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A genomic framework to assist conservation breeding and translocation success: A case study of a critically endangered turtle

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Abstract

Conservation breeding programs are an effective approach to addressing biodiversity loss. Captive populations are managed to maintain genetic diversity, yet there remains an "implementation gap" in effectively translating molecular genetic data into management. Technological advancements are facilitating rapid generation of genetic data, increasing accessibility for breeding programs. In 2010, Frankham and colleagues proposed a six-stage process for establishing successful conservation breeding and release programs. Here, we describe the conservation breeding program for the critically endangered Bellinger River turtle (*Myuchelys georgesi*) and characterize the value of genetic sampling for informing management actions. By generating a chromosome-level genome and population genetic data, we investigated past and present diversity and assessed relatedness among captive founders. We present a framework modeled on Frankham and colleagues six stages to assist managers in implementing genetic data into actionable conservation strategies. This framework, and worked case study, for managers aims to better guide implementation of genetic approaches into conservation breeding programs.

KEYWORDS
Bellinger River turtle, conservation breeding, *Myuchelys georgesi*, population genetics

1 | INTRODUCTION

1.1 | The six stages of conservation breeding

Conservation breeding programs are a valuable method for managing threatened species (Conway, 2011, Grueber et al., 2019). Such programs can assist biodiversity conservation via scientific research, public education, and as genetic reservoirs for reinforcing dwindling wild populations (Ochoa et al., 2016; Pritchard et al., 2012). High-profile cases of successful conservation breeding and release programs include the black-footed ferret (*Mustela nigripes*) (Wisely et al., 2003), Californian condor (*Gymnogyps californianus*) (Ralls & Ballou, 2004), and the Arabian oryx (*Oryx leucoryx*) (Price, 1989). Despite these examples and other notable successes, including several in Australia (Andrew et al., 2018; Heinsohn et al., 2022; Scheele et al., 2021), conservation breeding programs remain an intensive and expensive management approach (Conde et al., 2011).

Frankham et al. (2010) described a six-stage process of establishing successful conservation breeding and release programs; (1) recognizing decline of the wild population and its genetic consequences; (2) founding one or more captive populations; (3) expanding captive populations to a secure size; (4) managing the captive population over generations; (5) choosing individuals for reintroduction; and (6) managing the translocated population in the wild. Priorities throughout this process include developing husbandry techniques, rapid reproduction, disease mitigation, and genetic management (Frankham et al., 2010). Traditionally, genetic management of captive populations has been based on pedigrees from studbook records and the underlying assumption that founders are neither related nor inbred, which is often not the case (Hogg et al., 2019; Lacy, 1987). This assumption means initial breeding events may result in inadvertent inbreeding and diversity loss for populations already experiencing limited genetic variation (Barrett et al., 2022; Frankham et al., 2017). Advancements in genetic sequencing technologies and bioinformatic tools are making it feasible to integrate molecular data into conservation breeding programs to determine levels of relatedness and genetic diversity metrics.

High-throughput sequencing has resulted in the generation of large amounts of data and the emergence of reference genomes for conservation management. Reference genomes provide data for a range of investigations including designing species-specific microsatellite markers for population analyses, developing targeted single nucleotide polymorphism (SNP) panels, aligning and calling reduced representation sequencing (RRS) data within the same or closely related species, exploratory genome-wide analyses and high-resolution functional gene investigations such as complex immune gene families (Brandies et al., 2019; Galla et al., 2018; Peel et al., 2022). The data output by high-throughput sequencing often requires interpretation from experts in the field of genomics resulting in a "research implementation gap" (Taylor et al., 2017). Translational research is an interdisciplinary approach to conservation that seeks to bridge the gap between scientific knowledge and practical applications (Enquist et al., 2017). To maximize interdisciplinary contributions to conservation breeding programs, there is growing responsibility for scientists to engage with managers directly to implement research findings into management. Here, we present a case study that epitomizes Frankham and colleagues six-stage process of establishing a conservation breeding and reintroduction program and show how the integration of a multidisciplinary approach has benefited a critically endangered turtle species.

1.2 | Our case study species

Turtles are among the most threatened vertebrate taxa globally (Van Dyke et al., 2018). Over 20% of turtle species are listed as Critically Endangered by the International Union for the Conservation of Nature (IUCN; McCallum, 2021), with declines greatly reducing turtles' contributions to ecological processes and food webs (Chessman et al., 2020). Pleurodira, a 200-million-year-old suborder of freshwater turtles found only in Australia, New Guinea, Africa, and South America remains highly under-represented in the peer-reviewed literature. Within this suborder, the Bellinger River turtle (*Myuchelys georgesi*) is a critically endangered species in the family Chelidae. The species is a medium-sized omnivorous turtle with a current known distribution that is restricted to a 60 km range of the Bellinger catchment in north-eastern New South Wales (NSW), Australia (Figure 1A) (Cann et al., 2015; Zhang et al., 2018). The Bellinger catchment and several small freshwater catchments in NSW remain isolated and relatively untouched, which has facilitated unique habitat specialization and

