



Extremely low genetic diversity and small N_e in the Critically Endangered Nangur Spiny Skink (*Nangura spinosa*) of eastern Australia

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Received: 4 November 2025 / Accepted: 15 July 2026
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Abstract

Globally, many species have small, fragmented populations and hence genetic factors become a primary threat to their persistence. The Critically Endangered Nangur Spiny Skink (*Nangura spinosa*) inhabits vine thicket in Nangur and Oakview National Parks in south-east Queensland, Australia. The two populations are naturally isolated and are now very small, with census estimates of <50 in the Nangur population and ~1000 at Oakview. Using a genome-wide SNP dataset, we conducted a genetic health assessment to provide management actions for species recovery. The populations form two distinct genetic groups, with high genetic differentiation. In contrast, there is high connectivity within each population, likely driven by regular dispersal over very short distances and infrequent movements over longer distances. The Oakview population had low heterozygosity ($H_{\text{obs}} = 0.139$; $H_{\text{exp}} = 0.142$; mean individual $F = 0.116$) and contained 72% of the SNPs. The Nangur population had very low heterozygosity ($H_{\text{obs}} = 0.114$; $H_{\text{exp}} = 0.112$; mean individual $F = 0.289$). N_e estimates for the populations were very small: ~16–43 for Nangur and ~66–179 for Oakview. Genetic health metrics for the Oakview population are comparable to other Critically Endangered Australian skinks, but are much worse for Nangur. The results for the Nangur population indicate high levels of inbreeding, high relatedness, and very low individual heterozygosity. Poor genetic health is likely already contributing to fitness loss and declines, especially for the Nangur population. We recommend within-population translocations and/or managed breeding in captivity to slow genetic erosion. Additionally, genetic rescue is urgently required, via crosses between the populations. This study is a clear example of how genetic data can assist species recovery.

Keywords *Nangura spinosa* · Nangur Spiny Skink · Conservation genetics · Single nucleotide polymorphisms (SNPs) · Threatened species management

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Introduction

In species conservation, factors identified as most threatening to species tend to be environmental: habitat loss, invasive species, climate change, and disease (Gillespie et al. 2020; Geyle et al. 2021; Ward et al. 2021; Hogue and Breon 2022). However, for species that are already threatened by these factors, genetic processes can become just as important (Ralls et al. 2018; Kardos et al. 2021; Bail et al. 2026). The populations of threatened species are typically small and isolated, and the outcome of this is a potentially rapid loss of genetic diversity and an increase in inbreeding. These changes can be referred to as a reduction in genetic health. We define the term genetic health as ‘the genetic characteristics of a population that directly affect extinction

risk and survival', those being primarily genetic diversity and inbreeding (Bail et al. 2026). Poor genetic health can have large and direct impacts on the reproductive fitness of individuals, and leaves populations with less adaptability and resilience to environmental changes (Keller and Waller 2002; Frankham 2010, 2015). Consequently, extinction risk is much higher for threatened species with poor genetic health. If genetic issues are present in a threatened species but go ignored, then the benefits of addressing other ecological and demographic threats (e.g., bushfires or invasive species) may be limited (e.g., Westemeier et al. 1998; Hufbauer et al. 2015; Trask et al. 2019; Encinas-Viso et al. 2024). Thus, a holistic and evidence-based plan for species recovery must address both genetic and non-genetic factors to be effective (Frankham 2005; Ralls et al. 2018; Kardos et al. 2021; Bail et al. 2026).

Global efforts are underway to standardise the reporting of genetic health metrics (e.g., Kriesner et al. 2019; Hoban et al. 2022), and to include them in international agreements such as the Convention on Biological Diversity (Hoban et al. 2022; Frankham 2022; O'Brien 2022). A comprehensive genetic health assessment should evaluate several key metrics of genetic health at the population-level. These include genetic diversity and inbreeding, but also related metrics such as genetic connectivity, kinship, and effective population size (N_e ; Bail et al. 2026). These three additional genetic factors influence both the interpretation of genetic diversity and inbreeding metrics, as well as the possible conservation actions that can be taken. Genetic connectivity is important because population isolation prevents beneficial gene flow from maintaining genetic diversity (Frankham et al. 2017; Reyne et al. 2023). Patterns of connectivity are driven by dispersal and reproduction of individuals and, ultimately, determine inter- and intra-population structure (e.g., Bertola et al. 2023; Hartley et al. 2023). Kinship measures which individuals or populations are most related (i.e., genetically similar) to each other. Genetic management strategies that minimise mean kinship throughout a population are likely to maintain higher levels of genetic diversity (Frankham et al. 2017; Pavlova et al. 2023). N_e is useful because it predicts rates of genetic change (i.e., rate of genetic diversity loss for small populations) and therefore provides useful decision-triggers for prioritising genetic management (Frankham et al. 2014; Waples 2022). Together, these five metrics—genetic connectivity, genetic diversity, inbreeding, kinship, and N_e —represent an overall summary of the state of genetic health in a population and provide direction for managing it (see Bail et al. 2026 for further detail).

Once a species' genetic health is assessed, a range of cost-effective conservation actions become possible. The goal of genetic management is to maintain or restore a species' genetic health to levels that limit further population

decline or enable recovery. A primary goal is identifying whether and how urgently genetic management is needed based on the levels of genetic diversity, inbreeding, and N_e . This can contribute to triaging conservation efforts across populations, species, and even ecosystems (e.g., Kriesner et al. 2019). If a population does require management, genetic health can be restored by improving connectivity through habitat restoration between isolated populations, using a genetic health assessment as the blueprint (e.g., Bertola et al. 2023). However, habitat connectivity may not be possible in the short-term, in which case individuals can be translocated into (or captively bred with) the at-risk population to improve genetic health. This is known as 'augmented gene flow' and can provide large and immediate benefits (i.e., a 'genetic rescue') to the subsequent generation of crossbred individuals (Frankham 2015; Ralls et al. 2018; Fitzpatrick et al. 2020). Genetic data is invaluable in developing an augmented gene flow strategy as well as monitoring its success in subsequent generations (Taylor et al. 2024). In populations that are intensively managed, as is the case for captive colonies, it is possible to slow the rate of loss of genetic diversity and improve species' fitness by optimising which individuals have access to which mates using genetic measures of kinship (e.g., Pavlova et al. 2023). Despite the potential gains from including genetics in conservation, most threatened species still lack genetic health assessments (Ralls et al. 2018; IPBES, 2019).

