disappearing at rates 100–1000 times faster than the natural background rate (Ceballos et al., 2015; Pimm et al., 2014). Human impacts reduce and fragment populations, making species more vulnerable to loss of genetic diversity through genetic drift and inbreeding. These processes erode genetic diversity and reduce adaptive potential through a self-reinforcing decline known as the extinction vortex (Blomqvist et al., 2010). Understanding the evolutionary processes that drive gene flow, population structure and ultimately the genetic health of populations is a critical challenge for managers, especially where human alteration to the environment presents unique challenges for species conservation (Stockwell et al., 2003). Meanwhile, conservation breeding programs have become a widely used conservation strategy to safeguard endangered species ex situ, whilst facilitating population augmentation and reintroduction in the wild (Conway, 2011).

Genomics is rapidly becoming a vital tool for management of threatened species (Khan et al., 2016; Hogg et al., 2022; DeWoody et al., 2022) and is particularly valuable in CBPs where pedigree records are often incomplete or absent. Increased access to genomics, owed to reduced sequencing costs and more accessible analytical tools, means that genomic management of threatened species is more feasible than ever (Hogg et al., 2025). However, despite widespread recognition of the importance of

genetic diversity, conservation policies do not always provide guidance on how to achieve this in practice (Cook and Sgrò, 2017). In many cases, modern genomic methods have been slow to integrate into conservation management, mainly due to limited funding and access to expertise (Taylor et al., 2017). Academic and industry collaboration is becoming an increasingly effective pathway to bridge this gap (Shaw et al., 2024).

Historically, taxonomic bias has influenced conservation initiatives in favour of mammals and birds, especially conservation breeding and reintroduction programs (Harding et al., 2016). Meanwhile, of all vertebrate classes, amphibians have the highest rate of decline and extinction, with the 2022 Global Amphibian Assessment indicating that at least 40.7% of species globally are threatened with extinction. Critically, with the status of many species still unknown, this could be even higher. Overall, recent studies estimate that up to 222 amphibian species have disappeared since the industrial revolution, with an estimated 2,873 species globally threatened in 2023 (Luedtke et al., 2023). Among the main drivers of these declines are climate change, habitat loss, invasive species, and the global pandemic of Chytridiomycosis, a disease caused by Chytrid fungus (*Batrachochytrium dendrobatidis, Bd*) (Berger et al., 1998; Scheele et al., 2019). At least 501 species have experienced declines due to Chytridiomycosis, and the effects have been greatest in large-bodied, range-restricted anurans in wet climates in the Americas and Australia (Scheele et al., 2019).

While frogs' relatively high fecundity, small body size, and lack of captive-learned behaviours make them an ideal group for CBPs (Balmford et al., 1996; Griffiths and Pavajeau, 2008), the threat of Chytridiomycosis makes reintroduction and translocation particularly challenging (Scheele et al. 2021). For CBPs to contribute meaningfully to the long-term persistence and recovery of a species, they must retain sufficient genetic diversity to maintain adaptive potential in the face of ongoing threats (Willoughby et al., 2017). For example, genetic variation at the major histocompatibility complex (MHC) has been linked to Bd resistance and reduced susceptibility in frogs (Radwan et al., 2010; Trujillo et al., 2021). Therefore, safeguarding a species, as well as its genetic diversity and adaptive potential is the primary goal of most CBPs.

The Southern Stuttering Frog (*Mixophyes australis*) is a large terrestrial frog species endemic to temperate forests of the eastern coast of NSW, Australia. This species was recently described and assessed as endangered under IUCN Red List threat criteria due to its restricted extent and area of occupancy (below 2,000km^2) and the recent decline of the species from the southern two-thirds of its range, attributed primarily to the effects of Chytridiomycosis (Mahony et al., 2023). The conservation status of this species is currently awaiting formal listing under the Environmental Protection and Biodiversity Conservation (EPBC) Act and the IUCN Red List. Since 2022, the species has been part of an active CBP with founders sourced from multiple locations (Harrison et al 2026). This species provides an ideal case study to address questions about the genomic management of founder populations and integration of genetic knowledge and evolutionary processes into conservation breeding decisions.

Herein we explore how genomic tools can be used to inform conservation decisions in management of *M. australis* in- and ex-situ. Specifically, we aimed to: 1) illustrate how evolutionary mechanisms and patterns of gene flow among wild populations can inform management decisions in conservation breeding and reintroduction; 2) explain how genomic measures of diversity can be interpreted in context of species conservation and adaptive potential; and 3) demonstrate how genomic relatedness estimates can inform mate-pairing decisions in the absence of pedigree data.

1. Methods

1.1 Study species and location

The Southern Stuttering Frog (*M. australis*) is endemic to temperate forests of the eastern coast of NSW between Macleay River in the north and Victoria in the south. The Southern Stuttering Frog belongs to one of the oldest frog genera in Australia and likely had a higher diversity when the continent was more temperate in climate (Palazzesi and Barreda, 2007; Brennan et al., 2024). Mahony et al. (2023) provide a comprehensive description of this species' morphology, advertisement call, distribution, habitat, and ecology and identify four geographic sub-populations (named S1 to S4) (Fig. 1). Using historic records, they identify population declines and suggest that the species has become locally extinct south of Sydney, which corresponds to a disappearance from the southern two thirds of its distribution in the past 30 years (Mahony et al., 2023) (Fig. 1). The last remaining known population south of Sydney existed in Macquarie Pass National Park near Albion Park. In 2005, 20 tadpoles were captured from this population and transported to Melbourne to Melbourne Zoo and the Amphibian Research Centre, Werribee, Victoria. Chytridiomycosis has been implicated in the Macquarie Pass population's decline, as 12 free-living individuals collected in 2006 tested positive for Chytridiomycosis and the population has since disappeared in the wild (Daly and Craven, 2011).

In 2022, a CBP for *M. australis* was established at Symbio Wildlife Park in Helensburgh, New South Wales (NSW), with the aim of securing an insurance population and reintroducing the species into their historic range. Founders for the program were sourced as tadpoles from the wild. Two hundred total individuals were collected, 100 from each of two separate creek systems (hereafter called populations A and B), in Bowman State Forest, NSW. A high proportion of the founders tested positive for Chytridiomycosis, but were treated and cleared of the disease (Harrison et al., 2026). Further details of the establishment of the CBP and husbandry protocols are outlined in Harrison et al. (2026).

1.2 Sample collection and filtering

We obtained tissue samples from Symbio Wildlife Park. This dataset, referred to as the "conservation dataset" contains 73 samples from the CBP's founding population (A = 35, B = 37) sourced in 2022 (66 adults and 7 tadpoles (F1), of which 33 have since been retained for conservation breeding) and five samples of tadpoles from a reintroduced population (R1) in Tapitallee Creek on the South Coast, NSW. The founders of this reintroduced population were sourced from creeks in Bowman (corresponding to

the same source populations as the CBP at Symbio) and another population in Copeland that has since crashed. The R1 population was introduced prior to the establishment of Symbio's CBP and samples used in this dataset are tadpoles descended from the founders. The "wild dataset" was obtained from Mahony et al. (2023), which contains 55 *Mixophyes australis* samples (populations S1-4) from across the extant range (between Port Macquarie and Sydney), and four *M. australis* from some of the extinct range (Between Sydney and Nowra).

For genomic analysis a dataset of single nucleotide polymorphisms (SNPs) was produced using Diversity Arrays Technology (DArTseq; Canberra, Australia). DArTseq use a proprietary genome complexity reduction technique that uses restriction enzymes (in this case PstI and SphI), optimised for the target species, to fragment DNA. Sequencing is carried out on an Illumina Hiseq2500 and subsequent SNP calling and quality scoring is performed using custom software 'DArTsoft', as outlined in Kilian et al. (2012). The wild and conservation populations were co-analysed to establish the genetic diversity and evolutionary relationships of the CBP in context of the species. However, because the conservation and wild datasets were generated at different times using different SNP discovery panels, analyses were performed separately wherever possible, and direct comparisons between datasets should be interpreted with caution (described in supplementary section A). Where cross-dataset comparisons are presented, metrics were standardised as far as possible to minimise the effects of ascertainment bias (described below).

Datasets were filtered in RStudio v2024.04.2 (R Core Team, 2024) using 'dartRverse' v1.0.6 (Gruber et al., 2018; Mijangos et al., 2022). In all analyses, SNPs were filtered sequentially as follows: loci with reproducibility below 0.99 were removed, followed by loci with an average read depth below 10x or above 100x (to remove loci with poor coverage or potential paralogs), loci with a call rate below 90%, individuals with more than 50% missing data, secondary SNPs (retaining the SNP with the highest reproducibility per sequence tag), and loci with minor allele frequency below 1/(2N) (i.e. singletons). Finally, loci rendered monomorphic after the filtering steps were removed. The individual call rate threshold was intentionally kept lenient in all analyses to avoid losing entire populations, particularly the southern population (S4) which had low sample sizes. Deviations from this default pipeline for specific analyses are described in supplementary section B: Table S3. The data was also checked for the presence of sex-linked loci with 'dartR.sexlinked' package (Robledo-Ruiz et al. 2023) and none were found.