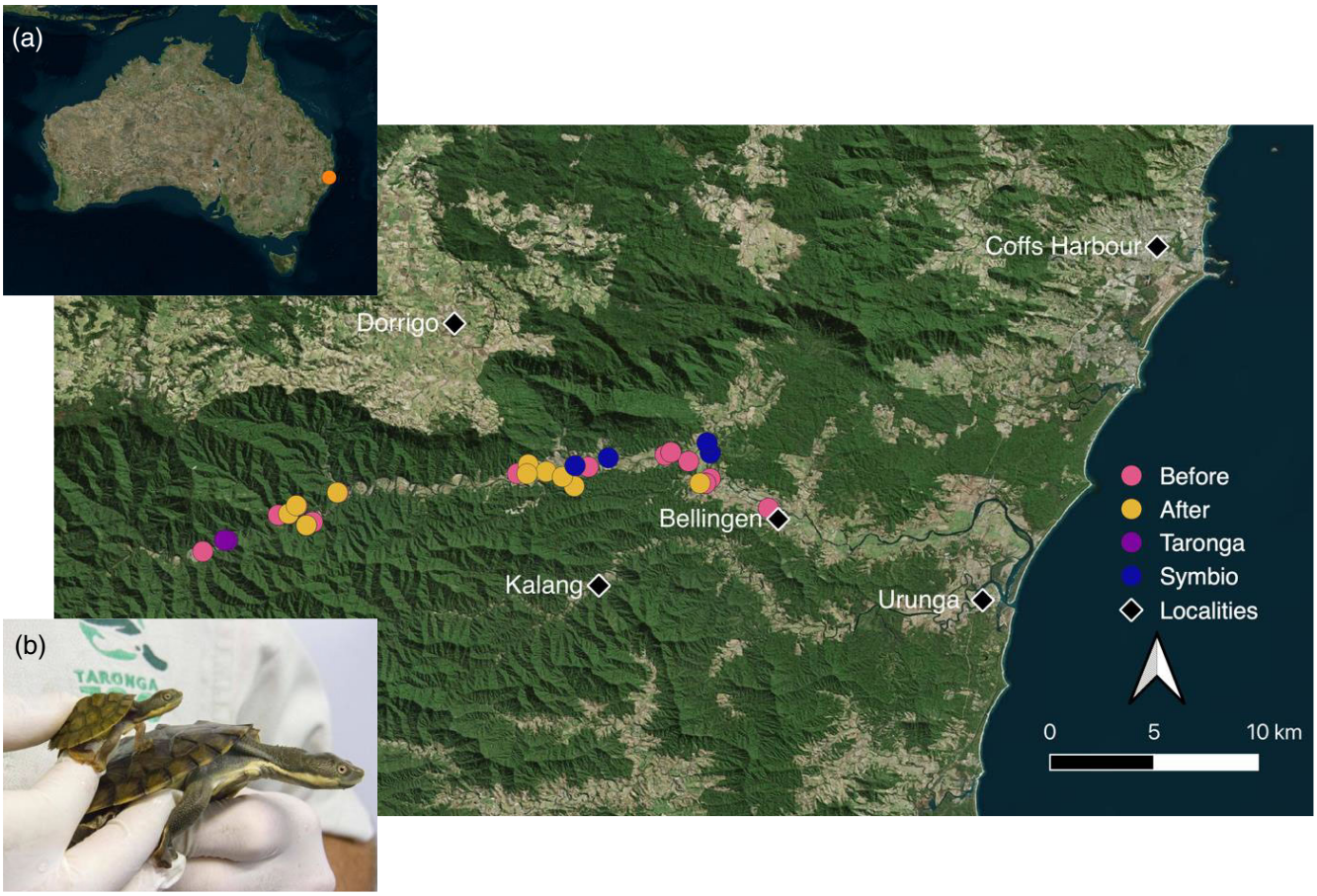

FIGURE 1 (A) An inset of Australia with a map of the Bellinger River basin showing the locations of historic (Before: 2007, pink) and contemporary (After: 2015–2020, yellow) samples, including the founder collection locations for the two conservation breeding programs (Taronga: 2015, purple; and Symbio: 2017, blue) (NSW Department of Climate Change, Energy, the Environment and Water (DCCEEW) (unpublished data) sample points. Note that the sample locations have been obscured as *M. georgesi* is listed as a Category 2 species in the DCCEEW sensitive species data policy. (B) A captive *M. georgesi* hatchling and adult. Photo: Amy Russell.

catchment-specific speciation (Spencer et al., 2014). *M. georgesi* has adapted to up-stream regions of the Bellinger River, preferring deeper waterholes surrounded by bedrock (Spencer et al., 2014). Although a relatively untouched catchment, the only known population is threatened by riparian habitat loss as a result of private landownership, predation by introduced and native predators, reduced water quality, hybridisation due to human mediated dispersal of the Murray River turtle (*Emydura macquarii*) and a novel disease outbreak (Chessman et al., 2020; Georges et al., 2018; Spencer et al., 2014; Zhang et al., 2018).

In 2015, a species-specific nidovirus resulted in the death of more than 90% of individuals, with mortalities occurring mostly among adults (Figure 1B) (Chessman et al., 2020, Zhang et al., 2018). During this time, it is estimated that the population declined from approximately 3000 to less than 150 individuals (Chessman et al., 2020; Spencer et al., 2018). To date, there has been limited evidence of individuals recovering from the disease and no records of breeding in the river since the outbreak. Additionally, knowledge on how the outbreak affected the species' distribution throughout the river remains limited due to accessibility constraints.

Species recovery is currently managed by NSW Department of Climate Change, Energy, the Environment and Water (DCCEEW) and includes a conservation breeding program that commenced in 2015, with a captive colony founded at Western Sydney University that was later relocated to Taronga Conservation Society Australia, Mosman, Australia ($N = 16$ individuals). The Taronga population was founded from emergency intakes, with seven females and nine males collected from two sites in the upper reaches of the Bellinger River where the virus had not yet reached (Figure 1A). This was followed by a second intake in 2017 to Symbio Wildlife Park, Helensburg, Australia ($N = 19$) (Figure 2A). The Symbio population was founded post-virus from six females and 13 males collected from four sites in the lower reaches of the river (Figure 1A).

