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CONTRIBUTED PAPERS

Longitudinal monitoring of neutral and adaptive genomic diversity in a reintroduction

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Abstract

Restoration programs in the form of ex-situ breeding combined with reintroductions are becoming critical to counteract demographic declines and species losses. Such programs are increasingly using genetic management to improve conservation outcomes. However, the lack of long-term monitoring of genetic indicators following reintroduction prevents assessments of the trajectory and persistence of reintroduced populations. We carried out an extensive monitoring program in the wild for a threatened small-bodied fish (southern pygmy perch, *Nannoperca australis*) to assess the long-term genomic effects of its captive breeding and reintroduction. The species was rescued prior to its extirpation from the terminal lakes of Australia's Murray-Darling Basin, and then used for genetically informed captive breeding and reintroductions. Subsequent annual or biannual monitoring of abundance, fitness, and occupancy over a period of 11 years, combined with postreintroduction genetic sampling, revealed survival and recruitment of reintroduced fish. Genomic analyses based on data from the original wild rescued, captive born, and reintroduced cohorts revealed low inbreeding and strong maintenance of neutral and candidate adaptive genomic diversity across multiple generations. An increasing trend in the effective population size of the reintroduced population was consistent with field monitoring data in demonstrating successful re-establishment of the species. This provides a rare empirical example that the adaptive potential of a locally extinct population can be maintained during genetically informed ex-situ conservation breeding and reintroduction into the wild. Strategies to improve biodiversity restoration via ex-situ conservation should include genetic-based captive breeding and longitudinal monitoring of standing genomic variation in reintroduced populations.

KEYWORDS
adaptive genetic diversity, Australian fish, conservation genomics, ex-situ population management, population genomics, Murray-Darling Basin, Percichthyidae, restoration threatened species

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Resumen: Los programas de restauración a manera de reproducción ex situ combinada con reintroducciones se están volviendo críticos para contrarrestar las declinaciones demográficas y la pérdida de especies. Dichos programas usan cada vez más la gestión genética para mejorar los resultados de conservación. Sin embargo, la falta de monitoreo a largo plazo de los indicadores genéticos posteriores a la reintroducción evita que se realicen evaluaciones de la trayectoria y la persistencia de las poblaciones reintroducidas. Se rescató un pez de talla pequeña (percha pigmea del sur [*Nannoperca australis*]) previo a su extirpación de los lagos terminales de la Cuenca Murray-Darling en Australia para después reproducirlo

en cautiverio con información genética y reintroducirlo. Realizamos monitoreos anuales o bianuales de la abundancia, aptitud y ocupación en vida silvestre durante once años, además de un muestreo genético posterior a la reintroducción. Analizamos los datos genómicos de los grupos originales rescatados, los nacidos en cautiverio y los reintroducidos. Nuestro objetivo era evaluar los efectos genómicos a largo plazo de la reproducción en cautiverio y la reintroducción de esta especie. Esto reveló baja endogamia y el sólido mantenimiento de la diversidad genómica neutral y adaptativa durante varias generaciones. Encontramos una coherencia entre la tendencia creciente en el tamaño de la población efectiva de la población reintroducida y los datos de campo que demostraron el restablecimiento exitoso de la especie. Nuestro estudio proporciona un raro ejemplo empírico de cómo el potencial adaptativo de una población localmente extinta puede mantenerse durante la reproducción de conservación ex situ genéticamente informada y su reintroducción. Las estrategias para mejorar la restauración de la biodiversidad por medio de la conservación ex situ deberían incluir la reproducción en cautiverio basada en la genética y el monitoreo longitudinal de la variación genómica actual de las poblaciones reintroducidas.

PALABRAS CLAVE
Cuenca Murray-Darling, diversidad genética adaptativa, especie amenazada, gestión poblacional ex situ, genómica de la conservación, genómica de la restauración, peces australianos, Percichthyidae

INTRODUCTION

Ex-situ breeding and population reintroductions are important tools for biodiversity conservation (Attard et al., 2016a; Seddon et al., 2007) that are becoming increasingly popular as human development and climate warming accelerate habitat loss and population extinctions (He et al., 2016). Limited information is available about the fate of most reintroduced populations as postreintroduction monitoring is often neglected (Bell et al., 2019; Frankham, 2010; Schwartz et al., 2007). Furthermore, most conservation breeding efforts do not incorporate genetic monitoring of released captive-bred individuals and of their wild-born offspring (Attard et al., 2016a; Schwartz et al., 2007). This prevents the collection of genetic data to inform on the ecological success (e.g., survival and recruitment) and evolutionary potential (e.g., levels of inbreeding and adaptive diversity) of conservation reintroductions.

Genetic diversity is intimately tied to evolutionary fitness and provides the means for populations to respond and adapt to novel and changing environments (DeWoody et al., 2021; Frankham, 2010; Lande & Shannon, 1996; Stange et al., 2021), such as those experienced during reintroductions (He et al., 2016). Genome-wide diversity is generally expected to decrease across generations in ex-situ conservation programs due to inbreeding, genetic drift, and adaptation to captivity (Frankham, 2010; Ouborg et al., 2010). The greater effect of drift relative to selection in small populations leads to the loss of standing genetic variation and reduction of adaptive potential (Whitlock, 2000; Willoughby et al., 2015). Nonetheless, fitness can be recovered in the wild if enough genetic diversity has been maintained and fitness is sufficient for re-establishment (Frankham, 2008). These issues highlight the importance of implementing scientifically rigorous practices when establishing ex-situ breeding and conservation reintroductions, such as (1) selecting appropriate source populations for captivity with high standing genetic variation (a proxy for neutral and adaptive genetic diversity) (DeWoody et al., 2021; He et al., 2016; Stange et al., 2021), (2) using multiple separate breeding units (pairs or groups) to generate a diversity of families (Wang, 2004), (3) managing crosses within units to minimize inbreeding and maximize genetic diversity (Attard et al., 2016a), (4) using captive environments that simulate natural habitat, and (5) rapidly reintroducing individuals to avoid adaptation to captivity (Brown & Day, 2002; Witzenberger & Hochkirch, 2011).