1.3 Population structure

We assessed population structure in wild and conservation populations to determine the degree of genetic differentiation among sampling locations and to inform designation of management units for the CBP. To this end, we analysed both datasets together (reported in supplementary section A) and independently.

For preliminary visualisation of population structure, we used a principal coordinates analysis created in R using the function 'gl.pcoa' provided in the package 'dartRverse' (Gruber et al., 2018; Mijangos et al.,

2022). PCoA is valuable to determine clusters of genetically similar groups, and was used here for visualisation purposes. For estimation of differentiation between populations within wild and conservation datasets, we used the fixation index F_{ST} calculated pairwise with 100 bootstraps using the 'gl.fst.pop' function, which implements the method outlined by Tills (1977). Wright (1965) defines FST values < 0.05 low, 0.05–0.25 moderate to high and > 0.25 very high differentiation. We further tested differentiation between populations by running a fixed differences analysis using the function 'gl.fixed.diff'. An accumulation of fixed differences between two populations indicates a lack of gene flow and can thus differentiate species or operational taxonomic units (OTUs) (Georges et al., 2018).

To test for gene flow between wild populations we tested for isolation by distance (IBD) which determines if genetic differences increase with geographic distance (Rousset, 1997). A strong and significant IBD relationship indicates that gene flow is distance-dependent and deviations from this relationship can indicate barriers to gene flow or population discontinuities (Diniz-Filho et al., 2013). We ran an IBD test using the function 'gl.ibd' which calculates genetic and Euclidean distance matrices and tests for significance using a Mantel test (Mantel, 1967; Rousset, 1997).

To examine patterns of gene flow we used a combination of population clustering analyses. These analyses aim to find how many genetic clusters (K) exist in the dataset and what proportion of each individual's genome originates from each cluster (Pritchard et al., 2000; Moorjani and Hellenthal, 2023). We used STRUCTURE (Pritchard et al., 2000), a Bayesian method that has several model parameters that may be used to optimise and improve inference. STRUCTURE is sensitive to the assumption that loci are unlinked, in Hardy-Weinberg equilibrium and in linkage equilibrium (LE) (Pritchard et al., 2000), so we further filtered our dataset to remove linked loci (Table S3). We ran STRUCTURE in R using the function 'gl.run.structure'. We used the admixture model (Individuals have mixed ancestry) testing for K = 1–10 with 50,000 steps, 20,000 burn-in and 3 replicates. We ran this twice, with and without the additional parameter of correlated allele frequency. The default model assumes that the allele frequencies in each population are independent and draws from a distribution that is specified by lambda (λ = 1) (Pritchard et al., 2000). The correlated allele frequencies model instead considers the allele frequencies in a hypothetical "ancestral" population which may work better for recently diverged groups (Falush et al., 2003). Using these results, we determined the optimal K using an Evanno plot created using the function 'gl.evanno', this method estimates the optimal K by analysing the rate of change in likelihood between successive K values. We then plotted the results with the CLUMPP method, using the algorithm 'greedyLargeK' and 100 iterations, implemented in the function 'gl.plot.structure'.

We also ran sNMF (sparse non-negative matrix factorization), an alternative method that uses least-squares optimization with regularization to estimate ancestry coefficients and allele frequencies and implements a cross-entropy test to determine the best K. This method is less flexible as it does not have the parameter options of STRUCTURE but is faster and more robust for large datasets with violations of Hardy-Weinberg equilibrium and linkage equilibrium, which is the case in wild populations with structure and inbreeding. Thus, we used the full dataset without filtering for linkage disequilibrium or Hardy-Weinberg equilibrium (Frichot et al., 2014). We ran sNMF in R using the 'LEA' package (Frichot and

François, 2015). To optimise the sNMF analysis, we first ran 18 tests with different combinations of the tolerance and alpha parameters. We estimated the cross-entropy for each run and selected the one with the lowest, as smaller values indicate better algorithm outputs and estimates (Frichot et al., 2014). Using the run with optimal parameters, we estimated cross-entropy values for numbers of populations (K) between 1–12 and selected the value for K with the lowest cross-entropy. This process was automated in a custom function 'run_snmf' available at github: kamryn-carter/Mixophyes-australis-project.

1.4 Genetic diversity

Diversity analyses were used to assess levels of genetic health in both conservation and wild populations using the combined dataset with relevant standardisations to reduce bias (described in supplementary section B). We calculated three complementary measures of heterozygosity to allow cross-comparison and account for different sources of bias. First, conventional genetic diversity metrics (observed heterozygosity (H_O), expected heterozygosity (H_E) and the inbreeding coefficient (F_{IS})) were calculated using the function 'gl.report.heterozygosity' in the dartRverse package. Heterozygosity calculation can be susceptible to bias; to reduce this, each population was filtered and analysed independently, as explained in supplementary section B (Sopniewski and Catullo, 2024). Second, because SNPs include only polymorphic sites, which can bias heterozygosity estimates (Schmidt et al., 2021; Sopniewski and Catullo, 2024), a proxy for autosomal expected and observed heterozygosity was calculated by adding an additional argument 'n.invariant' to the function gl.report.heterozygosity. This calculation takes into account an estimate of the number of invariant loci in the dataset provided by the function 'gl.report.secondaries' which estimates the number of invariant sites by modelling the counts of SNPs per sequence tag as a Poisson distribution. Third, we calculated standardized heterozygosity using the custom R function 'GENHET'. This is an alternative metric that adjusts for the heterozygosity expected at each locus so that individuals are compared on a common scale. Standardised heterozygosity results are presented as a ratio relative to the population mean (therefore values > 1 indicate above-average heterozygosity, and < 1 indicate below-average) (Coulon, 2009).

As alternative diversity metrics, we estimated allelic richness (A_R), private alleles (P_A) and effective population size (N_E). We calculated A_R per population in R using the 'allel.rich' function in the package 'PopGenReport' (Adamack and Gruber, 2014). Allelic richness quantifies the number of alleles at each locus, standardised for sample size using rarefaction and averaged across all loci (Hurlbert, 1971; El Mousadik and Petit, 1996). In comparison to heterozygosity, A_R estimates how many alleles exist overall, rather than the overall balance of allele frequencies, which makes this metric more sensitive to rare alleles and thus a good indicator of a population's adaptive potential (Kanaka et al., 2023). Private alleles (P_A) are alleles that are unique to a population (i.e. not found in any other population sampled). They can be a good indicator of genetic differentiation and uniqueness of populations (Slatkin and Takahata, 1984). We estimated this using the function 'gl.report.pa' for the wild population only, as values were not comparable across the two datasets. Effective population size is a concept that quantifies the number of breeding individuals in an idealised population with the same level of inbreeding or gene frequency divergence under random genetic drift as the real population (Wright, 1938). We estimated N_E using the

software NeEstimator V2 (Do et al., 2014). We exported files from R using the function gl2genepop and the software was run externally in a version compiled using Linux. Both wild and conservation datasets were analysed independently using the linkage disequilibrium method and allowing random-mating.

1.5 Relatedness

We estimated relatedness to identify the level of kinship among the conservation populations A + B (N = 73) and identify pairings of related individuals to quantify the risk of inbreeding and inform future mate pairings to maximise genetic diversity. Alongside this, we aimed to compare three common methods for relatedness estimation to test for consistency among them.

Relatedness is the expected proportion of alleles shared identical-by-descent between two individuals. Two alleles are identical-by-descent if they are inherited from a common ancestor without any mutation in between. Allele frequencies in the population are used to determine whether allele sharing is due to chance or shared ancestry (Weir et al., 2006). The relatedness coefficient (r) measures the proportion of identical-by-descent alleles between two individuals, averaged across the genome. Values range between 0–1, with parent–offspring / full siblings having r = 0.5 on average (full sibling estimates show greater variance than parent-offspring pairs), and half-siblings having r = 0.25 (Lange, 2002; Goudet et al., 2018). The kinship coefficient (Φ) measures the probability that a randomly drawn allele from each of two individuals is identical-by-descent, and this is equivalent to half the relatedness coefficient (Φ = r/2). We report Φ throughout, as it is directly comparable to the coefficient of kinship used in traditional pedigree-based methods (Boehnke and Cox, 1997).