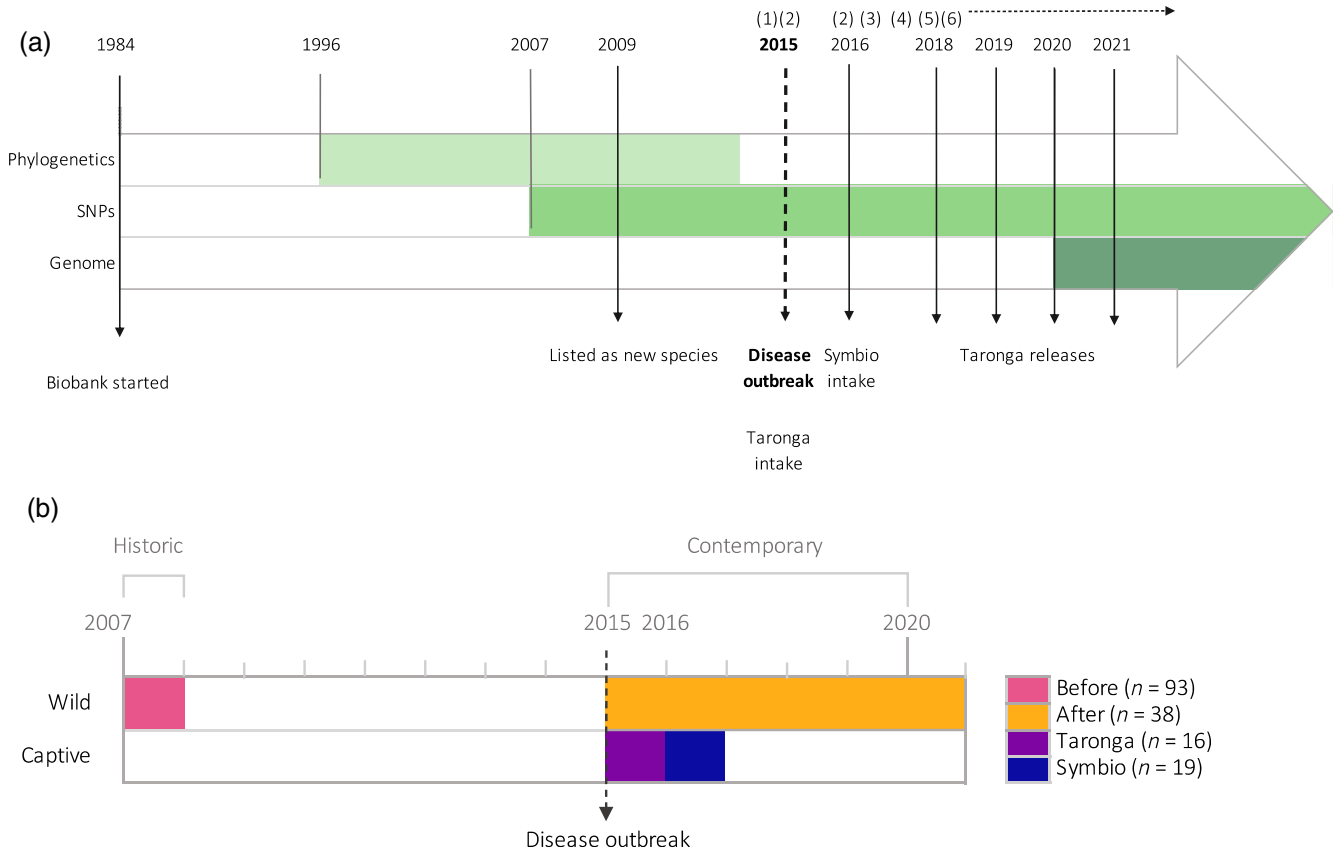

FIGURE 2 (A) Timeline of the collection and implementation of genetic data into *M. georgesi* management including disease outbreaks, founder intakes and captive release events. Numbers in brackets above the timeline indicate completion of Frankham et al. (2010) six-stage process for conservation breeding and reintroduction programs; (1) Recognizing decline of the wild population and its genetic consequences; (2) Founding one or more captive populations; (3) Expanding captive populations to a secure size; (4) Managing the captive population over generations; (5) Choosing individuals for reintroduction; and (6) Managing the reintroduced population in the wild. (B) Historic and contemporary samples used for comparative analyses of wild and captive individuals.

The species recovery program mirrors the principles of Frankham et al.'s six-stage process (Figure 2A) and has developed successful husbandry, breeding, and disease mitigation protocols (Taronga Conservation Society Australia, 2023). While Frankham's stages were written in 2010 for reintroductions, the underlying principles can be applied to various conservation translocation types, including conservation introductions and reinforcements (IUCN/SSC, 2013). Genetic sampling of the species dates back to 1986 (Figure 2A) (Georges & Adams, 1992) but the conservation breeding and release program initially had limited genetic data. This lack of data for genetic management of the species could potentially have long-term implications on the retention of genetic diversity and population viability for the species.

Here, we generate a comprehensive genetic toolkit for the Bellinger River turtle, translating our genetic findings into management recommendations for the conservation breeding and release program (Table 1). To achieve this, we assembled the first chromosome-level genome for the suborder Pleurodira and aligned population genetic data to (1) investigate historic and contemporary diversity and differentiation, (2) identify levels of founder relatedness within and between the Taronga and Symbio populations, and (3) develop an easy-to-follow framework for managers to translate research into management actions.

2 | METHODS

2.1 | Reference genome

Comprehensive details of genome assembly and annotation are provided in the Data S1. In summary, we conducted high molecular weight DNA extractions from the heart tissue of a male *M. georgesi* using the Nanobind Tissue Big DNA kit following the manufacturer's protocol (Circulomics, Pacific Biosciences, California, United States of America). PacBio HiFi Single-Molecule Real-Time (SMRT) bell libraries were sequenced across

TABLE 1 A framework for integrating genetic data into conservation breeding management, mirroring Frankham et al. (2010) six-stage process of establishing successful conservation breeding and reintroduction (or other conservation translocation) program.

Stage	Genetic input	Management outcome	*M. Georgesi* program
(1) Recognizing decline of the wild population and its genetic consequences	• Biobanking • Reference genome generation • DNA sampling of declining population (blood/tissue)	• Setting genetic foundations and acquiring samples for subsequent steps • Baseline analyses investigating wild population genetic diversity, differentiation and temporal changes wrought by the decline	• Tissue samples were collected in 1986 (A. Georges; Figure 2B) and biobanked at The University of Canberra • **A chromosome-level reference genome has been assembled and annotated for downstream analyses** • Commencement of annual surveying and sampling of wild population by NSW DCCEEW • **Baseline analyses of genetic metrics (H_S, H_E, H_O, F_{IS}, A_R, P_A,N_E, F_{ST})**[a]
(2) Founding one or more captive populations	• 20–30 contributing founders • DNA sampling of all founders • DNA sampling of contemporary wild individuals	• **Identifying founder relationships for baseline studbook data** • **Ensuring no hybrids or introgressed individuals in captivity** • **Ensuring captive populations are representative of wild diversity**	• Two captive populations established from 35 individuals (Taronga Conservation Society and Symbio Wildlife Park) • Tissues collected from wild and founding individuals (NSW DCCEEW; Figure 2B) • The founders gathered by NSW DCCEEW and various institutions were sourced from opposite ends of the species distribution and are housed separately • Genetic analyses identified the presence of hybrids which were then removed
(3) Expanding captive populations to a secure size	• Establishing a studbook with known founder relatedness • DNA sampling of each generation • DNA sampling of new founder intakes	• Provides data for stage 4	• Tissue collected from F1 for parentage analyses (Georges, 2020) • Development of studbook • Breeding program successfully increased numbers from 35 to ca. 299 individuals (including releases)
(4) Managing the captive population over generations	• Maintaining a studbook with known founder relatedness • Breeding between captive populations • Introducing genetic material via new founders	• **Maintaining/increasing genetic diversity** • **Minimizing MK**[a] • Monitor for genetic drift • Increasing genetic diversity with new individuals • Ensuring individuals are representative of wild diversity	• Continuation of progeny sampling • **Analyses to monitor genetic metrics (H_S, H_E, H_O, F_{IS}, A_R, P_A, N_E, F_{ST})**[a] • **Implementation of founder relatedness results by breeding individuals with low MK** and integrating genetic data into studbook
(5) Choosing individuals for reintroduction/ release	• Results from analyses in steps 2–4, that is, reintroduction/ translocation cohorts with wide ranging diversity	• Reintroduce/translocate genetically diverse individuals • Insight on where to reintroduce/ translocate based on wild diversity and population structure	• Retrospective integration of genetic diversity results from this study to inform reintroduction/translocation decisions

(Continues)

TABLE 1 (Continued)

Stage	Genetic input	Management outcome	*M. Georgesi* program
(6) Managing the reintroduced (or translocated) population in the wild	• Routine DNA sampling of wild population • Knowledge of captive and wild genetics from earlier stages	• Maintain wild diversity post-release • Ensure wild is not 'swamped' by different genotypes from a single captive population • Ensure captive-released animals are breeding with wild animals and contributing to the next generation	• Annual sampling of wild population by NSW DCCEEW • **Genetic diversity of wild and captive populations undertaken in this study**

Note: Bold text highlights the genetic contributions of our study. The steps in the *Myuchelys georgesi* case study were not undertaken chronologically at each stage, as we have retrospectively integrated genetic inputs into the program. We suggest that other conservation breeding programs make efforts to follow the order we recommend.

[a]NSW DCCEEW New South Wales Department of Climate Change, Energy, the Environment and Water; MK Mean kinship.