Our longitudinal genomic monitoring focuses on a restoration initiative for the southern pygmy perch (*Nannoperca australis*: Percichthyidae). This is a small-bodied fish (<85 mm) from south-eastern Australia typically found in small streams and wetlands with sheltered microhabitat and abundant aquatic vegetation (Lintermans, 2007). The species is well studied from an eco-evolutionary perspective, and is the target of multiple government- and community-based restoration efforts at both local and interjurisdictional scales. Southern pygmy perch has an evolutionary history influenced by historical variation in hydrology and climate (e.g., Unmack et al., 2011; Buckley et al., 2021) and has evolved reproductive strategies, genomic adaptations, and gene expression plasticity in response to contemporary environmental heterogeneity (e.g., Morrongiello et al., 2010; Morrongiello et al., 2012; Brauer et al., 2016; Brauer et al., 2017). Due to their small size, localized habitat preference, and limited dispersal capacity, southern pygmy perch populations are extremely vulnerable to excess diversion of water for irrigation and to habitat degradation and fragmentation (Lintermans, 2007; Hammer et al., 2013; Attard et al., 2016a; Brauer & Beheregaray, 2020). The species recently experienced major demographic declines and local extinctions across its range in the Murray-Darling Basin (MDB) and is listed as endangered in the state of South Australia (where this work took place), with the MDB lineage considered vulnerable under national Environment Protection and Biodiversity Conserva-

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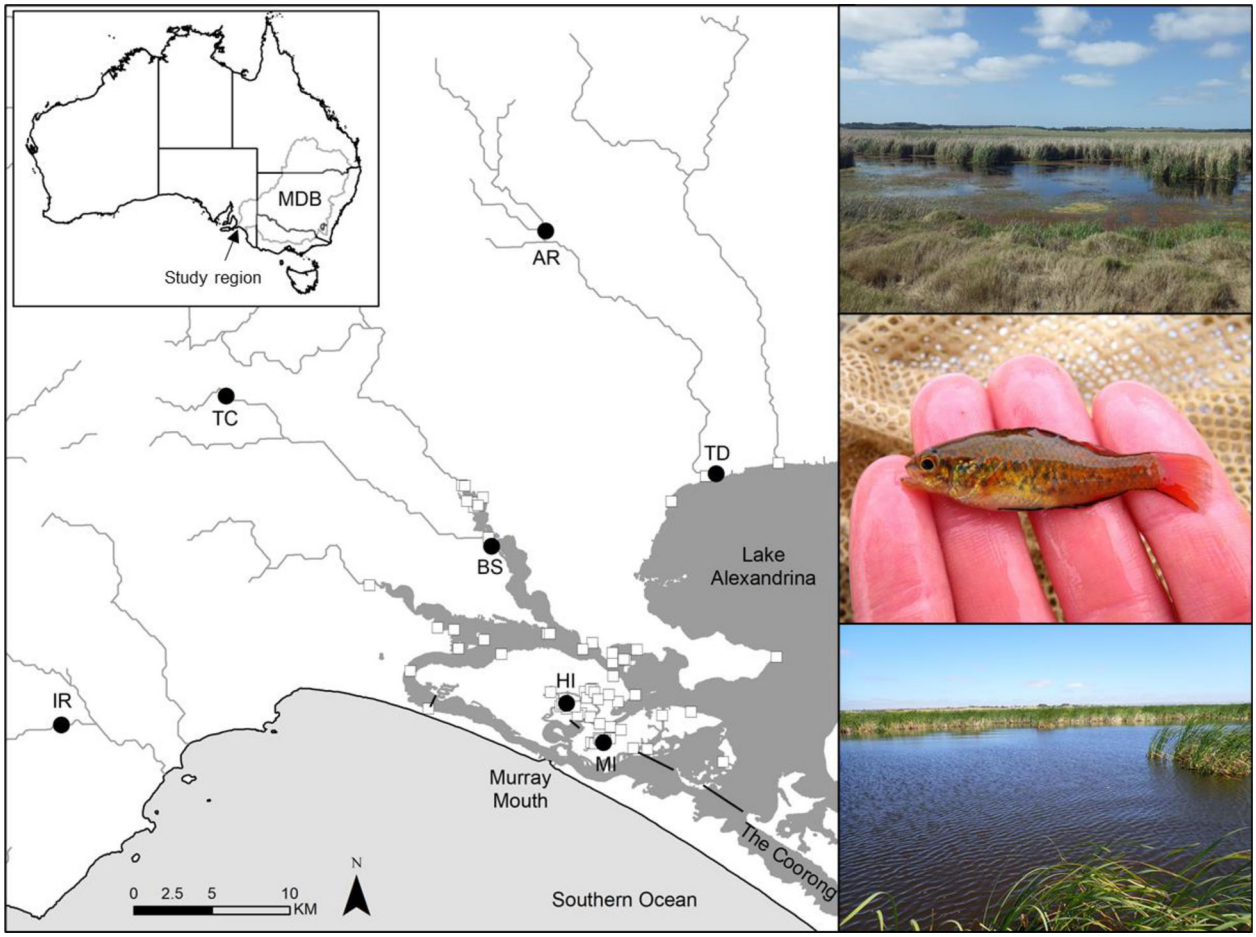

FIGURE 1 Map of monitored sites (white squares) and sampling locations for southern pygmy perch (*Nannoperca australis*) in the lower Murray River, South Australia. The Lower Lakes population was sampled from Hindmarsh Island, Mundoo Island, Turvey's Drain and Black Swamp. The adjacent populations were sampled from the Angas River, Inman River, and Tookayerta Creek. Photographs show southern pygmy perch and its representative habitat. Sampling sites: AR = Angas River, BS = Black Swamp, HI = Hindmarsh Island, IR = Inman River, MI = Mundoo Island, TC = Tookayerta Creek, and TD = Turvey's Drain

tion (EPBC Act) legislation (Hammer et al., 2013; Beheregaray et al., 2021).