Multiple relatedness estimators exist that differ in their underlying assumptions and performance under different conditions. We calculated and compared three different methods to estimate relatedness. First using the function ‘gl.grm’ in dartRverse which implements the additive relationship matrix approach developed by Endelman and Jannink (2012). This estimator computes expected covariances of marker genotypes using formulas based on allele frequencies (Endelman and Jannink, 2012). Second, we used the function ‘gl.run.EMIBD9’ and allowed inbreeding in the model. This is implemented through the external software EMIBD9. EMIBD9 is a likelihood estimator that uses an expectation maximization (EM) algorithm based on the maximum likelihood expectations to estimate both allele frequencies and identical-by-descent from genotype data which is much faster to run than similar methods. This method can estimate relatedness in both inbred and non-inbred cases and has reduced bias due to small sample sizes and has higher accuracy in populations that include many closely related individuals (Wang, 2025). Third, we used COANCESTRY, which implements seven different relatedness estimators using both moment and likelihood methods (Wang, 2011). From COANCESTRY we used the TrioML estimator as it accounts for inbreeding and tolerates a random sample and has also been shown to have the lowest variance and highest correlation with the true mean in simulated examples (Hogg et al., 2019).

We also ran the same analyses using a filtered dataset containing just the 26 individuals retained for the CBP (this group contains 12 individuals from population A and 14 individuals from population B and consists of 12 males and 14 females altogether). Using the function ‘gl.grm.network’ we visualised

relatedness as a network using the Fruchterman-Reingold layout, setting the kinship threshold to 0.062 (average kinship value of first cousins derived from Speed & Balding (2015)) to display pairwise relatedness at the level of first cousins or higher as a link between two nodes. This method includes an adjustment that calculates coefficients relative to an estimate of average inbreeding coefficient for the population, following the approach by Goudet et al. (2018), enabling relationships to be interpreted in the context of overall relatedness within the population.

2. Results

2.1 Population structure

2.1.1 Conservation and reintroduced populations

For the conservation population, across all analyses, we found limited evidence of genetic structuring and very little differentiation between conservation populations A, B and the reintroduced population R1. Within this dataset, the PCoA showed minor clustering of individuals, consistent with population structure (Axis 1 = 4.3%, Axis 2 = 4%) and very little separation between conservation populations A and B and the reintroduced population R1 (Fig. 2). The minor clustering observed in the PCoA is attributable to related family groups, as these clusters disappear if the data are filtered for parent-offspring relationships (Fig. S9). The F_{ST} results were consistent with the PCoA, with differentiation < 0.1 between A, B and R1 (Table 1). In addition, there were 0 fixed differences between these populations (Table S4).

Table 1
Table of F_{ST} values between of conservation populations A, B and reintroduced population R1. Bottom triangle shows F_{ST} values and top triangle shows P-values (100 bootstraps).

A	**A**	**B**	**R1**
	-	**0**	**0**
B	0.018	-	0
R1	0.075	0.079	-

2.1.2 Wild remnant populations

For the wild dataset, the PCoA revealed some genetic structuring among northern populations: S1-3 (henceforth referred to collectively as ‘northern population’), and differentiation between the northern population and southern population: S4 (henceforth referred to as southern population;(Fig. 3). Axis 1, explaining 12.7% of variation, differentiates the northern and southern populations with S1and S2 populations closely clustered and S4 most differentiated. Axis 2, explaining 8.4% of variation, differentiates populations S1 and S2 from S3. Axis 3, explaining 4.9% of variation differentiates

populations S1 and S2 (Fig. S10). Populations S1 and S2, despite being further apart (~ 60km) are more closely clustered than S2 and S3 (~ 30km apart).

These results were consistent with estimated FST values (Table 2) where differentiation between northern populations (S1-3) was moderate to high (0.05–0.25) and differentiation between northern populations (S1-3) and the southern population (S4) was very high (> 0.25).

Table 2
Table of F_{ST} values for wild populations. All values are highly significant (P < 0.0001).

S1	**S1**	**S2**	**S3**	**S4**
	-			
S2	0.12	-		
S3	0.22	0.13	-	
S4	0.32	0.30	0.44	-

The IBD test showed a strong correlation between individual genetic and geographic distance, particularly in the northern populations where pairwise comparisons between individuals cluster as a gradient along the line of best fit (Fig. 4). The significant Mantel statistic (R^2 = 0.71, P < 0.05) indicates isolation by distance is a realistic model to describe gene flow among populations. However, the southern population does not appear to follow this relationship, as pairwise comparisons between individuals from S1-S3 and individuals from the southern population (S4, in light green) show consistently large mean genetic distances that appear as a horizontal line across the top of the graph (Fig. 4). This pattern suggests restricted gene flow between the southern and northern populations, rather than simple distance-dependent isolation.

Results of a population clustering analysis using STRUCTURE with correlated frequency found three genetic clusters (K = 3). STRUCTURE recovered S2 and S3 as genetically similar, S1 as slightly distinct and S4 as markedly distinct (Fig. 5). In comparison, the STRUCTURE analysis with uncorrelated frequency found only two genetic clusters (K = 2) with S2 and S3 occupying one cluster and S1 and S4 appearing admixed (Fig. S11). The sNMF analysis also found three clusters (K = 3). Among northern populations, this analysis clustered S1 and S2 as most similar and S3 as most differentiated, although with substantial admixture between them, indicating shared ancestry and gene flow between populations. In contrast, the southern population appears distinct with lower levels of admixture (Fig. 5). When ancestry coefficients from sNMF are plotted on a map, northern populations appear as a gradient of admixture, showing a clear pattern of gene flow, while differentiation between northern and southern populations indicates a potential barrier to gene flow around the Newcastle region (Fig. 5). Across both sNMF and STRUCTURE (correlated frequency) analyses, the southern population S4 appeared as genetically distinct from the northern populations (Fig. 5), although it should be noted that one individual

(ABTC7124) is the only representative from the extant population surviving in the Watagans National Park area (-33°.03’S, 151°.33’E). This individual appears admixed in both analyses but aligns closest with individuals which represent populations south of Sydney that are now extinct.

2.2 Genetic diversity

To determine how genetically diverse wild and conservation populations are we report several indices of genetic diversity. These include three complementary measures of heterozygosity (conventional (H_O, H_E), autosomal (H_O, H_E), and standardized (H_O), alongside allelic richness (A_R), effective population size (N_E), and private alleles (P_A)) (Table 3). We note that the cross-dataset comparisons presented here are not directly comparable, as conservation and wild datasets were ascertained on different SNP panels, and should thus be interpreted with caution.

For conventional estimates of heterozygosity, S3 had the highest H_O (0.328) and S4 had the lowest (0.157) of the wild populations. Symbio’s conservation population (A + B) has a moderate H_O (0.289) and is roughly similar between populations A and B (Table S5). The reintroduced population (R1) appears the most heterozygous (H_O=0.399) although this may be biased due to low sample size (n = 3). The three heterozygosity measures broadly agreed in identifying S4 as the least heterozygous population, and R1 as the most heterozygous where data were available. However, the relative ranking of S1, S2, S3 and A + B varied depending on the measure: S3 ranked highest among wild populations for conventional H_O, S1 ranked highest for autosomal H_O and H_E, and A + B ranked highest overall for standardised heterozygosity. Autosomal H_O and H_E was consistently several orders of magnitude lower than conventional estimates across all populations and was approximately four- to six-fold lower in S4 compared to the other wild populations.

Of the wild populations, S4 had the highest level of inbreeding (F_{IS}=0.599), followed by S1 (F_{IS}=0.332), while S2, S3, A + B and R1 had low inbreeding (F_{IS} ~0.100). A + B had the highest A_R overall and S1 had the highest of the wild populations. Similar to heterozygosity estimates, S3 had the highest N_E followed by S2. A + B had a low N_E, however when related (> 0.15 mean kinship) individuals were filtered out, N_E increased by almost 40-fold (2776, SD = 2544.3- 3053.9). S2 had the highest number of P_A, followed by S1 and S4 had the least, indicating that S1 and S2 samples carry more unique diversity.