Abbreviations: H_S, Standardized heterozygosity; H_E, Expected heterozygosity; H_O, Observed heterozygosity; F_{IS}, Inbreeding coefficient; A_R, Allelic richness; P_A, Private alleles; N_E, Effective population size; F_{ST}, differentiation.

two SMRT cells on the PacBio Sequel II at the Australian Genome Research Facility (Brisbane, Australia). We assembled the HiFi genome using Hifiasm v.0.16.0 (Cheng et al., 2021), and scaffolded it using Hi-C data produced on a Illumina Novaseq 6000 and the YaHS v.1.1 scaffolding pipeline (Zhou et al., 2022). We extracted RNA from brain, liver, and spleen tissue of a female *M. georgesi* using the Qiagen Rneasy Mini Kit (Qiagen, Hidlen, Germany) following the manufacturer's protocol. Transcriptomes were sequenced at the Ramaciotti Centre for Genomics (The University of New South Wales, Sydney, Australia) on an Illumina NovaSeq 6000 S1 flow cell as 100 bp paired-end reads. We annotated the genome with FGENESH++ using a global transcriptome assembly that was generated from the brain, liver, and spleen transcriptomes (Table S1).

2.2 | Genetic analyses for management

Two-week river-wide, randomized, stratified surveys were conducted in April of 2007 and October and November of 2015, 2016, 2019, and 2020 by the NSW DCCEEW. October and November coincided with the beginning of the breeding season, at which time gravidity of adult females could be confirmed. Turtles were identified using scute notching, and their carapace width and length were measured. They were also weighed, bled or had skin biopsied, body condition checked, and swabbed for viral screening (Chessman et al., 2020). DNA samples were collected by extracting blood from the jugular vein or by removing part of the trailing webbing of the clawless toe on the hindfoot (Georges et al., 2018).

Blood and skin biopsies from 166 individuals were collected across 33 sites during surveys (2007, 2015–2020) for the purposes of population genetic analyses (Figures 1A and 2A, Table S5). The population genetics samples were stored in 75% ethanol at −20°C in the University of Canberra Wildlife Tissue Collection (GenBank UC < Aus>). As described in Georges et al. (2018), samples were sequenced over multiple runs using high coverage DArTseq™ (Diversity Arrays Technology PL, Canberra, Australia), a form of RRS. We aligned raw DArT sequences to the repeat masked genome generated in this study (Figure S2, Table S2) and called SNPs using Stacks v2.61 (Catchen et al., 2013; Rochette et al., 2019). The 'populations' module was then run with the following parameters: minimum samples per population 30% (−r 0.3); minimum minor allele frequency (MAF) of 0.01 (--min_maf 0.01); and --write_random_snp.

To partition our data, we investigated genetic clusters using a variational Bayesian framework in fast*STRUCTURE* v1.0 (Raj et al., 2014) and visualized the results using DISTRUCT v1.1 (Rosenberg, 2004). $K = 1$–4 clusters were tested based on the demographic characteristics of the species including population size and range (Figure S3). We used the fast*STRUCTURE* "chooseK.py" script to decide the optimal K. As no genetic clusters were detected (optimal value of K was one), we partitioned the SNP dataset output by the 'populations' module according to collection time relative to the disease outbreak and current location (Figure 2B). These predetermined groups were: (1) wild individuals sampled before the disease outbreak in 2007, $N = 92$ (hereafter "Before"); (2) wild individuals sampled after the disease outbreak between 2015 and 2020, $N = 38$ (hereafter "After"); (3) Taronga founders sampled in 2015, $N = 16$; and (4) Symbio founders sampled in 2017, $N = 19$. Variant filtering was carried out on three datasets: (i) all groups consisting of the four predetermined groups

($N = 166$), (ii) wild groups only (Before and After; $N = 131$), and (iii) captive populations only (Taronga and Symbio; $N = 35$). The SNP datasets were filtered on minimum average read depth (>2.5×), coverage difference between the reference and alternate alleles (<80%), call rate (>75%), retention of loci with heterozygosity <80%, and reproducibility (>90%) calculated using 51 technical replicates performed by DArT. Where samples were grouped across different sequencing plates, we found no evidence to suggest that batch effects influenced our results (Figure S1).

To investigate variation in the 'wild' and 'all groups' datasets, we undertook exploratory principal coordinates analyses (PCoA) using Euclidean distance via the R v4.3.0 package dartR v1.9 (Gruber et al., 2018; R Core Team, 2023) and visually inferred putative genetic differentiation between groups using eigenvalues in adegenet v2.1.3 (Jombart, 2008). We also applied a PCA to the dataset using the glPca() function in adegenet. We calculated pairwise fixation indices (F_{ST}) between all groups using hierfstat v0.5–11 (Goudet 2005). To identify relationships between geographic distance and genetic distance we used the Before dataset to perform an isolation-by-distance (IBD) mantel test in dartR using the gl.ibd() function with 999 permutations.

We calculated standardized heterozygosity (H_s) using the genhet function in R (Coulon, 2010) for all groups (where 1 is the average and so a value greater than 1 is more diverse than average); observed (H_o) and expected heterozygosity (H_E) using GenAlEx v6.5 (Peakall & Smouse, 2006) and visualized individual H_o distributions by group using the boxplot() functions in R. We calculated autosomal H_o and H_E (Schmidt et al., 2021) by re-running the Stacks 'populations' module with the parameter: minimum samples per population 75% (−r 0.75), and without the parameters: minimum minor allele frequency (MAF) of 0.01 (--min_maf 0.01); and --write_random_snp to retain both variant and invariant loci (Schmidt et al., 2021). We filtered the Stacks output on minimum average read depth (>2.5×), coverage difference between the reference and alternate alleles (<80%), call rate (>75%), and reproducibility (>90%) calculated using 51 technical replicates performed by DArT. Loci on sex chromosomes (scaffold 4) were removed from the dataset (Martinez et al., 2008). The resulting 11,208 loci (at both variant and invariant sites) were used to calculate autosomal H_o and H_E using GenAlEx v6.5 (Peakall & Smouse, 2006).

We performed a t-test to test whether H_s was significantly different between wild groups before and after the disease and used the Bartlett's test of homogeneity of variances base function to test whether individual observed heterozygosity was significantly different from expected heterozygosity in R. We calculated inbreeding coefficients (F_{IS}) and the associated 95% CI using the diveRsity v1.9.9 package (Keenan et al., 2013) and PopGenReport v3.0.7 (Adamack & Gruber, 2014) to calculate allelic richness (A_R) in R. We calculated the number of private alleles in each group compared to all other groups and pairwise private alleles between groups using the gl.report.pa() function in the dartR package. To calculate molecular relatedness, we ran simulations in COANCESTRY v1.0.1.10 (Wang, 2011) to determine the most appropriate moments estimator as per Hogg et al. (2019). We selected TrioML for final analyses. We set COANCESTRY parameters to account for inbreeding, with the number of reference individuals and bootstrapping samples set to 100 for all groups and between captive groups. We calculated mean kinship (MK) by dividing the TrioML value by two, representing the average relationship of each animal to all others within the sample set. We estimated MK for individuals within each group (MK_{WITHIN}) and between captive groups ($MK_{BETWEEN}$) using the captive dataset. We calculated effective population size (N_E) for wild groups using NeEstimator v2.1 (Do et al., 2014) with values reported for the no singleton alleles analysis and the associated jack-knifed 95% confidence intervals (Jones et al., 2016). We excluded captive groups from N_E analyses due to small sample sizes resulting in infinite confidence intervals.