In the Lower Lakes region of the MDB (Figure 1), the evolutionarily divergent and locally adapted population of southern pygmy perch (Brauer et al., 2016; Cole et al., 2016; Brauer et al., 2017; Buckley et al., 2021) was extirpated during the Australian Millennium Drought (a period of severe water shortages from 1997 to 2010) (Hammer et al., 2013). In 2007, 65 individuals were rescued and brought into captivity just prior to complete desiccation of their habitat (Hammer et al., 2013). They formed the founding population for a captive breeding program that utilized microsatellite DNA to establish brood groups that effectively maintained genetic diversity and kept inbreeding levels low in the F1 generation (Attard et al., 2016a). Following the return of water to the Lower Lakes region in late 2010, approximately 1350 offspring of the first captive generation were reintroduced to three former habitats in spring 2011 and autumn 2012 (Hammer et al., 2013). Monitoring of the reintroduced Lower Lakes population (and of other reintroduced small-bodied fish species; details in Hammer et al., 2013) took place between 2011 and 2021, with those field-based efforts forming the basis of this study.

The relatively short lifespan (3–6 years) and time to maturity (within 1 year) of southern pygmy perch (Lintermans, 2007), as well as existing knowledge about genomic regions underpinning hydroclimatic adaptation in the Lower Lakes population and across the species range (Brauer et al., 2016; Brauer et al., 2017), make it an ideal system for studying how genome-wide variation, adaptive diversity, and inbreeding change during a conservation reintroduction. We achieve that here by combining abundance, fitness, and occupancy data from longitudinal monitoring with genomic data from the original wild-rescued population, the captive population, and from multiple generations since reintroduction in the Lower Lakes. Samples from adjacent upstream populations of southern pygmy perch (Brauer et al., 2016; Cole et al., 2016) were also included to assess the possibility of gene flow into the Lower Lakes population since reintroduction. This study provides a rare opportunity to assess the long-term genomic consequences and the adaptive potential of a captive breeding and reintroduction program derived from a small number of founders. It exemplifies challenges and benefits of longitudinal genomic monitoring and is expected to promote and advance the genetic management of threatened species targeted by ex-situ conservation programs.

METHODS

Field monitoring, demographic data, and genomic sampling

Field surveys in the Lower Lakes region focusing on small-bodied freshwater fishes have occurred since 2002 (Hammer et al., 2013). Following reintroductions, the monitoring and sampling of the southern pygmy perch population was undertaken at least annually from 2011 to 2021 at the three reintroduction sites, and in another 20–35 formerly inhabited sites. Based on the annual autumn data, three key features of the population were examined, namely abundance (total number captured in a year during standardized sampling, see Wedderburn et al., 2022), proportion of young-of-the-year (YOY) fish in the catch, and naïve occupancy (the proportion of sites where the species was detected). Genetic sampling (fin clips) was undertaken on a total of 12 subpopulation samples ($n = 352$ individuals) representing four genetically distinct populations (Brauer et al., 2016; Cole et al., 2016) in the lower Murray catchment, South Australia (Figure 1). The Lower Lakes population of southern pygmy perch inhabits fringing wetland habitat over the large (649 km^2) connected Lake Alexandrina, whereas its nearest adjacent populations are found in three partially isolated tributaries of this catchment system: the Angas River, Inman River, and Tookayerta Creek (Whiterod et al., 2015). Individuals were collected under relevant ethics approval and research permits (Flinders AWC permit E313; ME99029527; ME99029705; ME9903013; ME9902959). Adult fish were fin clipped, measured, and returned live to their collecting site with tissue preserved in 100% ethanol and stored at −80°C until DNA extractions. The temporal samples include the wild-rescued breeders, two generations of captive-born populations (11 family groups representing the F1 and one representing the F2), and six samples representing mixed-age cohorts of the Lower Lakes reintroduced population collected between 2014 and 2019. Each F1 family group was produced from breeding two dams and two sires from the wild-rescued subpopulation; these were selected to avoid using inbred brooders and ensuring low estimated pairwise relatedness within breeding groups (details in Attard et al., 2016a). Additionally, each adjacent population was sampled in autumn 2019 (details in Tables 1 & Table 2, & Supporting Information).

DNA extraction, genomic libraries, and sequencing

DNA was extracted using a modified salting out process (Sunnucks & Hales, 1996). Extract quantity and quality was assessed using Qubit™ (Thermo Scientific), Nanodrop 1000 spectrophotometer (Thermo Scientific), and agarose gel electrophoresis. Double-digest restriction site-associated DNA (ddRAD) libraries were created using a ddRAD protocol (Peterson et al., 2012), modified as in Brauer et al. (2016) and sequenced as 150 base-pair paired-end reads on an Illumina HiSeq4000 at Novogene (Hong Kong). Detailed lab protocols are in Supporting Information.

TABLE 1 Demographic trends for the reintroduced southern pygmy perch (*Nannoperca australis*) population derived from fish monitoring in the Lower Lakes every autumn from 2011 to 2021

Year	Abundance	YOY (%)	No. of sites surveyed	Naïve occupancy
2011	0	0	21	0.00
2012	4	50	35	0.06
2013	1	100	35	0.03
2014	14	93	35	0.03
2015	37	81	26	0.08
2016	40	58	3 + 17(2)	0.30
2017	88	60	3 + 17(2)	0.35
2018	129	73	3 + 17(2)	0.45
2019	46	52	3 + 20(2)	0.39
2020	71	66	3 + 20(2)	0.43
2021	114	39	3 + 20(2)	0.35

Note: Abundance represents the total number of *N. australis* captured in the year. The proportion of young-of-the-year (YOY [%]) in the catch provides a general measure of recruitment success at the end of the season. Two replicate surveys of 17 or 20 sites (to account for probability of detection) were conducted from 2016 onward except at three sites. Naïve occupancy is the proportion of sites where the species was detected to indicate the extent of occurrence of *N. australis* within the Lower Lakes region.