*Table 3: Table of diversity indices of populations sampled. Including sample size (n), observed heterozygosity (H_O), expected heterozygosity (H_E), inbreeding coefficient (F_{IS}), autosomal heterozygosity, standardised heterozygosity, allelic richness (A_R), effective population size (N_E) and private alleles (P_A). *sample size was too low to estimate, **values were not comparable due to ascertainment bias.*

pop	S1	S2	S3	S4	A + B	R1
n	6	22	22	5	72	3
H_O (+- SE)	0.269 (0.004)	0.294 (0.003)	0.328 (0.004)	0.157 (0.005)	0.289 (0.003)	0.399 (0.004)
H_E (+- SE)	0.374 (0.002)	0.306 (0.002)	0.331 (0.003)	0.381 (0.002)	0.301 (0.002)	0.380 (0.001)
F_{IS} (+- SE)	0.332 (0.008)	0.106 (0.007)	0.092 (0.008)	0.599 (0.011)	0.106 (0.007)	0.108 (0.008)
H_O (+- SE) Autosomal	0.00019 (0.0002)	0.00013 (0.00013)	0.00012 (0.00015)	0.000031 (0.000083)	0.00010 (0.00010)	0.00043 (0.00024)
H_E (+- SE) Autosomal	0.00028 (0.00020)	0.00014 (0.00013)	0.00012 (0.00014)	0.000074 (0.00014)	0.000097 (0.000106)	0.00041 (0.00020)
Standardised heterozygosity (+-SD)	0.951 (0.107)	0.931 (0.095)	0.771 (0.065)	0.246 (0.048)	1.141 (0.130)	*
A_R	1.34	1.32	1.24	1.13	1.37	*
N_E	*	101.4 (96.6-106.7)	484.4 (379.3-668.4)	1.7 (1.6–1.8)	69.6 (69.5–69.7)	*
P_A	264	334	159	111	**	**

2.3 Relatedness

Relatedness analyses estimated kinship (Φ) between individuals and mean kinship for populations in the conservation dataset. Mean kinship represents the average relatedness of individuals within the population or the 'background relatedness'. Estimates of mean kinship within populations did differ across methods (Table 4), where GRM and TrioML had almost identical values around 0.015 (difference of 0.001), indicating each population has a background relatedness equivalent to second cousins. EMIBD9 estimated a higher mean kinship around 0.1 indicating the populations have a background relatedness equivalent to half siblings. For mean kinship estimates across the whole dataset (A + B combined), GRM and TrioML found a lower background relatedness (~ 0.007) equivalent to third cousins and largely unrelated. Meanwhile EMIBD9 found mean kinship very similar to within population estimates (0.092), equivalent to half-siblings.

Table 4
Within population mean kinship (Φ) values and standard deviation (SD) for conservation populations estimated using 3 alternative methods: GRM, EMIBD9 and TrioML. Values range from 0-0.5. E.g. Parent–offspring / full siblings: Φ = 0.25, Half-siblings: Φ = 0.125, Unrelated: Φ ≈ 0.

A	N	GRM (+-SD)	EMIBD9 (+-SD)	TrioML (+-SD)
	35	**0.013 (0.053)**	**0.097 (0.025)**	**0.012 (0.049)**
B	37	0.017 (0.059)	0.095 (0.029)	0.016 (0.054)
A + B	72	0.008 (0.041)	0.092 (0.020)	0.007 (0.037)
Breeders	26	0.007 (0.038)	0.010 (0.022)	0.007 (0.036)

For comparison of individual pairings, we report kinship coefficients for each method, for all possible pairings across populations A + B (Appendix: pairwise_kinship_comparison_all.csv). A correlation scatterplot matrix tested for consistency across methods (Fig. S12) finding that all methods were consistent in the ordering of pairwise relatedness (e.g. highly related individuals were always reported as highly related) but differences occurred in the slope of the correlation. At low relatedness, EMIBD9 tended to report higher estimates than GRM and COANCESTRY where GRM and TrioML give values around 0-0.05, EMIBD9 gives values around 0.05–0.1. At intermediate relatedness GRM, EMIBD9 and TrioML estimates are mostly linear but EMIBD9 estimates were generally higher. At high relatedness GRM tended to give the highest values, often around 0.3 (> 0.25 parent-offspring relationship), whereas EMIBD9 did not estimate any pairings with Φ > 0.25.

For the dataset filtered for individuals retained for conservation breeding (‘breeders’), mean kinship estimates for GRM and TrioML methods were very similar to estimates from the whole population (A + B), indicating that the breeders capture a representative sample of relatedness among the whole population (Table 4). In contrast, estimates from EMIBD9 were much lower than for the whole population (0.010 compared to ~ 0.095). Using this filtered dataset we again report kinship coefficients for each method (Appendix: pairwise_kinship_comparison_breeders.csv). We found EMIBD9 tended to estimate lower levels of relatedness, for example: where GRM and TrioML gave a value ~ 0.25, EMIBD9 gave a value ~ 0.15, however the ranking of relatedness was still consistent. According to both TrioML and GRM estimates, the dataset of breeders contains 8 parent-offspring/sibling relationships (~ 0.25), one half sibling (~ 0.125) and two first cousins (~ 0.062). Relatedness within this group was visualized using a network (Fig. 6). This network is based on results from the GRM method but includes an adjustment that calculates coefficients relative to the baseline level of inbreeding, enabling relationships to be interpreted in the context of overall relatedness within the population. These results aligned more closely with EMIBD9 estimates (zero parent-offspring/sibling relationships, six half-sibling relationships, two first cousin relationships).

3. Discussion

Conservation breeding programs have become a key strategy to safeguard genetic diversity and adaptive potential of endangered species ex situ, and also provide individuals for reintroduction and population augmentation to contribute to species recovery in the wild (Pritchard et al., 2012; Scheele et al., 2021; Pan et al., 2025). Mate-pairing design and management of multiple insurance populations are central challenges in these programs. Here, we integrate genomics and evolutionary biology into conservation to inform the management and breeding of Australian Southern Stuttering Frogs (*M. australis*). Our analyses show some population structuring, particularly between northern and southern populations separated by the Hunter River and find the southern populations, several of which are now extinct, to be genetically eroded. We provide estimates of relatedness for founders of the CBP and provide recommendations for the genetic management and conservation of wild and conservation populations.

3.1 Patterns of gene flow and differentiation in conservation and wild populations

Identifying population structure and defining management units is a fundamental step in designing a successful breeding program (Carrillo et al., 2015). Among the wild populations there is some genetic divergence, particularly between the northern (S1-3) and southern populations (S4) which are separated by the Hunter River in the Newcastle region. Our analyses indicate a lack of contemporary gene flow across this region. However, within this dataset is only one sample from the extant S4 population in the Watagans, between Newcastle and Gosford, which limits our assessment of genetic connectivity. Additional samples from S4 included in this dataset are from now extinct populations that are > 150km from the nearest extant population. More samples from the Watagans population are required to reliably test the level of genetic connectivity between this and the northern S1-3 populations and further assess the risk of outbreeding depression from interbreeding northern and southern populations.

Although we find genetic structuring among the northern populations (S1-3), structure and IBD analyses indicate genetic connectivity between them. We conclude that these differences are likely not large enough to cause outbreeding depression (Frankham et al., 2011) and suggest that structuring may be caused by fragmentation of habitat. However, experimental tests of combining ability should be conducted prior to integrating population mixing into the breeding program (Byrne and Silla, 2022). Among populations of *Mixophyes*, it is likely that river drainage systems can both facilitate gene flow via movement of tadpoles or inhibit gene flow by acting as geographic barriers. Many frog species rely on rivers for dispersal, yet, in many cases, rivers are the primary drivers of population structure, especially in fragmented, high-altitude environments (Zancolli et al., 2014, Fibla et al., 2023). For terrestrial species that move primarily over land, major rivers, human settlements and flow modification can impede dispersal (Zancolli et al., 2014; Churko, 2016). Peek et al. (2020) found that river modification by humans explained patterns of isolation and loss of genetic diversity in a river breeding frog species. Conversely, rivers can act as corridors, increasing gene flow between populations and maintaining genetic diversity (Frankham, 2015; Ralls et al., 2018). Alterations to mechanisms of dispersal may increase inbreeding

and reduce genetic diversity, putting the species at higher risk of local extinction. Such effects have been reported in another NSW frog species, namely *Rawlinsonia littlejohni* (formerly *Litoria littlejohni*) where large-scale damming may have increased genetic isolation and loss of genetic diversity (Stock et al., 2023).

We suggest that securing strong meta-populations in-situ should be prioritised by maintaining and increasing protection of the critical parks and reserves that serve as genetic strongholds for populations of the species (particularly S2 and S3). We also emphasise the importance of maintaining the hydrology of the region. Rainforest-dependent species are expected to persist after fire events where rainforest regenerative capacity is intact (Keith et al., 2022). However, repeated fire, drought, and hydrological instability may exceed resilience thresholds for the habitats that support species such as *M.australis*. Identifying priority refugia and ensuring exclusion of fire from immediate habitat zones is therefore critical for long-term persistence of *M. australis*.

3.2 Inbreeding and diversity in wild and conservation populations

Maintenance of genetic diversity is an essential consideration in managing conservation and wild populations of endangered species. Although not a direct comparison, the diversity measures of conservation and wild populations we recover here are similar, indicating that the sampled populations used to establish the CBP are genetically healthy. Conventional heterozygosity values for the wild and conservation populations are naturally inflated due to our calculation being made with SNP data, which contains only polymorphic sites compared to autosomal heterozygosity which is calculated from both polymorphic and non-polymorphic sites (Schmidt et al., 2021). This issue is exacerbated by ascertainment bias (described in supplementary section A); however, we have addressed this by following a robust filtering strategy and consistently accounting for bias in our interpretation of results. Therefore, the diversity estimates we present here provide a baseline estimate from which future studies can compare, to monitor an increase, maintenance or decline in diversity. Future indications of a loss of diversity may represent irreversible loss that reduces the adaptive potential of the species.