3 | RESULTS

3.1 | Reference genome

The final genome assembly was 2.0 Gb in size, consisted of 129 scaffolds, had a contig N50 of 56.12 Mb, and scaffold N50 of 123.4 Mb (Figure S2A, Table S2). BUSCOv5.2.2 identified 95.4% complete vertebrata genes, 94.9% of which were single copy and 0.5% were duplicated, 2.1% were fragmented, and 2.5% were missing (Figure S2A, Table S2). The genome statistics and distinct chromosome-length scaffolds (Figure S2B) confirm chromosome level-completeness. We used this genome to align and call variants using the population genetics data. Comprehensive details of genome assembly and annotation are provided in the Supplementary Material (Figure S2).

3.2 | Genetic analyses for management

We aligned the high density DArTseq data to the reference genome. Our initial analyses showed evidence of an *E. macquarii* ($N = 1$), *M. georgesi* and *E. macquarii* hybrid

($N = 1$), and introgressed ($N = 2$) individuals in the wild groups (Figure S4) (Georges et al., 2018). In accordance with earlier findings, we did not detect hybrids in either captive population having been identified and removed when the populations were founded (Georges et al., 2018). The *E. macquarii*, *M. georgesi* and *E. macquarii* hybrid, and introgressed individuals were removed from the dataset and SNPs were re-called. The re-called dataset yielded a Stacks output of 2172 SNPs. Refiltering on SNP calls for each group in R resulted in reduced representation datasets of 460 genome-wide SNPs in the 'all groups' dataset, 473 genome-wide SNPs in the 'wild' dataset, and 227 genome-wide SNPs in the 'captive' dataset. Our exploratory PCoA using the wild only dataset revealed minor levels of genetic structuring between Before and After individuals, with up to 3.5% of variance explained by PCo I and 2.5% by PCo II (Figure 3A). The addition of captive individuals in the second PCoA, revealed clustering of captive groups with Before individuals (Figure 3B). Up to 5.3% of the variation was explained by PCoI, and PCo II (3.7% variation) with both axes primarily separating out After individuals (Figure 3B). An almost identical clustering result was seen when a PCA was applied to the dataset (data not shown).

We found no notable differences in H_s between captive and wild groups (Table 2A) and no significant differences in the wild across time ($t = -0.902$, $df = 128$, $p = 0.369$). SNP and autosomal H_O and H_E was similar across all groups (Table 2A, Figure S5) with significantly higher observed than expected heterozygosity across all groups, indicating an excess of heterozygotes. There were no statistically significant levels of inbreeding (F_{IS}) observed as confidence intervals encompassed zero for all groups (Table 2A). A_R was also similar with no distinct differences across groups (Table 2A). P_A were only present in the Before group when comparing across all groups (Table 2A). Pairwise P_A numbers were consistently higher for Before and lowest for Taronga and Symbio, respectively (Table S3). MK_{WITHIN} ranged from 0.019 ± 0.048 to 0.048 ± 0.150, with the highest value observed in the After population (Table 2A). $MK_{BETWEEN}$ for Taronga and Symbio using the captive dataset was 0.012 ± 0.040, where 0.1250 is the equivalent of a half-sibling relationship. N_E estimates for Before and After were 148.9 (120.4–191.8) and 11.9 (8.2–17.1) respectively (Table 2A). F_{ST} values between all groups ranged from 0.005 (0.001–0.009) to 0.027 (0.020–0.034), with statistically significant F_{ST} observed between the Before and After groups (Table 2B). Our IBD mantel test found no correlation between geographic distance and genetic distance in the species ($r = 0.364$, $p = 0.258$).

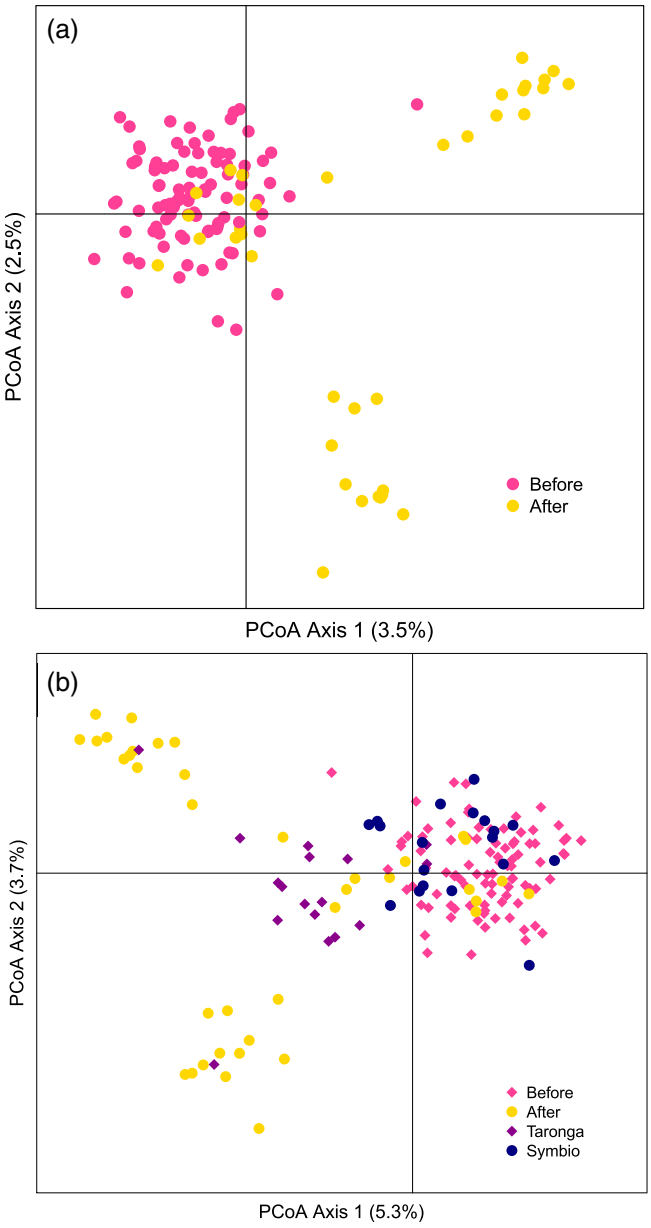

FIGURE 3 (A) Principal coordinates analysis (PCoA) of wild individuals before and after the disease outbreak ($N = 131$) using 474 genome-wide SNP markers. (B) PCoA of genome-wide diversity of all individuals ($N = 166$) using 460 SNP markers.

4 | DISCUSSION

4.1 | A worked example of how conservation managers can apply the framework

Please refer to Table 1 for a summary of the framework for integrating genetic data into conservation breeding management, particularly in relation to the genetic input, management outcomes and how this applies to our case study.

TABLE 2 (A) Population genetic indices of our sample groups, including standardized (H_S), observed (H_O), and expected (H_E) SNP and autosomal heterozygosity, inbreeding coefficient (F_{IS}), allelic richness (A_R), private alleles (P_A), mean kinship within populations (MK_{WITHIN}), and effective population size (N_E).