Bioinformatics and single nucleotide polymorphism filtering

Sequences were assessed for quality with Fastqc v0.11.8 (Andrews, 2010) before mapping to a recently assembled southern pygmy perch reference genome (Sandoval-Castillo & Beheregaray in review). Single nucleotide polymorphisms (SNPs) were called using dDocent (Puritz et al., 2014) and filtered using VCFtools (Danecek et al., 2011). Genotype error rate was calculated using 23 pairs of replicate samples. As inferences about population structure and genetic diversity can be biased by loci not conforming to neutral expectations (Luikart et al., 2003), we defined neutral and candidate adaptive SNP datasets. We used a previously characterized adaptive SNP dataset for southern pygmy perch that are statistically associated with hydroclimatic variation (Brauer et al., 2016), as well as Bayescan (Foll & Gaggiotti, 2008) to detect F_{ST} outlier SNPs. The candidate adaptive SNPs were then removed from the population genomic dataset to create a putatively neutral SNP dataset. Both datasets were thinned to only retain SNPs separated by more than the average fragment length of 500 bp to minimize the effects of linkage disequilibrium. Details about bioinformatics and filtering are in Supporting Information.

Data analysis

Genomic diversity, inbreeding, relatedness, and effective population size (Ne)

The number of alleles (Na), observed (Ho) and expected heterozygosity (He), and population-level inbreeding coefficient

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TABLE 2 Categories and subcategories of southern pygmy perch (*Nannoperca australis*) used for genomic analysis

Category (sampling year)	Subcategory	Individuals retained	Total number of individuals per population/cohort
Original wild-caught breeders (2007)	–	19	19
Captive F1 (2011)	Family group A	11	109
	Family group B	11	
	Family group C	10	
	Family group D	9	
	Family group E	11	
	Family group F	14	
	Family group G	7	
	Family group H	10	
	Family group I	11	
	Family group J	4	
	Family group K	11	
Captive F2 (2013)	–	14	14
Reintroduced population	R1: Spring 2014	10	105
	R2: Spring 2015	7	
	R3: Autumn 2017	7	
	R4: Autumn 2018	35	
	R5: Summer 2018/19	42	
	R6: Autumn 2019	4	
Adjacent populations (Summer 2018/19)	Angas River	8	24
	Inman River	6	
	Tookayerta Creek	10	

Note: Number of individuals retained after bioinformatics and genotyped at 10,877 SNPs.

(F_{IS}) were calculated for both the neutral and candidate adaptive SNP datasets using the R (RCoreTeam, 2019) packages adegenet and hierfstat (Jombart, 2008; Goudet, 2005). A Wilcoxon signed rank test was run using the R package stats to compare He and Na between years for the reintroduced population. Individual inbreeding coefficients (F) were also estimated for each subpopulation using the beta.dosage function of hierfstat. Pairwise relatedness was estimated using the triadic likelihood estimator (*r*) (Wang, 2007) within the R package related (Pew et al., 2015). Estimates of Ne were calculated for each sample using the LD method in NeEstimator V2.1 (Do et al., 2014). Minor allele frequencies were adjusted using the no singleton alleles option, and 95% confidence intervals estimated via jackknife (Jones et al., 2016).

Population structure

Pairwise F_{ST} and principal component analyses (PCAs) were used to test and visualize whether the reintroduced population may have diverged genetically from the wild-caught founders of the captive program, and to test for differentiation between reintroduced and adjacent populations. Pairwise F_{ST} were calculated for the neutral dataset in ARLEQUIN (Excoffier & Lischer, 2010) using 10,000 permutations to derive confidence intervals. Both the neutral and candidate adaptive SNP datasets were reduced to principal components using the R package vegan (Oksanen et al., 2019). Admixture (Alexander et al., 2009) was run using the neutral SNPs to assess connectivity between adjacent tributaries and the reintroduced population. Following an initial exploratory analysis with no prior population information, the supervised learning procedure was used with the wild-caught breeders, reintroduced F1s, and adjacent tributary populations evaluated as potential source populations. In both runs, K was assumed to be four ancestral populations (the three adjacent tributary and Lower Lakes populations).

RESULTS

Field monitoring and demography of the reintroduction

Across monitoring every autumn from 2011 to 2021, a total of 278 surveys were carried out at sites suitable to support the Lower Lakes population (65 unique sites overall and 30 sites annually surveyed). Following no detection soon after reintroduction in 2011, fish were recorded in every subsequent season from 2012, with the first wild-born generation apparent in March 2014. The species was initially detected in only 3–8% of surveyed sites (2012–2015) but showed substantial increase in occupancy (30–45% of surveyed sites) in subsequent years. During the same period, recruitment success fluctuated from moderate to high (YOY = 39–73%), thereby indicating a self-sustaining population (Table 1).

Neutral and adaptive genomic diversity

After bioinformatics filtering (Supporting Information), a total of 10,877 high-quality filtered SNPs were available for 271 individuals (from the initial sample of 352). No F_{ST} outliers among cohorts were detected with Bayescan (FDR < 0.1). A total of 159 of the 216 previously reported candidate adaptive SNPs (Brauer et al., 2016) were variable in the samples. The final neutral and adaptive SNP datasets consisted of 10,799 and 159 SNPs, respectively, across 271 individuals.