Northern populations have moderate heterozygosity, with a small heterozygote deficit evidenced by positive F_{IS} values. A positive F_{IS} value is usually interpreted as evidence of inbreeding, with ideal levels falling below a value of 0.05 (Frankham et al., 2019). However, F_{IS} can also be artefactually inflated due to the Wahlund effect, where unaccounted-for population substructure causes an apparent heterozygote deficit relative to Hardy-Weinberg expectations, even in the absence of true inbreeding (Garnier-Géré and Chikhi, 2013). Recent population declines may have increased chances of inbreeding, but elevated F_{IS} in S1 may also partly reflect population substructure, given these samples span a larger geographic distance than in S2 and S3. In contrast, S4's high F_{IS} is more likely to reflect true inbreeding, given its low N_E and consistently low heterozygosity across all measures, and its more geographically discrete sampling. Among the northern populations, S3 appears to be the healthiest genetically, with the highest effective population size (N_E) and observed heterozygosity (H_O). Population S3 likely represents a large

demographic population, meanwhile populations S1 and S2 are less diverse overall. However, the S1 and S2 populations are more unique, with more private alleles (P_A) and higher allelic richness (A_R), which indicates increased adaptive potential (Allendorf et al., 2012). This rare genetic variation could represent locally adapted alleles that may be worth capturing in future expansions of the CBP.

By comparison, the southern S4 population displays much more eroded genetic diversity, with low heterozygosity, extremely high F_{IS}, low A_R and P_A and critically low N_E, all consistent with the severe population decline observed over the last ~ 40 years. Four of the five individuals sampled from this population belong to now extinct populations (Daly and Craven, 2011) located more than 100km from the one individual representing an extant population in the Watagans, which may have inflated estimates of inbreeding due to the Wahlund effect. However, estimation of diversity metrics using one individual is not feasible, and further sampling is required to confirm the level of differentiation and diversity of this population. Regardless, this population likely falls below generally accepted conservation thresholds of N_E>50 for preventing inbreeding depression in the short term and N_E>500 for maintaining evolutionary potential in the long term (Franklin, 1980; Soule, 1980), particularly given more recent studies suggest these numbers should be at least doubled (Frankham et al., 2014). Following this framework, we propose that managers should be aiming to increase genetic diversity in *M. australis* populations, with S4 as a priority, and continue genetic surveys of populations over time to monitor changes in diversity.

3.3 Relatedness and breeding strategies to maintain diversity within the conservation breeding program

The maintenance of genetic diversity through informed mate-pairings in conservation populations of endangered species is critical to the success of these programs (Allendorf et al., 2012). Today, genomics is one of the most accurate methods for estimating relatedness in individuals with unknown parentage (Wang, 2016; 2025) and while relatedness analyses with large SNP datasets can be confounded by genotyping errors (e.g. allelic dropout), Attard et al. (2017), showed that despite this, SNPs still performed better than perfectly genotyped microsatellite data with set of tens of markers. In this study we estimated pairwise relatedness among individuals sampled for the *M. australis* CBP. Within the conservation breeding population, we find some related groups alongside a large proportion of unrelated individuals. These are promising results, indicating that there is variation in the population, and only minor inbreeding.

To determine which method was best suited to our dataset, our study also compared three different relatedness estimators. We found that, although their absolute relatedness values differed, the relative ranking of pairs from least to most related was largely consistent across methods. In particular, GRM and COANCESTRY's TrioML estimator showed strong agreement in both the full dataset and the subset of breeding individuals, suggesting they are relatively robust across different datasets. GRM may be preferable for routine analyses due to its speed and computational simplicity, whereas COANCESTRY's graphical interface and optimisation options may be more useful for some users for allowing greater control and ease of use. In contrast, the EMIBD9 estimator produced generally higher relatedness

estimates and showed greater variability between datasets, including a noticeable drop in mean kinship between the full population and the breeder subset. This likely reflects its design for small, highly inbred populations (Wang, 2025), meaning it should be used with caution in larger, more diverse datasets. The same effect can be problematic for the estimation of a relatedness network using the correction by Goudet et al. (2018) which decreases overall estimates of relatedness based on the average inbreeding of the population.

The discrepancies between relatedness estimators in identifying parent–offspring and sibling pairs highlight the importance of the choice of estimator (and its respective parameter options and corrections) for the dataset given. For conservation managers, this raises an important practical question of which estimator to rely on when making breeding decisions. Given the overall consistency of rankings across methods, our key recommendation is to focus less on absolute relatedness values and more on the relative ranking of pairwise relatedness when designing breeding pairings. A similar study comparing different relatedness estimators also concludes that no single optimal estimator exists for all datasets and recommends that researchers test candidate estimators for their systems by examining correlations with known pedigree relationships (Hauser et al., 2022). Goudet et al. (2018), provides recommendations of which relatedness estimator to use according to the context of the population however, further comparative work, using modern methods across a wider range of population sizes and structures, on datasets with known relatedness, would help clarify the strengths and limitations of each estimator and guide their appropriate application.

By characterizing the founder relationships of the breeding program, we have provided a basis for continued genetic management of *M. australis*. Translocated and conservation populations are prone to a loss of genetic diversity and inbreeding due to founder effects (Thavornkanlapachai et al., 2021). This can be combatted by sourcing individuals from several populations, creating a fully representative sample that encompasses wild diversity (Frankham et al., 2010). The CBP has recently collected individuals from an additional source population located in The Glen Nature Reserve (corresponding to S3) (see Harrison et al. (2026)) and additional genetic analyses are planned to assess relatedness and diversity of this population in the future. Following recommendations and guidelines provided in Byrne and Silla (2022), breeding experiments are planned to assess the effect of population mixing on frog viability to further inform the genetic management of the CBP.

Genetic rescue via gene flow from genetically diverse sources can be an effective way to improve fitness (Fitzpatrick et al., 2020; Pavlova et al., 2023). Therefore, establishing additional conservation populations sourced from different subpopulations will further strengthen the breeding program. An effective strategy for conservation breeding is to manage multiple semi-isolated subpopulations with controlled exchange. This approach ensures that all individuals have a balanced contribution to the gene pool and allows the introduction of new alleles while preventing panmixia (Fernández and Caballero, 2001). In *M. australis*, following a structured breeding strategy (incorporating both within-population and periodic between-population pairings) could increase genetic diversity while avoiding excessive homogenisation. Studies have demonstrated that a small number of migrants per generation (generally 1–10 but relative

to N_E of the population) reduces inbreeding and loss of heterozygosity whilst retaining local differentiation (Mills and Allendorf, 1996; Nathan et al., 2017). This mimics natural migration processes observed in wild populations (Garant et al., 2007).

3.4 Conservation recommendations and future research

This study contributes to the conservation of *M. australis* by describing the population structure and diversity of wild populations and using this information to provide recommendations for management. Our population structure analyses indicate that currently held conservation populations A and B are not significantly genetically distinct. Following the decision tree provided by Frankham et al. (2011), which predicts the probability of outbreeding depression between two populations, it is unlikely that interbreeding between these populations would cause outbreeding depression. Strategically designed mating pairs crossing individuals both within and between populations A and B, as well as newly sourced captive population C, are now planned to quantify the outcome of population mixing on offspring viability. The results of these breeding experiments will be combined with genomic data to make recommendations for the genetic management of the CBP into the future. In the present study we also quantify the relatedness between founders of the conservation breeding populations A and B of *M. australis*, providing managers with a framework for future breeding pairings to prevent inbreeding and maximise diversity. The findings we present here will also contribute to government policies which focus on conserving biodiversity across landscapes and maintaining the resilience of species within those landscapes (described in supplementary section D).

While we provide a basic understanding of the genetic diversity of *M. australis*, there are important avenues for further research. First and foremost, additional sampling and analysis of the extant population in the Watagans (corresponding to the northmost S4 individual) should be a high priority. Incomplete sampling means we cannot accurately confirm the level of differentiation and diversity of this population. Without this knowledge we cannot provide recommendations for the health of this population and whether it can be safely bred with northern populations without suffering from outbreeding depression. Once this can be determined, fitness studies, such as those involving controlled experimental crossing to test the effects of outbreeding depression can be conducted prior to reintroduction (Byrne and Silla, 2020; 2022). In addition, further surveys in the Blue Mountains region of NSW should be conducted to test for the occurrence of any remnant populations of *M. australis*. As reintroductions take place on the south coast, it will be increasingly important to document any genetically distinct sub-populations throughout the species historic range.

Finally, as reintroductions take place, continued genetic monitoring will prove valuable for monitoring the health of natural populations and ensuring reintroduced populations are viable and sustainable. Future genetic studies should make use of the reference genome recently published for *M. australis* (Tang et al., 2024) to increase the robustness of analyses using SNP data, or for applications in functional genomics (Brandies et al., 2019). Future studies integrating whole-genome resequencing data using the reference genome would also enable demographic reconstruction. Our findings may also enable future studies linking genetic diversity and disease resistance. Anecdotal evidence for northern populations growing

resistance to Chytridiomycosis after a rapid decline in the last 40 years may make *M. australis* a valuable study system for research into the disease, similar to studies conducted with Corroboree frogs (Kosch et al., 2019).