Group	*n*	H_S (±SD) SNP	H_O (±SE) SNP	H_E (±SE) SNP	H_O (±SE) Autosomal	H_E (±SE) Autosomal	F_{IS} (95% CI)	A_R	P_A	MK_{WITHIN} (±SD)	N_E (95% CI)
Before	92	1.007 (0.097)	0.290 (0.009)*	0.280 (0.008)	0.0180 (0.0007)*	0.0148 (0.0005)	−0.035 (−0.051 to 0.020)	1.727	11	0.019 (0.047)	148.9 (120.4–191.8)
After	38	0.990 (0.097)	0.286 (0.010)*	0.262 (0.008)	0.0170 (0.0006)*	0.0138 (0.0005)	−0.094 (−0.125 to 0.064)	1.688	0	0.048 (0.150)	11.9 (8.2–17.1)
Symbio	19	0.969 (0.102)	0.279 (0.011)*	0.252 (0.009)	0.0174 (0.0007)*	0.0136 (0.0005)	−0.181 (−0.149 to 0.075)	1.669	0	0.023 (0.072)	—
Taronga	17	1.009 (0.098)	0.293 (0.012)*	0.248 (0.009)	0.0165 (0.0007)*	0.0130 (0.0005)	−0.108 (−0.253 to 0.121)	1.658	0	0.027 (0.076)	—

	Before	After	Symbio
After	0.026 (0.020–0.031)		
Symbio	0.005 (0.001–0.009)	0.027 (0.020–0.034)	
Taronga	0.010 (0.004–0.016)	0.002 (−0.002–0.006)	0.019 (0.011–0.027)

Note: Significantly higher HO to HE denoted by "*". (B) Population differentiation (FST) including 95% CI between all groups.
Abbreviations: CI, 95% lower and upper confidence intervals; SD, standard deviation.

4.1.1 | Stage 1—Recognizing decline of the wild population and its genetic consequences

"*When recognising declines in wild populations, the collection and preservation of DNA samples, such as blood and tissue, in a biobank can provide essential genetic data for future research*" (Frankham et al., 2010). Through collection and preservation of DNA, we can also identify genetic consequences resulting from threatening processes through analysis of temporal data, as well as sample provision for development of a reference genome for neutral and adaptive genomic investigations (Sunde et al., 2022).

The reference genome for *M. georgesi* was created using tissue collected and stored in a biobank from 1986. The sampling by managers in 2007 and 2015–2022 (Figure 2B) has provided population genetic data for researchers to investigate the genetic consequences of the nidovirus outbreak and other threatening processes, including evidence of shifts in wild genetic diversity since the disease event (Table 2A, Figure 3A). We developed and utilized our reference genome during establishment stages 2 and 6 for Symbio and Taronga, respectively. If financial resources and collaborative opportunities are available, we recommend that a reference genome be developed during stage 1 for conservation breeding programs so genetic output can be utilized as early as possible and to improve the reliability of RRS data variant calls to improve downstream inferences (Shafer et al., 2017; Torkamaneh et al., 2016; Wright et al., 2019). We also recommend samples from the declining wild population be collected to provide baseline genetic data and to readily assess genetic consequences of population declines. This may encompass decreases in diversity, the risk of bottlenecks, the potential for inbreeding depression, and the occurrence of genetic drift. Addressing these issues early is crucial to prevent the need for more extensive interventions due to a potential delayed response in observable genetic changes.

We examined population genetic data from samples that were previously sequenced for earlier analyses (Georges et al., 2018) (Figure 2B). Using our reference genome and set of reliable genome-wide SNP markers, we found no significant differences in H_s between all groups ($p = 0.369$) indicating consistent levels of heterozygosity in the population since the nidovirus outbreak. SNP and autosomal observed heterozygosity were significantly higher than expected heterozygosity in all groups (Table 2A), suggesting that the population bottleneck caused by the mass mortality event has either not resulted in a corresponding genetic bottleneck or it is a signature of a bottleneck-induced heterozygosity excess, likely to be detectable for a few generations until a new equilibrium between mutation and drift is reached (Cornuet & Luikart, 1996). This is also suggested by the large drop in N_E. The relatively low genome-wide diversity compared to other species also suggests historical bottlenecks have already occurred in this species (Georges, 2020). It has also been suggested that associative overdominance (neutral loci becoming effectively

over-dominant as a result of disequilibrium with a locus under selection) may conserve genetic variation in small populations compared to expectations from neutral theory (Frydenberg, 1963; Gilligan et al., 2005; Rumball et al., 1994; Schou et al., 2017), potentially contributing to the significantly higher levels of observed to expected heterozygosity in all groups however, long-term monitoring is needed to confirm this. Notably higher SNP heterozygosity compared to autosomal heterozygosity estimates are due to the inclusion of invariant sites and the species small population size (Schmidt et al., 2021), with autosomal heterozygosity estimates likely providing a more accurate representation of low genome-wide diversity in the species (Schmidt et al., 2021). A_R was consistent across groups, with slightly lower values observed for the After, Symbio, and Taronga groups compared to Before (Table 2A). The lower number of unique P_A in the After group compared to Before highlights potential losses in alleles overtime, although discrepancies in sample size may not have captured the range of alleles currently present in the wild (Table S3). Although alleles present in the After group do not appear to be captured in captivity, each captive population contains multiple alleles not observed in the current wild population (Table S3). As there has been minimal evidence of wild clutches hatching since the outbreak, the After group may still reflect the diversity of the larger pre-disease population suggesting that genetic consequences of the outbreak may not be evident until post-disease F1 individuals can be analyzed. We have advised species managers that continual monitoring once wild individuals start reproducing will be crucial in identifying long-term trends.

In this case study, we demonstrate the value of biobanking during the early stages of a wild population decline, and we advocate for continual monitoring in threatened species including DNA samples collection and biobanking to capture temporal trends in genetic diversity.

4.1.2 | Stage 2—Founding one or more captive populations

"*When founding one or more captive populations, a fully representative sample that encompasses wild diversity is needed to maximise captive population viability*" (Frankham et al., 2010). Frankham and colleagues proposed that 20–30 contributing founders are sufficient to create a genetically diverse population that is representative of wild diversity, although molecular analyses are needed to confirm this hypothesis. DNA samples from, (1) all founders are needed and, if feasible, (2) contemporary samples collected across the wild population should be sequenced. The data generated from these samples can be used in stages 3 and 4 for, (1) diversity and founder kinship analyses that can inform management decisions to maximize diversity in captivity, prevent inbreeding and to detect hybrids, or introgressed, individuals among founders, and (2) contemporary wild samples that provide information to ensure that the genetic diversity of the wild population is reflected in captive individuals.