Levels of neutral and candidate adaptive genomic diversity for the wild-caught breeders that founded the ex-situ breeding program were retained in the F1 and also across all temporal samples of the reintroduced population. No significant differences in He or Na were observed between any of the captive or reintroduced samples (Figure 2, $p > 0.05$). The sample from

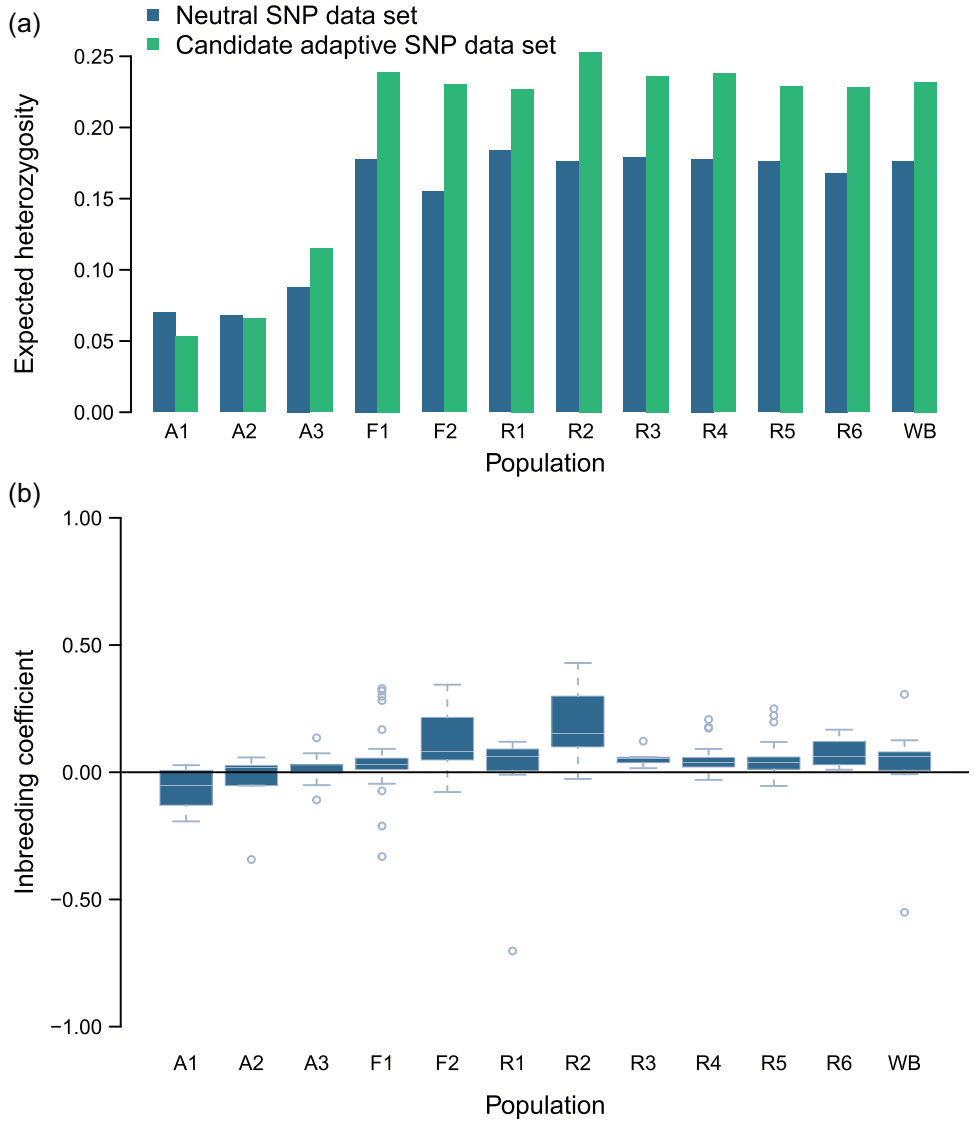

FIGURE 2 Levels of genomic diversity and inbreeding for southern pygmy perch (*Nannoperca australis*). Expected heterozygosity based on the neutral SNP and the candidate adaptive SNP datasets (a) and the individual inbreeding coefficient (F) values based on the neutral SNP dataset (b). Results are shown for the adjacent populations (A1: Angas River, A2: Inman River, and A3: Tookayerta Creek), captive populations (F1 and F2), captures from the reintroduced population (R1: Spring 2014, R2: Spring 2015, R3: Autumn 2017, R4: Autumn 2018, R5: Summer 2018/2019, and R6: Autumn 2019), and the original wild-caught breeders (WB) that founded the captive breeding program

autumn 2019 was excluded due to its small size ($n = 4$). In contrast, levels of neutral and candidate adaptive genomic diversity of the adjacent upstream populations were less than half than the Lower Lakes samples (Figure 2, $p < 0.001$).

Relatedness and inbreeding

Very few individuals with $r > 0.1$ (139 out of 2280 comparisons) were discovered in the Lower Lakes samples and no individuals were repeatedly sampled during monitoring (Supporting Information). Relatedness among wild-caught breeders ranged from 0 to 0.216 with one out of 171 (0.6%) comparisons with $r > 0.1$. No related individuals were included in the same captive breeding groups, as previously demonstrated with microsatellite data for a larger sample (Attard et al., 2016a). Within F1 family groups, there were 110 out of 469 (23.5%) comparisons with $r > 0.1$ (r ranged from 0 to 0.462). In the F2s, r ranged from 0 to 0.319 with 16 of 91 (18%) comparisons with $r > 0.1$. Relatedness within the R1 cohort ranged from 0 to 0.446 with two of 45 (4%) comparisons with $r > 0.1$. For the R2 and R3 cohorts, no related pairs of individuals were detected. Relatedness within the R4 cohort ranged from 0 to 0.495 with six of 595 (1%) comparisons with $r > 0.1$. For the R5 cohort, estimates ranged from 0 to 0.198 with four of 861 (0.5%) comparisons with $r > 0.1$. Pairwise relatedness values for the R6 cohort were all 0. Very few individuals with $r > 0.1$ were discovered in any of the adjacent populations and no repeated samples were discovered (Support-

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ing Information). For the Angas River, *r* ranged from 0 to 0.322 with only three of 28 (11%) comparisons with $r > 0.1$. Relatedness estimates for both the Inman River population and the Tookayerta Creek population were all 0.