Declarations

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Ethics approval

This research was conducted under scientific license number: SL101788 and ACEC Project Number 17/586: Project Title - Fauna assessments/Species Impact Statements/Recovery Plans and Ecology of Herpetofauna.

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Author Contribution

Conceptualization: P.B., A.S., J.P., D.E., G.D., R.M. Data curation: K.C., D.E., G.D., R.M., A.H., J.P. Methodology: K.C., R.S., D.E. Formal analysis and Investigation: K.C., R.S. Funding acquisition: K.C., G.D., R.M., A.H., J.P. Resources: G.D., R.M., A.H., J.P. Supervision: D.E., A.S., P.B. Validation: R.S., D.E. Visualization: K.C. Writing - original draft: K.C. Writing - review and editing: K.C., D.E., R.S., G.D., R.M., A.H., J.P., A.S., P.B.

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Data Availability

Raw sequence data is available in the sequence read archive (SRA) under project number: PRJNA1522155The code and datasets generated and analysed for this project are available in the Github repository: kamryn-carter/Mixophyes-australis-projectPlease note that geographic coordinates have been removed from the metadata and any enquiries on the threatened species Mixophyes australis should be directed to the Accountable Officer: Rachel Melrose (Rachel.Melrose@dcceew.nsw.gov.au).

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Figures

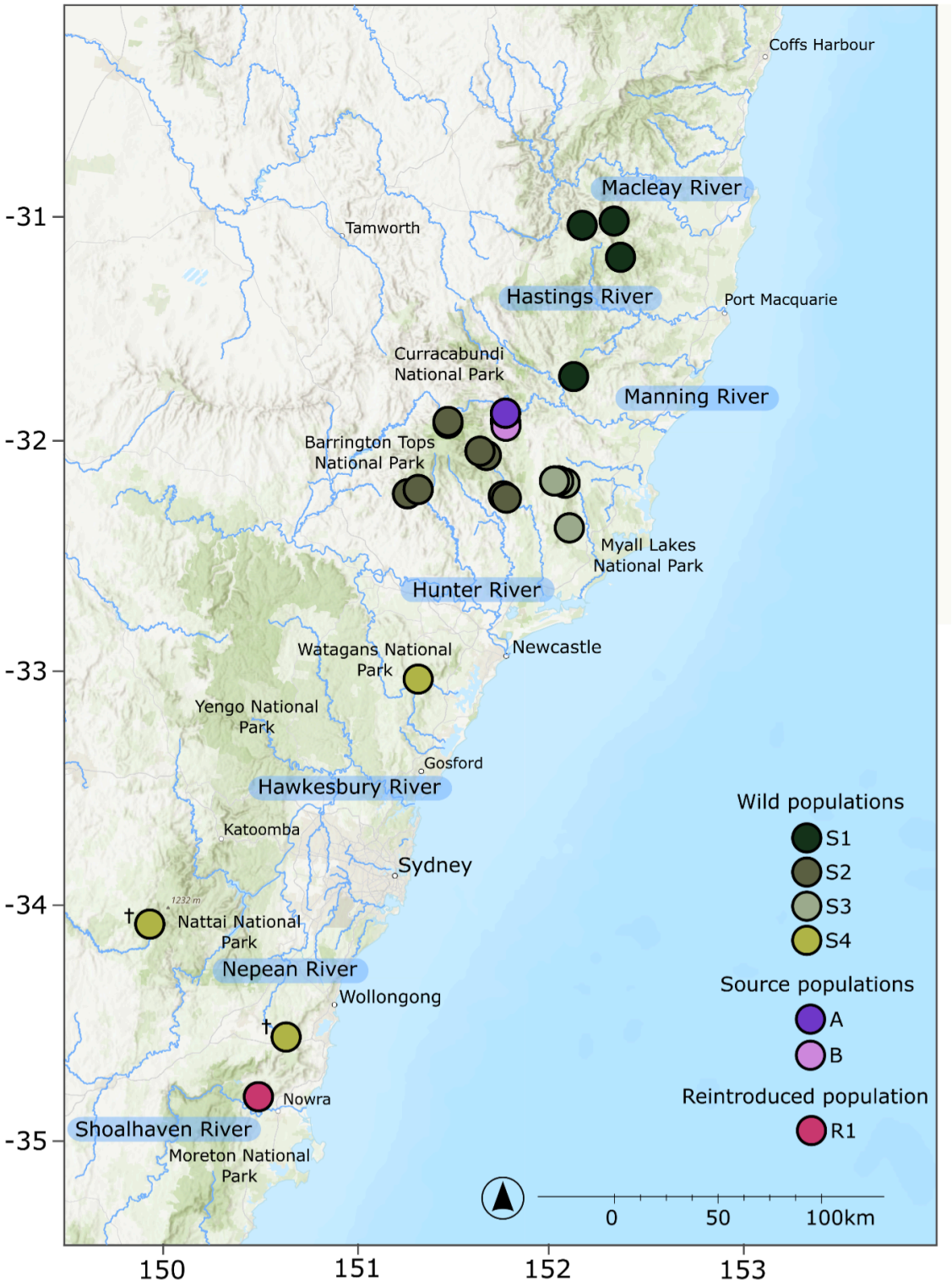


Figure 1

Map of individuals sampled. Wild populations from Mahony et al. (2023). Source populations A and B are locations of conservation breeding founding populations, R1 is a reintroduced population (introduced prior to the establishment of the conservation breeding program). † Indicates population is presumed extinct.

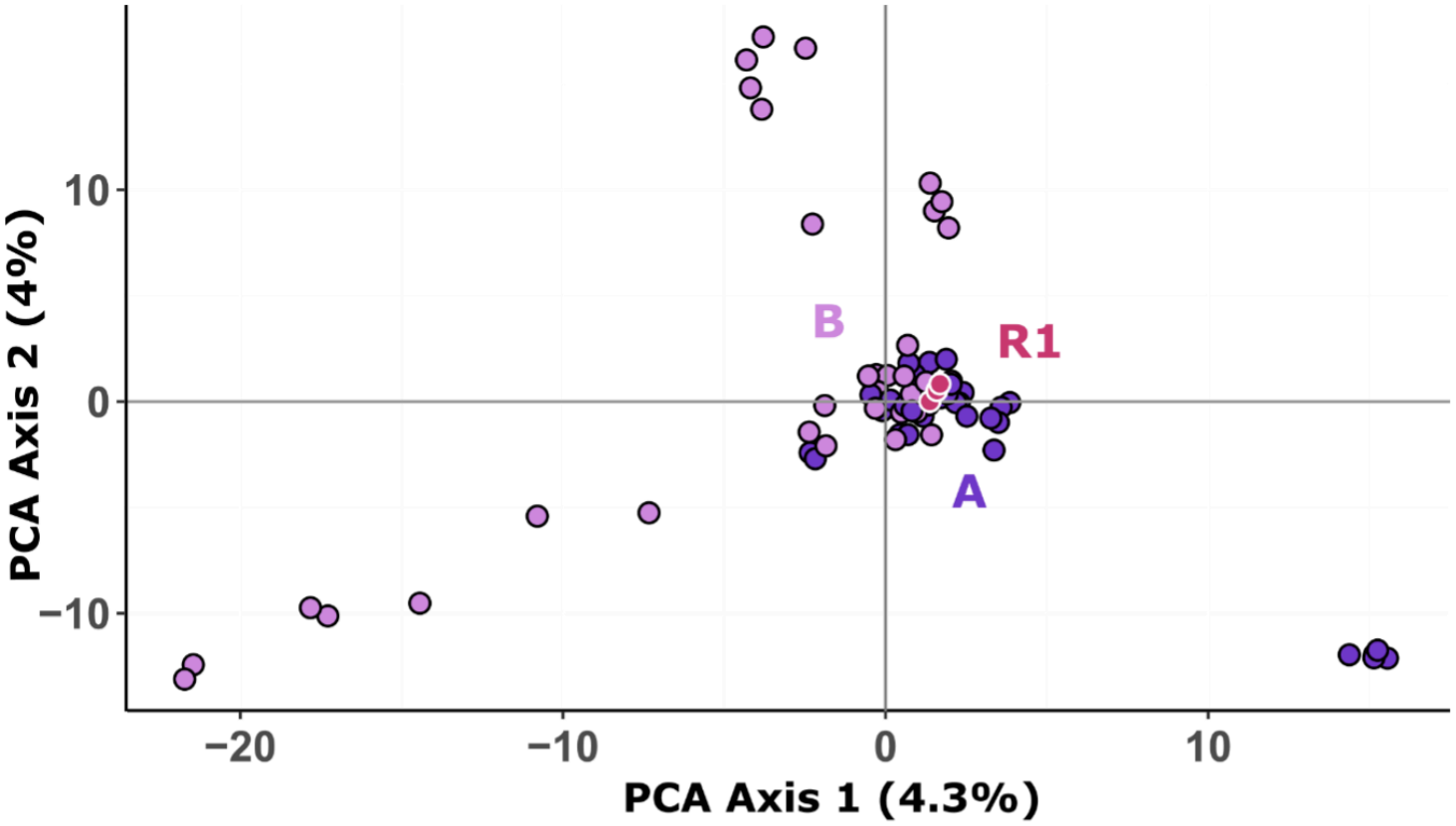


Figure 2

Results of a Principal Coordinates Analysis (PCoA) for conservation founding populations A and B, and the reintroduced population R1. Visual inspection shows little evidence of genetic structure or differentiation between populations (axis 1 = 4.3%, axis 2 = 4%) and external clusters correspond to related groups.