In line with stage 2 management outcomes, we observed minor but non-significant shifts in genetic structure since the outbreak, where Taronga and Symbio are shown to be most representative of historical diversity (Before) (Figure 3B). The clustering of captive groups with Before in the PCoA suggests that Taronga and Symbio represent the genetic profile of the once larger population which may be useful in reinforcing the current wild population. The small number of Taronga individuals that clustered with after suggests greater representation of current wild variation within Taronga that is consistent with the low F_{ST} values between the two groups (Table 2B). However, as only a small amount of variation is explained by the PCoA axes (<4%), long-term analyses of the offspring of the outbreak survivors will be useful in confirming these trends. MK_{WITHIN} in the wild is higher after the disease outbreak with Taronga and Symbio falling between historical and contemporary levels. Populations that sustain high MK_{WITHIN} over generations are expected to experience more rapid changes in allele frequencies and lower adaptation potential in future (Frankham, 1996). In this instance, the conservation breeding program will play a crucial role in strengthening the wild population post-bottleneck by providing opportunities to mate with unrelated individuals, mitigating future inbreeding.

In our case study, molecular analyses to assess genetic representation of the captive populations was conducted post-founding due to a lack of genetic resources at the time. Ideally, stage 2 should be implemented at founding to quickly address genetic concerns prior to breeding. In situations where genetic analysis cannot occur at the time of founding, we recommend collection of DNA from all founders for future analysis.

4.1.3 | Stage 3—Expanding captive populations to a secure size

"*During the expansion phase of conservation breeding, priority is on rapid population growth rather than intense genetic management*" (Frankham et al., 2010). During Stage 3, maintaining an accurate studbook is crucial but may not be effective enough to mitigate long-term

founder effects and prevent inbreeding depression when assuming founders are unrelated (Ivy et al., 2009; Ivy & Lacy, 2010). During this stage, DNA samples from each generation and new intakes are essential for molecular identification of relatives to prevent long-term founder effects and inbreeding in subsequent stages (Hogg et al., 2019). This is important in cases where paternity determination can be difficult, including group-housed enclosures, in species where females retain sperm (Gist & Jones, 1987; Sever & Hamlett, 2002), and when mixed parental clutches are present. This information allows for more informed genetic selection of mates and alleviates the impact of founder effects in subsequent generations. Additionally, this information allows managers to monitor novel genetics introduced into the population through new intakes and provides data for long-term monitoring of genetic drift.

Through expert collaboration and development of effective breeding protocols (Taronga Conservation Society Australia, 2023), the number of *M. georgesi* individuals in captivity has rapidly grown from 35 to ca. Two hundred and ninety nine individuals in 80 years, increasing the global population size from <150 to approximately 450 individuals. Throughout Stage 3, a studbook has been maintained to inform breeding, established before the availability of genetic data. Before our study, the DNA sequences of Taronga's founders and offspring were used for internal parentage analysis to inform the studbook (Georges, 2020). As the turtles are typically housed in groups where females have been observed to retain sperm, determining paternity through traditional means may sometimes be challenging with the absence of molecular information. Due to low numbers in the wild, there have been no new founder intakes to the captive program. In accordance with the species' conservation action plan (Jakob-Hoff et al., 2017), we anticipate DNA from new wild individuals adopted into the breeding program will be analyzed and integrated into the studbook (Table S4, Table S5).

Our case study and previous work demonstrates that rapid population growth can go hand in hand with the integration of traditional pedigree and molecular genetic data.

4.1.4 | Stage 4—Managing captive populations over generations

"*Loss of genetic diversity and inbreeding is exacerbated over generations in small captive populations*" (Frankham, 1995). *When managing captive populations over generations, focus shifts from rapid reproduction in Stage 3 to mitigating genetic issues in stage 4*" (Frankham et al., 2010). Samples collected in Stage 3 can be used to explore a standardized set of genetically measured Essential Biodiversity Variables in stage 4 (H_S, H_E, H_O, F_{IS}, A_R, P_A, N_E and F_{ST}) to provide comparable data for monitoring and management (Hoban et al., 2022). It may be preferable to undertake these analyses during Stage 3 to identify genetic changes early on and ascertain if genetic interventions are required, such as the introduction of new individuals to increase genetic diversity, or breeding between populations to reduce inbreeding (Hoffmann et al., 2021, Kinghorn and Kinghorn, 2021). In cases where founder relationships are unclear during initial breeding, it is essential to retrospectively incorporate these data to help inform future breeding decisions (Hogg et al., 2019), and to apply genetic principles such as not breeding individuals captured close together, until genetic data can be incorporated.

After population expansion and establishment of a studbook in Stage 3, *M. georgesi* managers sought genetic expertise for continued genetic monitoring of captive individuals. This was an opportune time as individuals only had one generation of captive ancestry, allowing our data to be proactively implemented to mediate future genetic issues. Given that the lifespan of *M. georgesi* likely surpasses 30 years and individuals typically do not reach sexual maturity until approximately 6–12 years of age, this may prove more challenging in species with shorter lifespans and short generation times. With access to genetic data, our analyses revealed consistent genetic diversity metrics in both Taronga and Symbio, with Taronga showing slightly higher diversity metrics throughout all analyses (Table 2A,B).

MK values are useful for determining how related individuals are within, and between, populations and for indicating, which animals should be considered for breeding/translocation to minimize relatedness and maximize genetic diversity (Frankham et al., 2017). When choosing breeding pairs, individuals with high MK estimates should not be paired, and breeding of their progeny should be carefully considered. To assist the stage 4 outcome of managing captive populations over generations using our MK analyses (Table S4, Table S5), we suggest breeding individuals that are on average less related to each other within Taronga and Symbio to prevent founder effects and inbreeding depression (Frankham, 2008; Frankham et al., 2010; Lacy, 1987). As we observed lower levels of MK between ($MK_{BETWEEN}$) Taronga and Symbio compared to within (MK_{WITHIN}) (Table 2A, Table S4, Table S5), we have also suggested to the recovery team and captive managers that breeding between Taronga and Symbio (when sexually mature) could result in a decrease in average kinship, a reduction in inbred individuals, and an increase in diversity

(Hoffmann et al., 2021, Kinghorn and Kinghorn, 2021), as observed in other species (del Mar Ortega-Villaizan et al., 2011; Thavornkanlapachai et al., 2021). Given that only Taronga individuals have reached reproductive maturity and animals born in captivity (F1) have yet to breed, genetic monitoring of each generation is necessary to detect any genetic drift in captivity (Gilligan & Frankham, 2003) in addition to diversity and MK monitoring. This is being implemented through genetic sampling of captive born individuals.

We demonstrate in our case study how the use of genome-wide SNP data can provide fine-scale insights to support mitigation of genetic issues in captive populations. The generation of a reference genome in this study will also provide support for future investigations into adaptive potential including immune gene diversity.

4.1.5 | Stage 5—Choosing individuals for reintroduction/release

To choose individuals most suitable for release, genetic information from stages 2–4 can be used to select individuals with wide-ranging levels of differentiation and/or low levels of relatedness. Before translocating captive individuals, it is essential to understand the genetic metrics of both the wild and captive population to prevent the introduction of identical or closely related genotypes from captivity into the wild, particularly when there may be more diverse individuals available in captivity (Hogg et al., 2020). Analyses of wild populations in stages 1–2 can be used to inform release site selection by releasing individuals at sites where genetic differentiation within a species or population is high and inbreeding or outbreeding depression is unlikely (Grueber et al., 2018; Nistelberger et al., 2023). We recommend performing this genetic admixture to maximize diversity in the wild but using populations that have exchanged genes within the last 500 years and where there is little evidence of local adaptation to minimize the risk of outbreeding depression (Frankham et al., 2011).