The individual-level inbreeding (F) was low across all of the Lower Lakes cohorts (Figure 2 & Supporting Information). The average F in the wild-caught breeders was 0.052 (−0.527 to 0.316). Low inbreeding coefficients were maintained in the F1 sample with an average of 0.039 (−0.329 to 0.331) and throughout the annual snapshots of the reintroduced population with an average of 0.059 (−0.751 to 0.441). The F2 cohorts showed a substantial increase in inbreeding compared to the F1s with an average F of 0.222 (0.066−0.430). Individual-level inbreeding was low in the adjacent populations. The Angas River, Inman River, and Tookayerta Creek populations had average F of −0.065 (−0.193 to 0.028), −0.045 (−0.343 to 0.058), and 0.011 (−0.109 to 0.135), respectively.

Effective population size (Ne)

There was an increasing trend in the reintroduced population size over time with Ne estimates of 24 (95% CI: 8.6–infinite), 278 (95% CI: 102.7–infinite), 356 (95% CI: 180.4−4713.8), and 778 (95% CI: 387.7−36,063.4) for the samples from spring 2014, autumn 2017, autumn 2018, and summer 2018/19, respectively. The Ne for the wild-caught breeders was 392.1 (95% CI: 73.3–infinite) (Supporting Information). The single-cohort F1 and F2 estimates of 30.6 (95% CI: 26.7−35.1) and 12.3 (95% CI: 7.9−21.3), respectively, represent the number of breeders (N_b) in the captive population (Waples et al., 2014) and are broadly consistent with the actual number of individuals that contributed to those cohorts (Attard et al., 2016a). The adjacent populations, spring 2015 and autumn 2019 cohorts, returned infinite Ne estimates, which are commonly reported for this method, and likely reflect increased sampling error associated with small sample sizes and low genetic diversity (Do et al., 2014).

Population structure

Genetic differentiation among the Lower Lakes cohorts was very low with all F_{ST} values below 0.07. If the reintroduced cohort from 2019 (R6) and the captive F2s are excluded due to small sample size and unrepresentative breeding groups, respectively, the F_{ST} values are all below 0.015. The three adjacent populations were all highly differentiated, both from one another and from the wild-caught breeders and the Lower Lakes population (F_{ST} ranged from 0.150 to 0.643) (Supporting Information). This pattern is supported by the PCA results based on neutral SNPs (Figure 3), where all Lower Lakes samples group together and adjacent populations appear as unique clusters. The candidate adaptive SNP dataset shows slightly different results with the Tookayerta Creek population grouping with the Lower Lakes cohorts (Figure 3). However, the Angas River and Inman River populations are again separate, both from

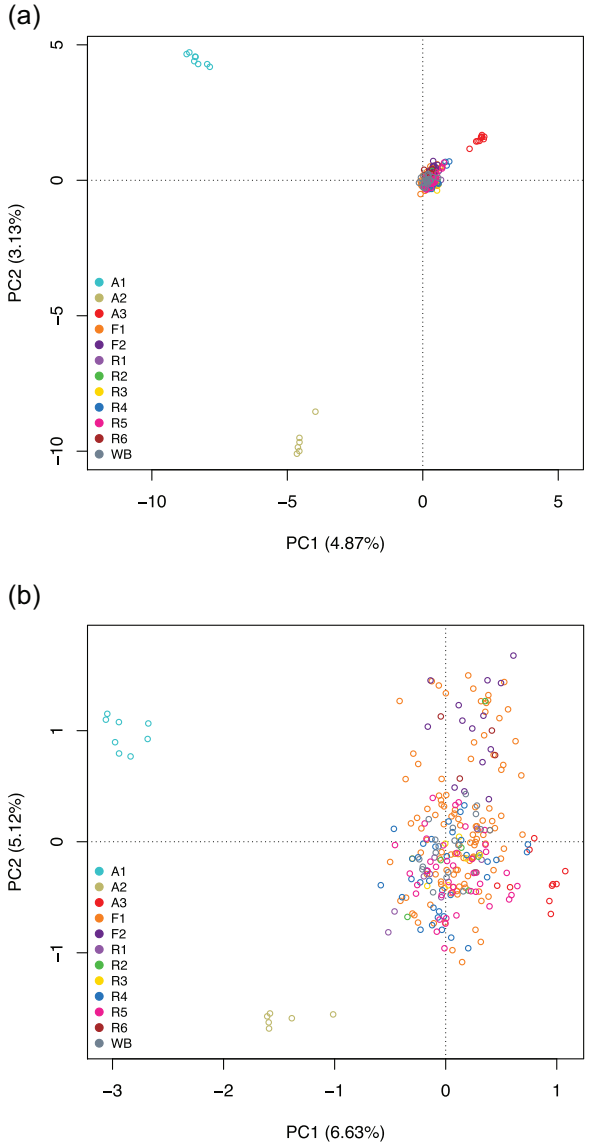

FIGURE 3 PCA plots for the lower Murray populations of southern pygmy perch (*Nannoperca australis*). (a) neutral SNP dataset and (b) candidate adaptive SNP dataset. Adjacent populations (A1: Angas River, A2: Inman River, and A3: Tookayerta Creek), Lower Lakes captive populations (F1 and F2), Lower Lakes captures from the reintroduced population (R1: Spring 2014, R2: Spring 2015, R3: Autumn 2017, R4: Autumn 2018, R5: Summer 2018/2019, and R6: Autumn 2019), and the original Lower Lakes wild-caught breeders (WB)

each other and from the Lower Lakes and Tookayerta Creek populations. The Admixture results support the differentiation between the three adjacent populations and the Lower Lakes population. These results also suggest some admixture from the Tookayerta Creek population into the Lower Lakes population, but none from the Lower Lakes population into any of the adjacent populations. The supervised learning-based hypothesis test further supported very low Tookayerta Creek ancestry within the Lower Lakes population, but provided no evidence for recent connectivity (Supporting Information).