The Nangur Spiny Skink (*Nangura spinosa*) is a medium-sized skink that inhabits burrows in semi-evergreen vine thicket (a type of 'dry rainforest') in a highly localized area of eastern Australia. It occurs as two populations: Oakview National Park (henceforth, 'Oakview') and Nangur National Park (henceforth, 'Nangur'; Fig. 1). These two populations are ~36 km apart and have been separated for at least hundreds of thousands of years by unsuitable dry habitats (Borsboom et al. 2010). There are many other patches of similar semi-evergreen vine thicket in the region, some of substantial area, but the species has not been detected in extensive surveys (Borsboom et al. 2010). It is not clear why the species is so localised—even at Nangur it is only known from about 1% of the remaining vine thicket area (Borsboom et al. 2010). The total area of occupancy for the species is < 5 km², with an estimated occupied area of 7.4 ha at Nangur and 480 ha at Oakview (Fig. 1; D. J. Ferguson et al., unpub. data). *Nangura spinosa* is listed as Critically Endangered in state (NCA 1992) and national legislation (EPBC Act 1999), and Endangered at the international level (IUCN; Hoskin et al. 2018). Surveys since 2005 have estimated the population in Oakview to be "at least in the high hundreds to possibly 1000 adults" (Borsboom et al. 2010; D. J. Ferguson et al., unpub. data), while the Nangur population was estimated to be 90 adults in 2015 and 44 adults

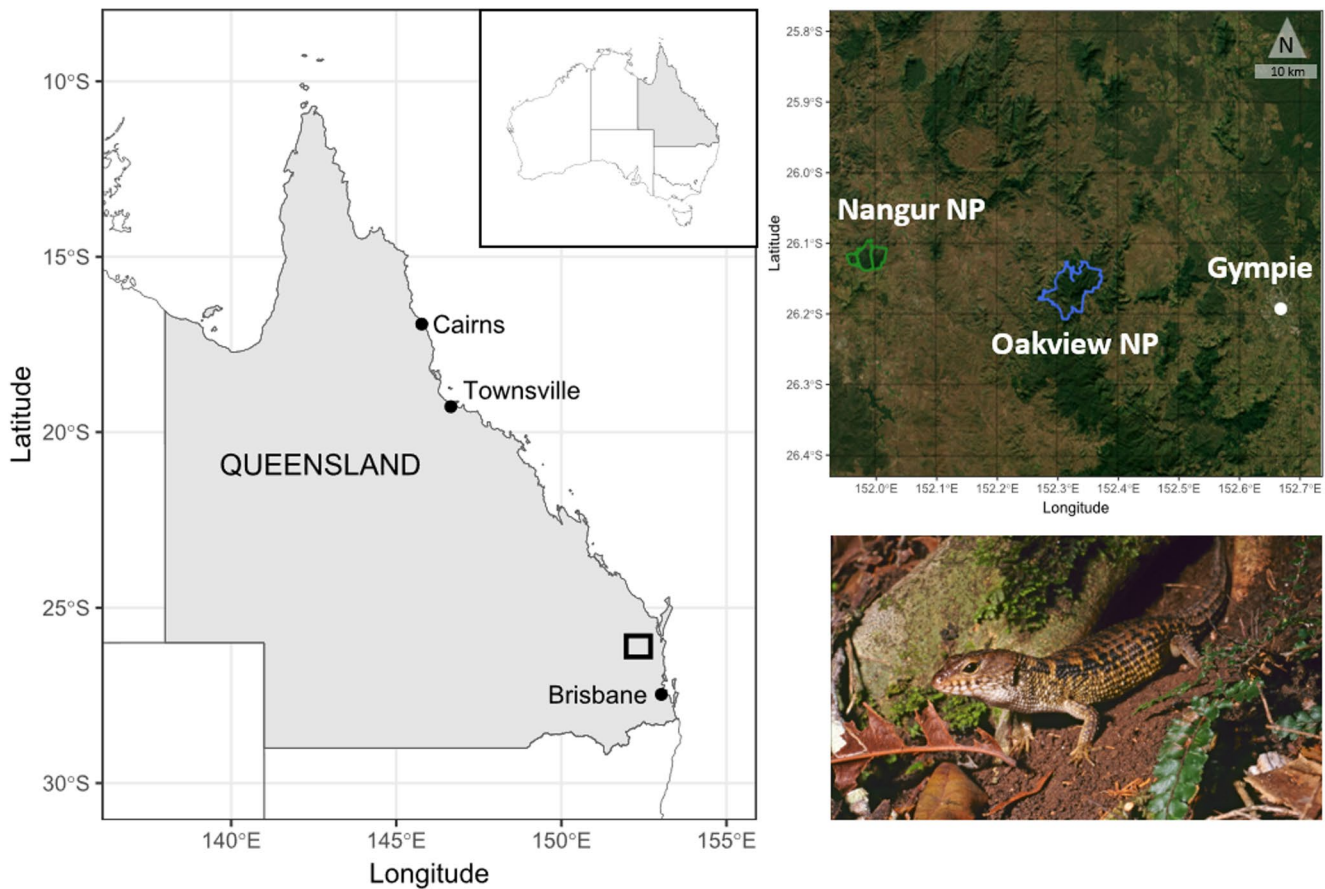


Fig. 1 Location of Nangur National Park and Oakview National Park (inset) in south-eastern Queensland, Australia. Photo of *Nangura spinosa* at burrow entrance (photo credit: Steve Wilson)

in 2020–2021 (D. J. Ferguson et al., unpub. data). Surveys at Nangur between 2001 and 2010 did not detect *N. spinosa* in some areas it was present in the 1990s, and therefore estimated a decline in occupied area from 23.8 ha to 7.4 ha (Borsboom et al. 2005, 2010). At Oakview there is evidence of ongoing declines in peripheral habitat areas over the last decade, with a greater than 100 ha reduction in occupied area (D. J. Ferguson et al., unpub. data).