For *M. georgesi*, the availability of contemporary wild samples meant that we could identify that the combined captive populations are representative of historical wild diversity before the outbreak (Figure 3B) (stage 2). As such, releases from both Taronga and Symbio will be essential in supplementing pre-disease diversity into the contemporary wild population. Additionally, our MK estimates provide data for breeding decisions in the captive breeding program and minimizing the release of inbred individuals into the wild (Table S4, Table S5). To date, site selection for release has not been genetically informed due to insufficient data and has instead been selected based on ease of access and ability to obtain landholder approvals.

Our case study supports the use of genetically informed translocations by providing insight on the genetics at different sites throughout the river (Figures 1 and 3B). For example, the release of Taronga and eventually Symbio individuals at the opposing end of the river from their collection site (Figure 1A) could facilitate admixture with genotypes in lower and upper regions, respectively.

4.1.6 | Stage 6— Managing the reintroduced population in the wild

Due to persistent threats in their natural environment, reintroduction of certain species or reinforcement of wild populations might not be possible for an extended period. However, over time, captive populations may undergo genetic changes that make them better suited to their captive conditions but less adapted to the challenges of the wild (Christie et al., 2012; Frankham, 2008). This adaptation to captivity can result in reduced fitness in the wild due to a loss of genetic diversity, the accumulation of deleterious mutations, or the fixation of alleles that are advantageous in captivity but not in the wild (Frankham et al., 2017; Lacy, 1987). To minimize genetic adaptation to captivity and ensure long-term viability of the remaining wild population (Crates et al., 2023; Frankham, 2008), it is commonly suggested that releases be carried out within a few generations (Williams & Hoffman, 2009). To evaluate the effects of conservation translocations on genetic diversity, it is crucial to have ongoing monitoring of the reintroduced or reinforced population, at least until the population is self-sustaining with an improved conservation status (IUCN/SSC, 2013). Apart from techniques like radiotracking, this requires the collection of DNA samples from the wild population over numerous generations and analyzing them for any changes. For example, this can be achieved by mirroring the temporal comparative analyses in this study (Table 2A,B).

It is recommended that 1–10 individuals every 2–3 generations is sufficient in increasing genetic diversity (Allendorf, 1983; Backus et al., 1995; Lacy, 1987; Lande & Barrowdough, 1987). Prior to reinforcing the wild population, the *M. georgesi* recovery program undertook measures including predator management, habitat restoration, water quality assessments, and community engagement to minimize environmental stressors (Jakob-Hoff et al., 2017). The program has conducted four rounds of reinforcements by releasing 82 F1 juveniles of unknown sex into the Bellinger River (Figure 2A), significantly boosting wild population numbers.

To evaluate the short-term survival and movement of the released individuals, a select number have been radio tracked. Additionally, annual surveys are conducted to sample animals, with DNA biobanked for future sequencing. Given that genetic changes may not be immediately apparent in a long-lived species, long-term monitoring with a focus on sampling new and juvenile individuals, is critical for detecting alterations in the species. Long-term genetic monitoring will also play a critical role monitoring levels of hybridisation and introgression which simultaneously threatens the persistence and locally adapted genetic identity of the species (Georges et al., 2018).

4.2 | Integration of genetic data into management actions

In combination with husbandry, breeding, and disease mitigation, incorporating genetic data throughout conservation breeding establishment and management is valuable for maximizing long-term viability in captivity and to guide conservation translocations to the wild. Here, we have provided a worked example of geneticists working with an established breeding program to answer genetic questions posed by managers using DNA samples they provided. We communicated our findings to managers during scheduled meetings and in-person workshops where our recommendations have been integrated into the species Conservation Action Plan (Jakob-Hoff, *unpublished*).

In summary, for the Bellinger River turtle we recommend that genetic diversity be maintained, or potentially increased, by:

i. Breeding individuals that are on average less related to each other within Taronga and Symbio to prevent founder effects and inbreeding depression.
ii. Breeding between Taronga and Symbio to minimize founder effects and inbreeding depression.
iii. Encouraging translocations from both Taronga and Symbio to ensure representation of genotypes from both captive populations in the wild.
iv. Alternating or varying release locations during each release round to facilitate genetic admixture between captive bred and wild individuals as there is some genetic differentiation between the wild and captive populations (Figure 3).

Here we aimed to provide a generalized checklist based on our own example of genetic data integration, which can be adapted for other conservation breeding programs. Integration of genetics into management activities may need to occur retrospectively depending on the establishment stage of the conservation breeding program when the genetic data is generated (Table 1). Instigating discussions between scientists and managers can facilitate productive dialogue, allowing for a better comprehension and adaptation of each other's work and tools (Hogg et al., 2017). This cyclical approach leads to ongoing improvement and enhancement of conservation strategies.

4.3 | Future directions

As emerging infectious diseases are causing rises in extinction risk (Blehert et al., 2009; Piotrowski et al., 2004), our capacity to understand genetics and genomics is also increasing. The DNA of disease-susceptible species provides valuable insight on species resilience, with genomics giving us the tools to unlock the answers. For *M. georgesi*, as translocation candidates are juveniles, there remains an unknown risk that once individuals mature, they will succumb to the virus in the wild, compromising the translocation program. Another unknown threat is whether the few adult survivors of the virus are genetically predisposed to resist the virus, or they merely avoided exposure (Zhang et al., 2018). To answer these questions, our high-throughput sequencing efforts and chromosome-level genome provides a valuable genomic tool for future functional gene research on *M. georgesi* and other Australian turtle species. Beyond neutral diversity investigations, high-throughput and genome-wide sequencing provides high-resolution data for immune-gene investigations, that rely on high-quality assemblies and genome-wide data (Peel et al., 2022). Each year the cost of sequencing and bioinformatic analyses becomes cheaper and more streamlined permitting studies like ours (Wright et al., 2019). Once streamlined sampling and basic genetic management become conventional practise in breeding programs, genetic research can progress towards higher resolution conservation efforts aimed at preserving the adaptive potential and functional diversity within captive programs.

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CONFLICT OF INTEREST STATEMENT

This submission is the original work of all authors. All other work is acknowledged in the manuscript. The authors declare they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The male *M. georgesi* reference genome assembly and all raw sequencing reads including the 3-tissue transcriptome RNA-seq reads are available from NCBI under BioProject PRJNA1003540.

ETHICS STATEMENT

Collection of samples was conducted in accordance with the conditions of NSW DCCEEW Animal Ethics Committee (AEC151201-3, AEC160503-01 and AEC180904-5) and Scientific Licenses (MWL00102467, SL101672 and SL10255), and the Taronga Conservation Society Australia Scientific License SL101204.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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