DISCUSSION

We investigated the long-term outcomes of an ex-situ restoration program by using longitudinal genomic data to assess changes in inbreeding and genetic diversity across multiple generations of a threatened freshwater fish. Low inbreeding and maintenance of both neutral and adaptive genomic diversity of the original founding population were observed in the first generation in captivity (F1) and, critically, across up to eight generations in the population reintroduced to the wild. In addition, an increasing trend in the effective population size estimates and capture data for the reintroduced population indicate successful and ongoing recruitment in the wild. Our longitudinal monitoring in the wild of the F1 and subsequent generations indicate that the benefits of a well-designed genetically informed captive breeding might be long-lasting and can promote persistence and adaptive potential of reintroduced populations.

Genomic diversity and inbreeding in a conservation reintroduction

Levels of putatively neutral and adaptive genome-wide diversity remained markedly similar across the various Lower Lakes cohorts of southern pygmy perch sampled between 2007 and 2019. The minor increase in genetic diversity from the 2007 wild-caught breeders into the captive F1s can be attributed to the design of matching unrelated family groups for the breeding program (Attard et al., 2016a). Inbreeding was also successfully maintained at very low levels across the Lower Lakes cohorts, with only four highly inbred (F>0.25) and 11 moderately inbred individuals (F>0.125) out of 105 captured fish. This is consistent with previous reports of negligible inbreeding in the wild-rescued Lower Lakes population (Attard et al., 2016a), including in comparisons relative to all known populations of southern pygmy perch (Cole et al., 2016). The relevance of a genetically informed breeding design is highlighted by the results for the captive F2s. Although the design for the F1 captive population was genetically guided, the F2 was randomly bred from a small subset of 37 individuals representing three family groups retained in captivity as insurance following the initial reintroductions. This resulted in substantial loss of diversity between the F1 and F2, as reported in an intergenerational microsatellite DNA study in captivity (Attard et al., 2016b). Our genomic results show that inbreeding in the F2 also increased markedly, with five highly inbred individuals (F>0.25) and another six moderately inbred individuals (F>0.125) out of 14 genotyped individuals. A rapid increase in inbreeding is often reported in captive populations (Woodworth et al., 2002; Frankham, 2008; Witzenberger & Hochkirch, 2011). Modeling based on genetic and demographic data found that reduced time in captivity increased the likelihood of reintroduction success, as long as a sufficient number of individuals were available for release (Robert, 2009). These findings are supported by empirical observations that loss of genetic diversity and antipredator traits takes place in long-term captive populations (>8 generations) (Kraaijeveld-smit et al., 2006). In steelhead trout (*Oncorhynchus mykiss*), a single generation of domestication impacted on the expression of hundreds of genes and resulted in 15% reduction in lifetime reproductive success compared to wild fish (Christie et al., 2012; Christie et al., 2016). Ensuring founder individuals are representative of the original population's diversity, are unrelated, outbred, and ensuring time in captivity is minimized are general recommendations for ex-situ breeding programs (Witzenberger & Hochkirch, 2011).

The southern pygmy perch breeding program followed these guidelines and minimized inbreeding and maintained genetic diversity in the captive F1 (Attard et al., 2016a). Although well guided by genetic management, the successful reintroduction reported here was also heavily reliant on the presence and availability of suitable habitat within a generation of wild extirpation. Accordingly, a focus needs to remain on wild habitat maintenance and restoration in tandem with genetic breeding and rescue techniques. Indeed, despite using similar genetically informed breeding design and reintroduction efforts (Attard et al., 2016a), attempts to reestablish the co-occurring Yarra pygmy perch (*Nannoperca obscura*) in the Lower Lakes resulted in failure (Beheregaray et al., 2021; Wedderburn et al., 2022). That species has much lower pre-European genetic diversity and more specific environmental requirements than southern pygmy perch (Brauer et al., 2013; Attard et al., 2016a; Wedderburn et al., 2022), which implies that there can sometimes be no replacement for wild habitat restoration.

Southern pygmy perch is known to display generally low levels of genetic diversity across its range (Brauer et al., 2016; Cole et al., 2016). This pattern, shared by several regionally co-occurring small-bodied habitat specialist freshwater fish species (Brauer et al., 2013; Sasaki et al., 2016; Lean et al., 2017), is often attributed to population declines following European colonization linked to loss and fragmentation of habitat, reduction of water quality, overharvesting of water for agriculture, and competition and predation by introduced species (Brauer & Beheregaray, 2020). We found remarkably low neutral and adaptive diversity in the upstream adjacent populations compared to the Lower Lakes population (e.g., genome-wide heterozygosity of 7% vs. 17%, respectively). Eco-evolutionary simulations and analyses of genomic data of isolated populations of southern pygmy perch from the upper reaches of the MDB indicate that enhanced drift can rapidly result in loss of genetic diversity and increased differentiation (Brauer & Beheregaray, 2020). This can also potentially explain the lower diversity and high genetic differentiation reported here for the adjacent populations. The higher diversity of the Lower Lakes population, on the other hand, is probably due to occasional asymmetrical downstream migration, a process that can influence genetic diversity in downstream sink populations (Morrissey & de Kerckhove, 2009). Accordingly, the Admixture results are consistent with historical admixture downstream but none in an upstream direction (Supporting Information), a hypothesis statistically inferred in an MDB-wide population genetic study of southern pygmy perch based on a larger sample (578 individuals and 45 localities) (Cole et al., 2016). Despite their low genetic diversity, there was no evidence of inbreeding in the adjacent populations, which is suggestive of mechanisms of inbreeding avoidance or

of other adaptive strategies. Interestingly, included in the repertoire of reproductive strategies reported for southern pygmy perch is diversified bet-hedging (Morrongiello et al., 2012). For this species, this takes place as an adaptive maternal response that maximizes the production and dispersal of eggs in relation to both predictable and unpredictable environmental variances (Morrongiello et al., 2012). Additional integrative field- and genomic-based studies are needed to test for associations among reproductive strategies, genetic diversity, and inbreeding in pygmy perch.