The main impact in the last two hundred years has been clearing of habitat, particularly for native timber harvesting, hoop pine plantations, and cattle grazing (EPBC Act 1999; Borsboom et al. 2010). *Nangura spinosa* was only described in 1993, so it is not possible to know whether this clearing removed entire populations. It is also hard to know how much it impacted the two known populations. It has been estimated that 89% of the species' potential habitat (semi-evergreen vine thicket) has been cleared in the vicinity of Nangur, and 17% has been lost around Oakview, resulting in increased habitat fragmentation (Borsboom et al. 2010). However, it is not known how much of these cleared areas were occupied by *N. spinosa*. Clearing of these patches

ceased with the establishment of the two national parks, but the species faces ongoing threats from invasive plants and animals, bush fires, and climate change (EPBC Act 1999; Borsboom et al. 2010; Chapple et al. 2019; D. J. Ferguson et al., unpub. data). On top of this, the population sizes are now at or below the recommended rule-of-thumb of 1000 adults (roughly equivalent to an N_e of 100) for short-term genetic health (Frankham et al. 2014). At these population sizes, the impacts of genetic erosion can accumulate to harmful levels in just a few generations (Frankham et al. 2014). As such, a genetic health assessment is an important step in preventing further decline and planning for recovery in *N. spinosa*.

Here, we assess the genetic health of *N. spinosa* to provide recommendations for improving the short- and long-term prospects for both populations. Our aim was to assess the five key genetic health metrics: genetic connectivity, genetic diversity, inbreeding, kinship, and N_e . We predicted no connectivity between the two populations, limited genetic connectivity between sampling areas within each population, and all other genetic health metrics to be poor for both populations (i.e., low genetic diversity, high

inbreeding, high kinship, low N_e). We further predicted that genetic health metrics would be noticeably worse for the very small Nangur population.

Methods

The methodology we followed for assessing the genetic health of *N. spinosa* can be summarised as follows. Small tissue samples were collected from individual skinks living in the two wild populations or in captivity. Genomic DNA was extracted and then underwent ddRAD library preparation and sequencing. We processed the raw sequencing data to generate three datasets each defined by the individuals included and the type of genetic markers used (SNP-only, or SNPs and invariant sites). We then applied further filters to the three datasets to improve their quality and to suit the assumptions of the population genetic analyses. Next, we analysed population structure and connectivity between and within populations using the SNP-only dataset. Finally, we employed all three datasets to measure genetic health metrics (population genetic diversity, individual inbreeding, and effective population size).

Field sampling

Tissue samples for sequencing were collected from *N. spinosa* individuals during survey and monitoring work by the Department of Environment, Tourism, Science and Innovation (DETSI). Most of the samples (83 samples) came from 2020 to 2021. These samples were taken from individuals at all known inhabited areas in Nangur, from six sites spread across the Oakview population, and from a DETSI managed captive population derived from individuals collected from Oakview in 2020 (henceforth, ‘captive population’). An additional 10 samples (5 from Nangur and 5 from Oakview) were collected in 2006 and 2007 as part of an earlier study (Borsboom et al. 2010). Exact sampling locations within the National Parks are kept confidential to protect against poaching or disturbance by people eager to see and photograph the species. Samples were obtained by cutting a small amount of tissue from the tail-tip, using sterile dissecting scissors. Upon collection, tissue samples were immediately placed in 95% ethanol and subsequently stored at -20°C .

A total of 93 individuals were included in this study: 33 from Nangur, 41 from Oakview, 14 from individuals that were moved to captivity from Oakview, and 5 individuals that were born in this captive population. All samples were subsampled and a piece of each was shipped on dry ice to the Australian Genomics Research Facility (AGRF) for Tissue DNA Extraction and Genotyping-By-Sequencing (GBS). Samples were duplicated for two of the individuals,

as technical replicates, meaning that a total of 95 samples were included in batch sequencing. Three other individuals (one from Nangur, two from Oakview) had duplicated sub-samples used for library establishment.

Reduced representation sequencing

Extraction of genomic DNA from tissue was performed using AGRF’s tissue DNA extraction service, which used the QIAamp 96 DNA QIAcube HT Kit with the QIAcube HT Prep Manager Software (AGRF 2025). This was followed by quantification of DNA quantity and concentration with a Promega Quantifluor System, and quality assessment with an Agilent GenomicTape. AGRF’s GBS approach was used for library preparation and sequencing which follows a ddRAD (double digest Restriction-site Associated DNA) based protocol (AGRF 2025; Peterson et al. 2012). The restriction enzymes PstI and HpyCH4IV were selected for double-digestion, using AGRF’s Standard Establishment (AGRF 2025). This service selects the best pair of restriction enzymes to produce sequence fragments that are not repetitive and that amplify well. Following double-digestion, genomic fragments were ligated with two NGS adapters (which facilitate sequencing), and 48 unique multiplexing barcodes with two additional index sequences (which link sequences to the sample they came from). Samples then underwent size selection (obtaining 280–342 bp sequence fragments) using Blue Pippin, and then an indexing PCR, followed by single-end sequencing on an Illumina NovaSeq6000 that generated approximately 100 bp reads.

SNP calling, filtering, and final datasets

Single Nucleotide Polymorphism (SNP) calling from the raw sequencing data took place on James Cook University’s High Performance Research Computing Cluster in a containerised environment of *Stacks version 2.62* (Catchen et al. 2011, 2013). Raw sequencing reads were received as FASTGZ (compressed FASTQ) files. This data was initially demultiplexed (i.e., reads were linked to the sample they came from) using the *Stacks* function *process_radtags*, which also corrected barcode errors of up to one nucleotide mismatch, and rejected reads of low quality or with uncalled bases (r, q, and c arguments, respectively).