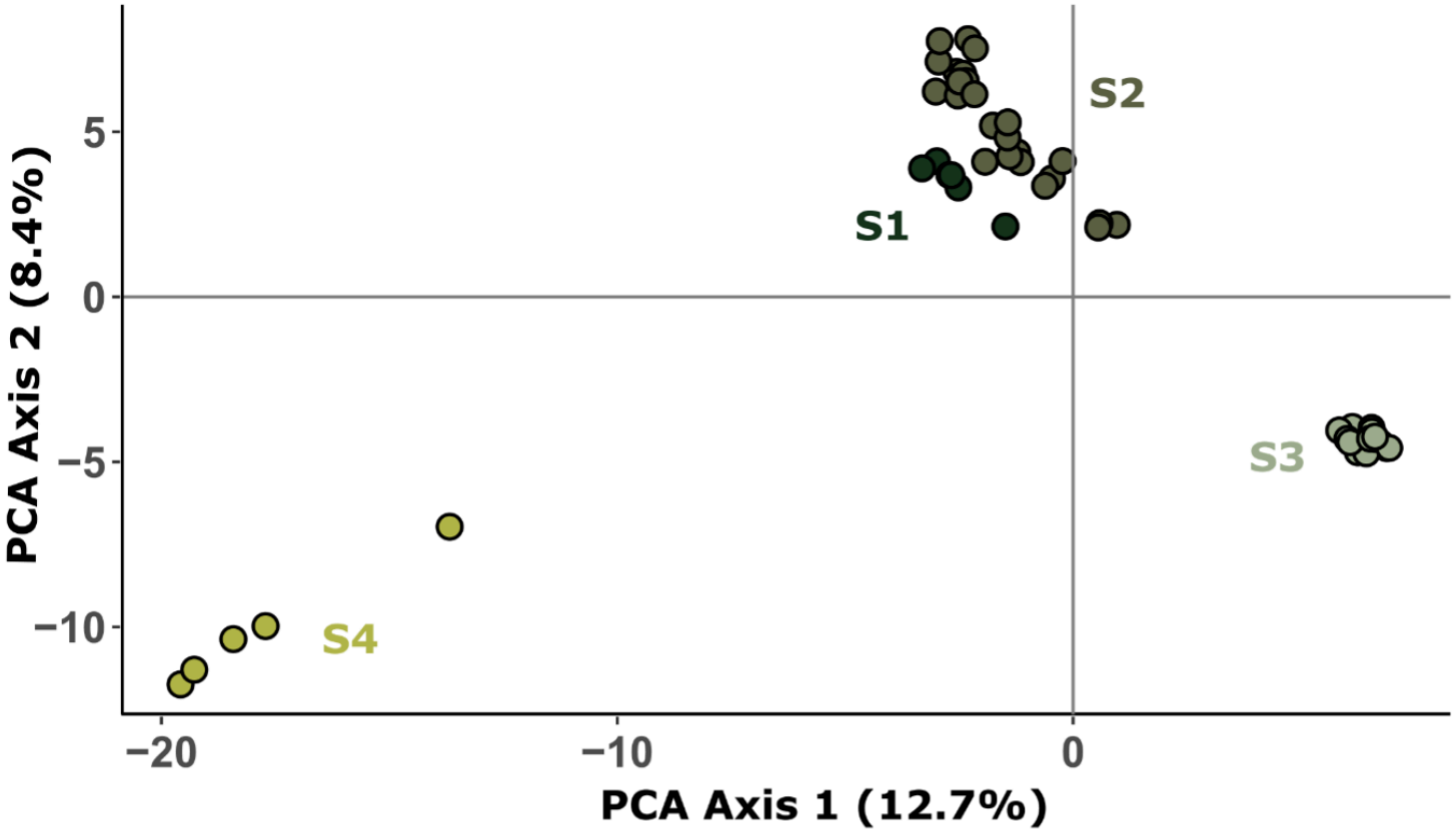


Figure 3

Results of a Principal Coordinates Analysis (PCoA) for wild populations S1-4. Visual inspection shows some genetic structure and differentiation between populations (axis 1 = 12.7%, axis 2 = 8.4%).

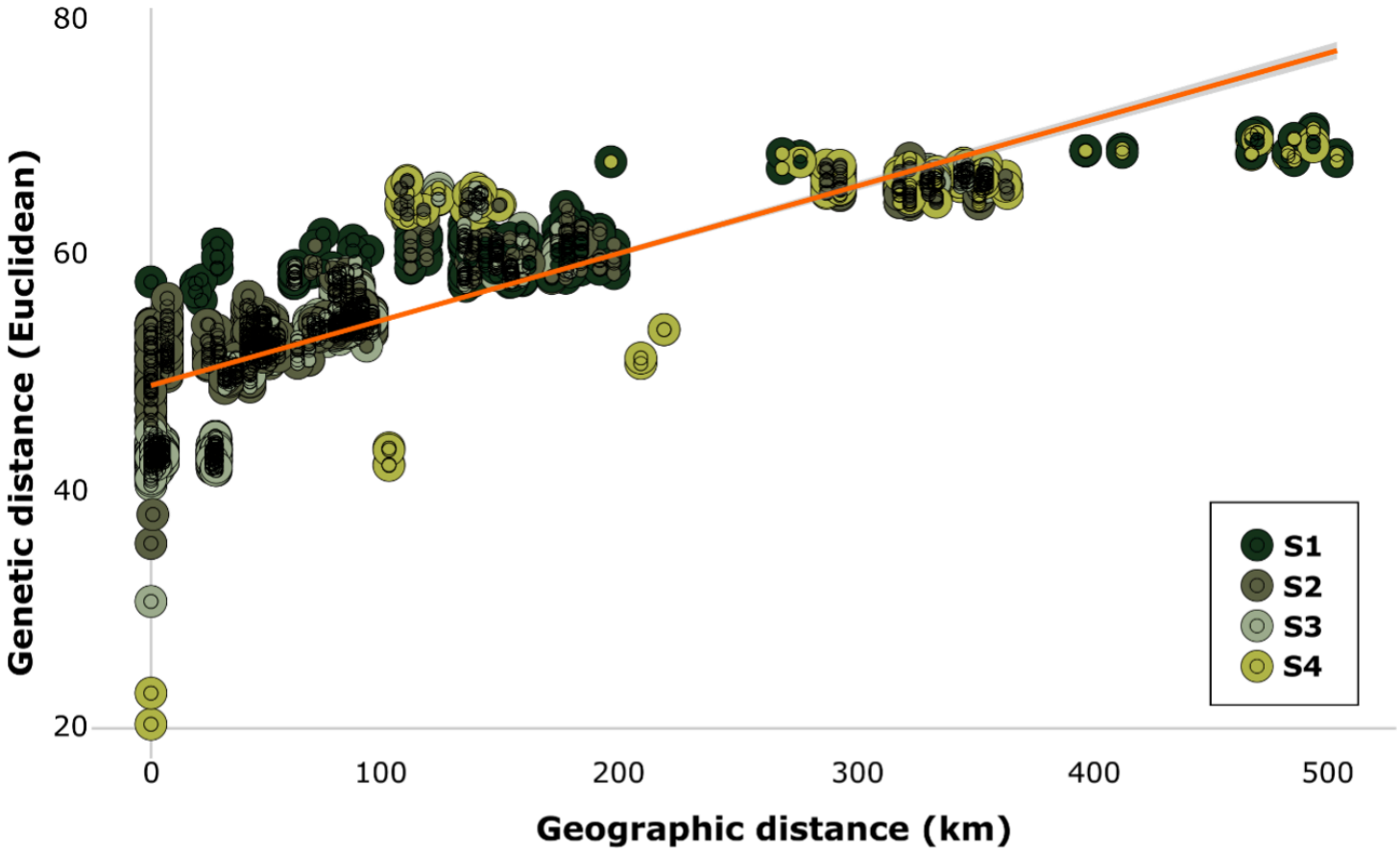


Figure 4

Plot of geographic versus genetic distance in wild populations. Each point represents a comparison between two individuals, with the colour of the inner and outer circles representing the populations those two samples belong to. Comparisons with S4 cluster horizontally across the top of the graph, indicating that genetic distance between S4 individuals and individuals from all other populations is high, irrespective of geographic distance.