Reintroduction persistence and adaptive potential despite a small founder population

The accelerated and underestimated rate of environmental degradation and biodiversity loss (Bradshaw et al., 2021), limited conservation resources, and insufficient information about indicators of population persistence for most threatened species imply that scientific generalizations are often needed to guide urgent management actions (Flather et al., 2011). One such generalization relates to the minimum population size required for conservation reintroductions to avoid inbreeding depression in the short term and for retaining evolutionary potential (Tracy et al., 2011; Frankham et al., 2014). Substantial evidence indicates that all captured Lower Lakes fish originate from the ~1350 reintroduced fish produced by the ex-situ captive population of 65 broodstock. The captured and original wild-rescued Lower Lakes individuals comprise a homogenous population with unique ancestry that is highly divergent from all adjacent upstream populations and from any other recorded population of southern pygmy perch (Unmack et al., 2011; Attard et al., 2016a; Brauer et al., 2016; Cole et al., 2016). In addition, we found no evidence of migrants or of recent and ongoing gene flow into the Lower Lakes, consistent with microsatellite-based parentage for 71 Lower Lakes fish captured until 2014 that were assigned as either F1 captive-born or as descendants of that captive population (Attard et al., 2016a). It is encouraging that, despite the potential for sexual selection and skewed parentage to decrease genetic diversity in southern pygmy perch (Morrongiello et al., 2010; Attard et al., 2016a), there was no evidence for accumulation of inbreeding or for loss of neutral and candidate adaptive genomic diversity in up to eight generations since reintroduction. Similarly, a gene expression study of wild southern pygmy perch showed that even small and isolated populations can respond to hydroclimatic selection, retain functional variation, and maintain evolutionary potential (Brauer et al., 2017). The knowledge accumulated from evolutionary, population genomic, and conservation studies of southern pygmy perch is consistent with the increasing number of empirical studies and meta-analyses that suggest that fragmented and declining populations (including some of very small size; 100s instead of 1000s individuals) are responding to multifarious natural selection and perhaps retaining adaptive potential (Koskinen et al., 2002; Fraser et al., 2014; Wood et al., 2016).

Although we report on the maintenance of genomic proxies of population persistence, the southern pygmy perch case study exemplifies well the paradigm of many small threatened populations that are highly vulnerable to extirpation due to nongenetic stochastic events (Lande, 1993). This nonmigratory species resides in seasonably variable wetlands and ephemeral waterways, and is a candidate for local and regional extinctions due to agricultural development and drought-associated habitat loss, especially under the more arid and hydrologically variable conditions predicted for southeastern Australia in the future (de Oliveira et al., 2019). A population viability analysis (PVA) for southern pygmy perch that considered a range of threats (e.g., isolation in nonpermanent habitats) found that reintroductions of 500 female adults over a 5-year period would probably be successful (Todd et al., 2017). The PVA also suggested that such populations can withstand some level of disturbance, especially if habitat allows the persistence of oldest age class individuals that showed the highest reproductive value (Todd et al., 2017). Environmental management of waterways and wetlands, including habitat restoration and reestablishment of riverine connectivity, in combination with conservation interventions (e.g., genetic rescue) would greatly assist in maximizing persistence and evolutionary potential of reintroduced populations.

The importance of longitudinal genomic monitoring postreintroduction

The dearth of genetic monitoring postreintroduction (Witzenberger & Hochkirch, 2011; but see Osborne et al., 2012) precludes the collection of valuable ecological and evolutionary information, such as genetic diversity, inbreeding, effective population size, wild recruitment, skewed parentage, and gene flow (Schwartz et al., 2007; Frankham, 2010). This is problematic as demographic parameters alone cannot determine the success of conservation programs. Even a large reintroduced population with effective recruitment may harbor low genetic diversity and high inbreeding (e.g., the Alpine ibex (*Capra ibex*): Grossen et al., 2018), reducing adaptive potential and increasing vulnerability to environmental change (Schwartz et al., 2007). Most genetic monitoring studies are still limited to snapshots in time and space (Schwartz et al., 2007; Habel et al., 2014), impacting on assessments of post reintroduction meta-population dynamics (Mathieu-Bégné et al., 2019) and the success of genetic rescue interventions (Frankham, 2015; White et al., 2018; Fitzpatrick et al., 2020). Where it has been implemented (e.g., Dowling et al., 2014; Osborne et al., 2012), long-term monitoring illustrates beneficial aspects of captive breeding programs, such as how rearing wild-caught larvae (as opposed to captive breeding) can help maintaining genetic diversity. The genomic basis for adaptation also remains understudied and thus is difficult to incorporate into reintroduction monitoring. Advances in ecological genomics will likely improve the understanding of mechanisms underlying adaptive responses to changing environments (Bossdorf et al., 2008; Ouborg et al., 2010; Alvarez et al., 2015) and identifying biogeographic patterns of vulnerability and resilience to environmental change

(Sandoval-Castillo et al., 2020). This knowledge can then be incorporated into reintroduction monitoring to ensure adaptive resilience is maintained in reintroduced populations.

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DATA AVAILABILITY STATEMENT

The neutral and candidate adaptive SNP datasets are available in Figshare: https://doi.org/10.6084/m9.figshare.c.5632612.v1

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SUPPORTING INFORMATION

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