The *Stacks* pipeline *denovo_map.pl* was then used to identify shared polymorphic loci across individuals. The pipeline identifies identical and similar reads within individuals to form a ‘stack’. Each stack of reads represents a putative allele in the population, which are then assembled into a putatively homologous locus (i.e., a specific sequence of DNA consisting of many base pairs that is found in most individuals). Ultimately, a catalogue of

loci is built, against which individuals can be compared. SNPs are then called from polymorphic loci shared across the population. This pipeline was run without the use of a reference genome, and following the guidelines set out by Rochette and Catchen (2017).

Optimising the appropriateness of the *denovo_map.pl* pipeline required the selection of three parameters, which are involved in trade-off between the number of polymorphic loci obtained for analysis and the amount of error introduced into the dataset (Paris et al. 2017; Rochette and Catchen 2017). These parameters were: the minimum number of identical reads required to form a stack (m); the maximum distance between two stacks required to join them as two alleles of the same locus (M); and the maximum distance allowed between loci across individuals required to join them as the same locus (n). Selection of these parameters followed the recommendations of Paris et al. (2017): m was left at 3 and the value of M and n were set to be the same, at a value that was determined using the method below.

The value of the M and n parameters was tested in a sensitivity analysis for the values 2–6. This was first done for each population, separately running them through *denovo_map.pl* to determine individuals that consistently had low read depths and call rates (Cerca et al. 2021). Following the removal of outlier individuals, the parameters were then tested using the entire dataset to determine the optimal value of M and n. The optimum value was assessed using two approaches. First, following the ‘r80 method’ of Paris et al. (2017), the optimum parameter value was one that maximised the number of polymorphic loci shared by 80% of individuals. We added the additional requirement that polymorphic loci have <60% heterozygosity to reflect an additional down-stream filtering step. Second, following the method of Bresadola et al. (2020), the optimum parameter value was one that minimised error rates between technical replicates, specifically for read depths that tended to have low error rates (10–60x). Error rates for this second approach were calculated in *TIGER* (Bresadola et al. 2020) using data obtained by running *denovo_map.pl* for each parameter value with one bi-allelic SNPs per locus, a maximum heterozygosity of 60%, and the requirement that each SNP is shared by 80% of individuals. The maximum heterozygosity filter is analogous to filtering by Hardy-Weinberg Proportions (HWP) in that it removes SNPs that have unrealistic proportions due to technical artifacts, sampling biases, or non-neutral processes. All other non-replicated individuals were then excluded before running *TIGER*. After parameter values were selected, *denovo_map.pl* was run for the entire dataset, and the *populations* module of *Stacks* was used to export to a VCF file and apply additional parameters.

Three genetic datasets were output from *Stacks*. The reason for having different datasets comes from the recommendations of Schmidt et al. (2021). They demonstrated that robust estimates of heterozygosity required the inclusion of all sites on all sequenced loci (‘genome-wide estimates’), not just SNPs on polymorphic loci (‘SNP estimates’). However, invariable sites provide no extra information to other analyses that rely only on SNPs. Furthermore, the inclusion of secondary SNPs (any additional SNP on a polymorphic locus after the first SNP) increases linkage disequilibrium. That is, the physical proximity between secondary SNPs is a source of non-independence that violates the assumptions of many population genetic analyses. Dataset 1 contained just one SNP per locus, and no invariant sites, to be used for all analyses except genetic diversity. Dataset 2 and 3 both contained all sites on all included loci (SNPs and invariant sites) and were both used to analyse genetic diversity parameters. The difference between Dataset 2 and 3 was that Dataset 3 contained the eight additional samples that were collected in 2006–2007, so that all individuals could be compared using the same SNPs (these individuals were not included in Dataset 1). Further quality control steps applied to each of the three datasets are described in detail below and outlined in Online Resources 7–9.

The VCF file for Dataset 1 contained only one bi-allelic SNP per locus (selecting the first of each locus to ensure reproducibility), with a maximum heterozygosity of 60%, and with a call rate of 100%. Following preliminary data exploration, Dataset 1 received the following further filtering steps in *VCFtools version 0.1.16* (Danecek et al. 2011): read depth was restricted at 10–60x; a minimum minor allele count was set to 3; one of each pair of individual replicates was randomly selected to be removed; and individuals that were suspected of contamination were also removed (determined by looking for unrealistically high individual heterozygosity or unrealistic levels of genetic similarity between individuals). The samples collected in 2006–2007 were excluded from Dataset 1 and 2 so they did not contribute to estimates for the current population (they were only included in Dataset 3).

Dataset 2 was filtered entirely in *Stacks* rather than also in *VCFtools*, so that the filters developed for Dataset 1 could be applied to monomorphic loci as well as the SNPs. To do this, the *Populations* module was used to remove the same individuals that were excluded from Dataset 1, maximum heterozygosity was set to 60%, and the minimum call rate was set to 100%. *Stacks version 2.62* does not have a built-in function to filter loci by read depth. Therefore, all bi-allelic SNPs (not just one per locus) were exported to a VCF file and filtered for read depth outside of the range 10x–60x. For every SNP that was filtered out, its entire locus was excluded from Dataset 2. Finally, a refined set of loci were

produced. All of the SNPs on these loci were exported to a VCF file and the number of remaining invariant sites was recorded.

Dataset 3 experienced the same dataset processing as Dataset 2 except the samples collected from 2006 to 2007 were left in as long as they passed other filtering steps.