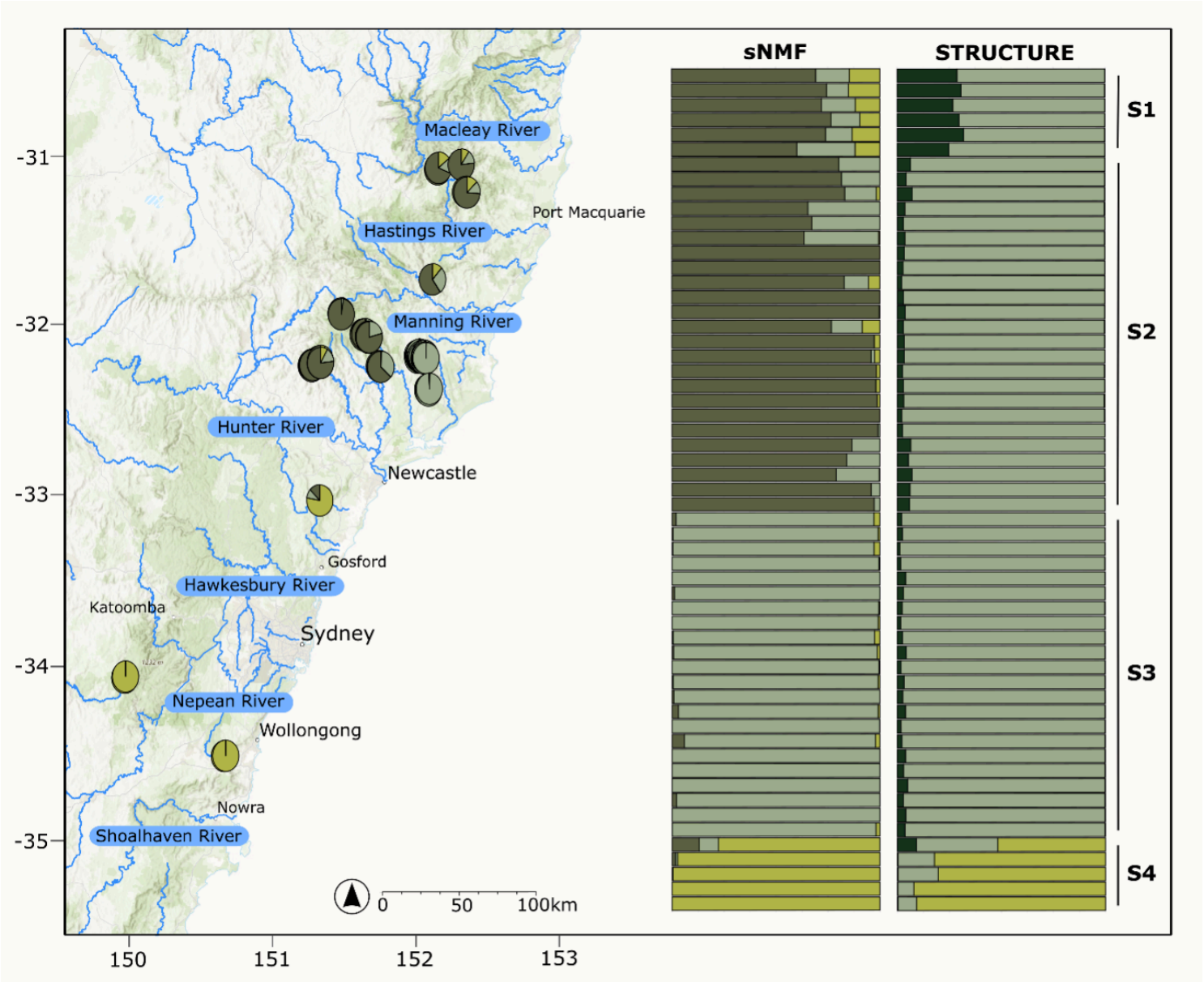


Figure 5

Left: Ancestry coefficients estimated from sNMF analysis plotted on a map of wild samples. Colours denote genetic clusters (K=3). Right: Ancestry coefficients for each individual estimated via two methods (left: sNMF, right: STRUCTURE) plotted as bar graphs. STRUCTURE analysis uses admixture model with correlated allele frequency. Colours denote genetic clusters estimated by each analysis (for both STRUCTURE and sNMF: K=3).

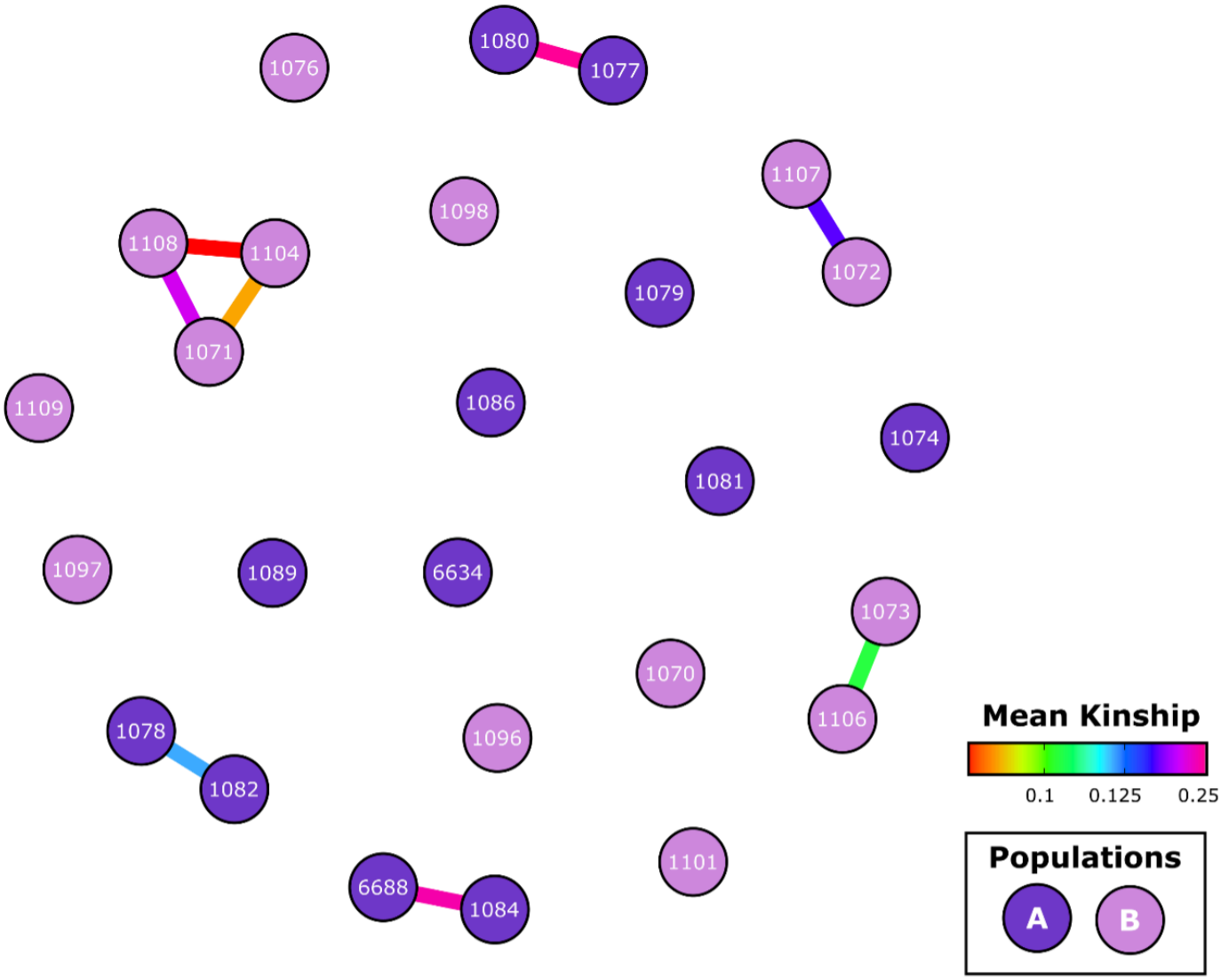


Figure 6

Network based on a genomic relatedness matrix (GRM) created using gl.grm.network which adjusts values to account for background genetic relatedness using the approach by Goudet et al. (2018). Relatedness threshold=0.062 and visualised using the Fruchterman-Reingold layout.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- MixophyespaperSupplementarymaterialdraft5.docx
- pairwisekinshipcomparisonbreeders.csv
- pairwisekinshipcomparisonall.csv