A common approach in population genetics is to randomly remove all but one individual from any groups of individuals with a first-degree relationship (such as parent-offspring relationships or between individuals with the same parent) to compensate for non-random sampling. However, this method has come under scrutiny because the presence of related individuals is itself a real biological signal, especially in small populations (Waples and Anderson 2017). To assess any effects of first-degree relationship filtering on downstream analyses, two approaches to dealing with these relationships were applied to Datasets 1 and 2. The first was to not filter any individuals due to relationships. The second approach was to remove one individual from each pair with a first-degree relationship. Juveniles and sub-adults were preferentially removed rather than adults if detected; in all other cases, the individual removed was randomly chosen. This strategy targets a major source of non-independence in the sampling design, which was the sampling of both adult females and their offspring when found together in a burrow. Although a less stringent filter is recommended as more robust by Waples and Anderson (2017), the absence of large groups of mutually connected first-degree relationships made that method impractical. In subsequent analyses, wherever Datasets 1 or 2 are mentioned, it implies that both filtering treatments (i.e., filtered for first-degree relationships or not) were analysed for that dataset, except where otherwise stated.

First- and second-degree relationships were inferred using *Colony* version 2.0.7.1 (Jones and Wang 2010) for Dataset 1, and assumed to apply to Dataset 2 and 3. SNP genotypes were converted from a VCF file to Colony input format using a custom R script, and the two populations were run separately. All individuals were entered as polygamous, with no candidate mothers or fathers provided. The software was run with both the inbreeding and outbreeding model, but there were no differences in any of the relationships that were inferred between these models. A 'medium' length run was used, with a sibship size prior, but no sibship scaling. The allelic drop-out rate was set to 0, and the genotype error rate was set to 0.001. Results were taken from the *Colony* 'pairwise dyad' outputs as these were most congruent with known relationships (i.e., between captive individuals).

All further analysis and filtering steps (including the actual filtering of first-degree relationships) took place in the *R* version 4.3.3 software environment (R Core Team

2024) using the *R Studio* version 2023.06.01.524 integrated development environment (Posit Team 2025). VCF files were converted to *genlight* format (Jombart 2008; Jombart and Ahmed 2011) in *R* using the *vcfR* (Knaus & Grünwald, 2016 2017) and *dartR* (Gruber et al. 2018; Mijangos et al. 2022) packages.

Testing for HWP followed the method of Waples (2015). This entailed an Exact Test of HWP for all SNPs. To assess whether a population was out of HWP across SNPs, the number of significant outliers was subjected to a binomial test with a false discovery rate of 0.05. To determine which outliers were also candidates to be removed from the dataset, a Benjamini-Hochberg false discovery rate correction was applied to all p-values (Benjamini and Hochberg 1995). Only SNPs that were out of HWP in both populations after this correction would have been removed (the 'Out All' option recommended by Pearman et al. 2022). However, no outlier filtering was used for the final results because: (a) outliers were not shared across populations, (b) there were very few outliers owing to earlier filtering steps, and (c) filtering them had a negligible effect on preliminary runs of downstream analyses. To determine whether a violation of HWP was due to heterozygote excess or deficit, a distribution of SNP F_{IS} values was plotted (Waples 2015). HWP testing was performed on Dataset 1, separately for each population, and for both first-degree relationship treatments, using the *gl.report.hwe* function from the *dartR* package. F_{IS} values were calculated using the *dartR* function *gl.basic.stats*. The results from Dataset 1 are assumed to apply to Dataset 2 and 3, and preliminary analysis of Dataset 2 and 3 demonstrated no differences in outcome.

Whole-species and within-population structure and connectivity

To investigate population structure and connectivity, analyses were performed on Dataset 1, with captive-born individuals filtered out so they did not distort the natural signal of the wild populations. Several different statistical approaches were used to investigate population structure and connectivity in *N. spinosa*. Briefly, these were: K-means clustering—to determine how many clusters best represent the data and what these clusters are; *netviewr*—to visually explore population structure and connectivity through connections between individuals; and Analysis of MOlecular VAriance (AMOVA)—to determine how much variance different a priori groupings explain. Allele Frequency Difference (AFD), F_{ST} , and mean genomic distance were calculated to provide further context to the differentiation between Nan-gur and Oakview populations. Finally, to investigate dispersal within populations, the genomic distance between pairs of individuals was compared to geographic distance, and the

radius of an average genetic neighbourhood was estimated for both populations.

K-means clustering was performed in *R* using the *ade-genet* function *find.clusters*, following Jombart et al. (2010). The function performs a principal component analysis to reduce the number of dimensions in the data and then performs K-means clustering iteratively for several possible numbers of clusters (K) up to the total number of individuals. For each value of K, the Bayesian Information Criterion (BIC) is calculated which represents the amount of deviance left in the data for each model of population structure. If there is an optimal value of K and it is greater than $K=1$, then that value will be preceded by a distinct drop in the BIC (Jombart et al. 2010). Following Raftery (1995), a change in BIC of more than 2 was required to evidence a difference between K values. K-means clustering was first performed on the entirety of Dataset 1, and then on Nangur and Oakview independently.

The *R* package *netviewr* (Steinig et al. 2016) was used to perform an unsupervised network clustering analysis. In this analysis individuals are allowed to connect to other genetically similar individuals that are (at most) each other's k^{th} nearest neighbour (k-NN). This requires a matrix of pairwise genomic distance values between individuals, which is calculated in *PLINK version 1.9* (Chang et al. 2015) with the *distance* argument set to 'square 1-ibs'. The *netviewr* analysis was run for values of k-NN from 1 to 60 and the resulting networks were plotted in *R* and visually explored. AMOVAs (Excoffier et al. 1992) were performed in *R* using the *poppr* function *poppr.amova* (Kamvar et al. 2014, 2015), using both the *ade4* (Thioulouse et al. 2018) and *pegas* (Paradis 2010) methods. Variance components reported were 'between-populations', 'between-individuals', and the error term 'within-individuals'. Phi-statistics were also reported. Populations were defined a priori for each AMOVA. Initially, to assess the amount of structure at a species-wide scale, 'population' was defined as Nangur and Oakview. Subsequently Nangur and Oakview were analysed separately to determine how important structuring was within these units. This meant defining 'population' based on the sub-networks that arose in the *netviewr* analysis and K-means clustering (Fig. 2; Online Resources 13–22). The significance of each AMOVA was tested with 1000 permutations using the function *ade4* function *randtest*.

The average pairwise genomic distance between Nangur and Oakview was calculated using a custom *R* script. F_{ST} was calculated in *R* using the *dartR* function *gl.fst.pop* using 100 bootstraps. Allele Frequency Difference (AFD), an F_{ST} analogue with a more intuitively linear relationship to population differentiation (Berner et al., 2019), was calculated using the *dartR* function *gl.report.pa*. Genomic distance is used here as a proxy for kinship, because kinship

is biased in small, inbred populations where allele frequencies from a non-inbred reference population are unavailable (Wang 2014). Two individuals with identical genotypes would receive a genomic distance value of 0, and individuals with different genotypes at all SNPs would be 1. Using the *PLINK* generated genomic distance matrix, the mean genomic distance between Nangur and Oakview was calculated, as was the mean genomic distance within each population. The pairwise genomic distance between individuals was compared to their geographic distance apart for each population. Geodesic distances were calculated from latitude and longitudes in *R* using the *geodist* function in the *geodist* package (Padgham and Sumner 2024). This was plotted using *tidyverse* packages (Wickham et al. 2019) in *R*, with 95% confidence intervals around a locally estimated scatterplot smoothing ('loess') term as a trend line that had a span (i.e., degree of smoothing) of 0.75.

In sexual organisms with low dispersal, a genetic neighbourhood represents an area around each individual that is theoretically panmictic and within which their parents are likely to originate (Wright 1946). The radius of a genetic neighbourhood is also theoretically twice the standard deviation of the parent-offspring dispersal distance (i.e., the distance between a parent's place of birth and its offspring's place of birth; Neel et al. 2013; Wright 1946). The presence of multiple genetic neighbourhoods in a population has also been found to downwardly bias estimates of effective population size (Neel et al. 2013; Nunney 2016). The radius of an average genetic neighbourhood in each of Nangur and Oakview was estimated using the *sGD* package (Shirk and Cushman 2011) in *R*, following the approach recommended by Neel et al. (2013), and Shirk and Cushman (2014). This involved using the *sGD* package to define a neighbourhood of radius r around each individual and calculating F_{IS} for the individuals in that neighbourhood. The value of r was increased iteratively from 400 m to 1600 m by steps of 100 m. Radii smaller than 400 m frequently had small sample sizes and were well below the point we were seeking. The best estimate for neighbourhood radius was that which best centred the distribution of F_{IS} values on zero.

Genetic health metrics

The levels of genetic diversity in Nangur, Oakview, and the captive population were estimated for Dataset 2 and 3 (the datasets containing both SNPs and invariant sites) with the *dartR* package. In this instance, the captive population is treated as all individuals living in captivity, including those born in the wild. Oakview also includes individuals that were born in the wild but have since been moved to the captive population. Observed and expected heterozygosity, F_{IS} , and percentage of SNPs that are polymorphic in

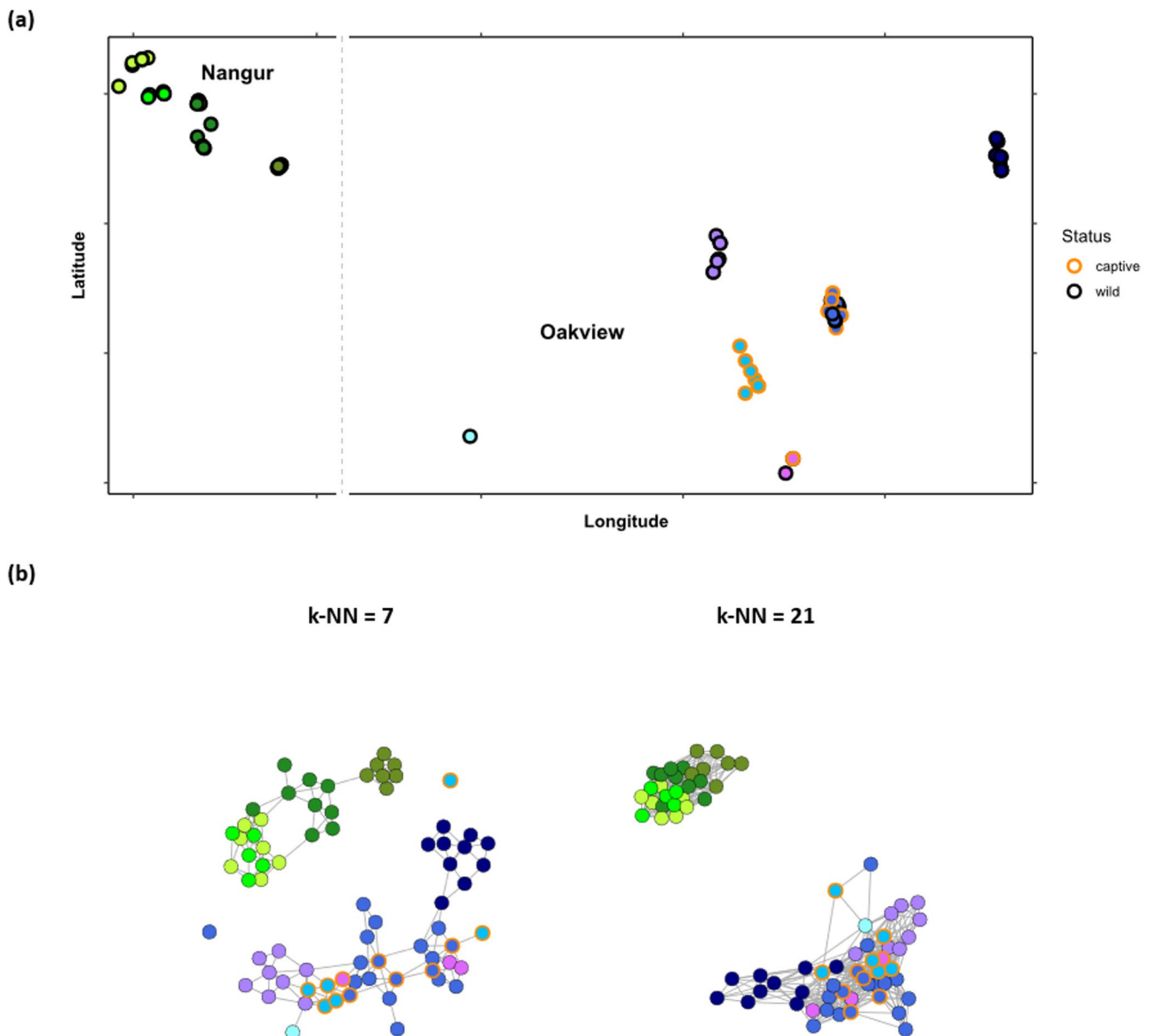


Fig. 2 Geographic mapping of *Nangura spinosa* individuals (a) and corresponding *netviewr* plots (b) demonstrating population structure and connectivity within the Nangur and Oakview populations for Dataset 1. Individuals have been colour-coded by rough geographic sampling area for comparison between (a) and (b). Individuals that have been captured and now form part of the captive population are

each population were calculated using the *gl.report.heterozygosity* function with the ‘pop’ method. ‘SNP’ estimates of observed and expected heterozygosity are estimated using only SNPs (excluding secondaries), ‘genome-wide’ estimates include both SNPs (including secondaries) and invariant sites in the calculations. The function *gl.report.pa* was used to calculate the percentage of SNPs that had private alleles in either Nangur or Oakview, and the number of SNPs with fixed allelic differences between Nangur and Oakview (i.e., fixed alleles).

highlighted in orange. Ticks along the x-axis represent approximately 2 km longitudinal distance (but note that there is a break in the x-axis between the Nangur and Oakview populations), ticks along the y-axis represent approximately 1.11 km latitudinal distance. *Netviewr* network plots show within-population structure at fine ($k\text{-NN}=7$) and coarse ($k\text{-NN}=21$) scales

Individual multiple locus heterozygosity (MLH) from SNP data is an estimate of the true genome-wide heterozygosity and is strongly correlated with genome-wide inbreeding (Kardos et al. 2015, 2016), so this was used as the main measure of an individual’s level of inbreeding. Individual MLH was calculated from Dataset 3 using the *dartR* function *gl.report.heterozygosity* with the ‘ind’ method. Results in Table 1 were analysed from Dataset 2, and populations were defined as for population-wide metrics of genetic diversity. A linear model was run to

Table 1 Metrics of genetic diversity and inbreeding from the genetic health assessment for *Nangura spinosa* calculated using Dataset 2: a refined dataset of 12,314 biallelic SNPs from a total of 3,742,811 sites (used for genome-wide metrics). The first-degree relationship filter has not been applied here (see Online Resource 30)

Metric ^a	Nangur	Oakview ^b	Captive Population ^b
Sample size	28	47	17
SNP H_{obs}	0.114 (0.183)	0.139 (0.168)	0.135 (0.182)
Genome-wide H_{obs}	0.000376 (0.0124)	0.000458 (0.0125)	0.000446 (0.0130)
SNP H_{exp}	0.112 (0.172)	0.142 (0.168)	0.133 (0.170)
Genome-wide H_{exp}	0.000370 (0.0118)	0.000467 (0.0126)	0.000436 (0.0124)
F_{IS}	0.00124	0.0301	0.00809
MLH	Q_1 : 0.108, Q_3 : 0.122	Q_1 : 0.135, Q_3 : 0.145	Q_1 : 0.135, Q_3 : 0.141
F	Q_1 : 0.243, Q_3 : 0.330	Q_1 : 0.099, Q_3 : 0.159	Q_1 : 0.127, Q_3 : 0.162
%Poly	41%	72%	51%
$A_{private}$	28%	58%	
A_{fixed}	1%		

^aShown are: observed heterozygosity, H_{obs} ; expected heterozygosity, H_{exp} ; population inbreeding (i.e., non-random mating) coefficient, F_{IS} ; the first (Q_1) and third (Q_3) quartiles of individual multi-locus heterozygosity, MLH; Q_1 and Q_3 of individual inbreeding, F; percentage of SNPs that are polymorphic, %Poly; percentage of SNPs that had private alleles in only one of the two populations, $A_{private}$; percentage of SNPs that have fixed differences between Oakview and Nangur, A_{fixed} . Standard deviations shown in brackets where appropriate. ^bOakview includes 12 individuals who are also included in the captive population

determine that individual MLH was not biased by individuals with poorer read depth, using the *R* function *lm* from the *stats* package (Online Resource 29).

Crow and Kimura (1970) showed that the inbreeding coefficient (F) measures the proportion that heterozygosity has been reduced from non-inbred heterozygosity (Kardos et al. 2016). Hence, a conservative estimate of F can be represented for each individual based on their MLH using Eq. [1], a rearrangement of the formula presented in Crow and Kimura (1970):

$$F_i = 1 - \frac{MLH_i}{MLH_0} \quad (1)$$

where MLH_i was the MLH of individual *i* and MLH_0 was the MLH of a non-inbred individual, which was approximated here as the maximum MLH value observed in *N. spinosa*. Because both populations of *N. spinosa* are small and likely inbred to some degree, the true values of F are likely higher than the estimates presented here (Wang 2014).

To estimate effective population size (N_e), the single-sample bias-corrected linkage disequilibrium (LD) method (Hill 1981; Waples 2006; Waples and Do 2010) was used on Dataset 1. Dataset 1 is more suitable for using LD to estimate N_e because the exclusion of secondary SNPs, which are retained in Dataset 2 and 3, reduces the physical linkage in the dataset. The analysis was implemented in *N_eEstimator v2.1* (Do et al. 2014), which was run from the *dartR* interface function *gl.LDNe*. Random mating was assumed in the estimation. To further reduce computational load and the amount of physical linkage in the dataset, five random subsets of 1000 SNPs were generated from Dataset 1. The LD method of estimating N_e was applied to each subset,

with a minimum minor allele frequency filter of either 0.01 or 0.05 and either of the two first-degree relationship treatments. That is, there were four possible combinations of filter applied to each of five random subsets. Jackknife 95% Confidence Intervals are presented for each replicate (Jones et al. 2016). Finally, the arithmetic mean of the five subsets was then calculated for each of the four combinations of first-degree relationship filter and minor allele frequency filter. The Nangur and Oakview populations were analysed separately.

Plotting figures and maps

Except for BIC plots (which used the package *adegenet*) and the *netviewr* plots (which used the package *netviewr*), plotting was achieved with the help of the *tidyverse* packages in *R*. Mapping data for national parks comes from the Queensland Government (2024), and maps of Australia were plotted with the aid of the *R* packages *ozmaps* (Sumner et al. 2001), *sf* (Pebesma 2018; Pebesma and Bivand 2023), and *leaflet* (Cheng et al. 2024). All map features, latitude and longitudes, and exact sampling locations of individuals within the National Parks have been anonymised in Figs. 2 and 5. The relative positions of individuals are to scale in these figures, with ticks along the x-axis representing approximately 2 km longitudinal distance and ticks along the y-axis represent approximately 1.11 km latitudinal distance. However, note the break in the x-axis so that the National Parks can be presented side-by-side, which was achieved using the *R* package *ggbreak* (Xu et al. 2021). In Fig. 5, the positions of individuals have been randomly jittered to reduce overplotting.

Results

SNP calling, filtering, and final datasets

Approximately 495 million single-end raw reads were generated by Illumina sequencing. Of these, 96% reads were retained by *process_radtags*, with a median of approximately 5 million reads retained per sample (Online Resource 1) across 95 samples. Reads were rejected due to lack of a missing restriction enzyme cut site (~66% of dropped reads), or missing barcode (~33%), with only approximately 1% dropped due to low quality (Online Resource 2). Three individuals were removed from the dataset due to consistently low read depths after processing in *denovo_map.pl* across parameter values. These samples were: a 2006–2007 sample from Nangur, a 2006–2007 sample from Oakview, and a 2020–2021 sample from the captive population. After these samples were removed, the modified “r80 method” (Paris et al. 2017) found that the optimum value of Stacks parameters *M* and *n* was 2 (i.e., these parameters maximised the number of loci and polymorphic loci assembled by Stacks; Online Resource 3). Error rates calculated by *TIGER* (Bresadola et al. 2020) were lowest at read depths between 10x and 200x (Online Resources 4–5). However, there were very few reads with greater depth than 60x. Between the read depths of 10x and 60x, error rate was lowest when *M* and *n* were equal to 2 or 3 (Online Resource 6). Hence, the parameter values of *M*=2, *n*=2, and *m*=3 were chosen for *denovo_map.pl*. Parameters were selected due to minimised error and maximised number of polymorphic loci, although these factors varied little across the tested parameter space. Running *denovo_map.pl* with the selected parameters resulted in 92,791 loci, 34,926 of which were polymorphic. The error rate for read depths between 10x and 60x was 0.00162. The mean locus length was 92.15 bp. The 60% heterozygosity filter removed <1% of the initial number of SNPs and reduced the datasets’ observed heterozygosity by approximately 6–7% (Online Resources 7, 8 and 9).

Three datasets were made to suit the differing requirements of genetic analyses. In particular, invariant sites and secondary SNPs are not desirable in many population genetic analyses, but are needed for robust estimation of heterozygosity. For this reason, Dataset 1 included only a single SNP per locus, while Dataset 2 and 3 also included invariant sites and secondary SNPs. While 80 individuals are the same across all three datasets, Dataset 3 differed from Dataset 1 and 2 in the inclusion of eight additional samples collected from 2006 to 2007. The Dataset 1 VCF file included 6,568 SNPs (2,968 in Nangur; 4,659 in Oakview) for 80 individuals (28 from Nangur, 52 from Oakview, including 12 that were born in Oakview but now live in captivity, and 5 individuals born in captivity). Twelve samples were removed

from Dataset 1: two that were technical replicates of other samples, two due to likely contamination, and the eight samples remaining of those collected in 2006–2007. Dataset 2 and 3 contained all nucleotide sites (variant and invariant) from each short-read locus, including secondary SNPs and invariant sites, rather than just one SNP per locus like Dataset 1. The Dataset 2 VCF file had the same 80 individuals as Dataset 1, but with 12,314 SNPs, with the same total number of sites as Dataset 1. The Dataset 3 VCF file included the extra 8 individuals from 2006 to 2007, making 88 samples with 11,306 SNPs. In Dataset 1 and 2, the total number of nucleotide sites across all loci was 3,742,811, including both invariant sites and SNPs. In Dataset 3, the total number of sites across all loci was 3,518,795. Further details on the number of SNPs and individuals remaining after each filtering step are included in Online Resources 7–9.

The first-degree relationship filter removed 10 individuals from Nangur and 12 individuals from Oakview (including five from the captive population). Further details of families and pairwise relationships inferred by *Colony* are discussed below. Nangur was found to be significantly violating HWP ($p < 0.001$) when no first-degree relationship filter was applied, with 6.8% of SNPs violating HWP. The distribution of SNP F_{IS} values was symmetrical, and there were equal numbers of SNPs with heterozygote deficits and excesses. There were nine outlier SNPs after correcting the Exact Test *p*-values using the Benjamini-Hochberg correction. After the first-degree relationship filter was applied, Nangur was not found to be significantly violating HWP ($p = 1$), with 3.2% of SNPs violating HWP, and only 3 outlier SNPs after the Benjamini-Hochberg correction. Oakview was not found to be significantly violating HWP, whether with ($p = 1$, 3.9% of SNPs violating HWP) or without a first-degree relationship filter ($p = 0.757$, 4.7% of SNPs violating HWP) applied. Oakview had 12 outlier SNPs after the Benjamini-Hochberg correction, and five when the first-degree relationship filter had been applied, but did not share any of these with Nangur.

Population structure and connectivity

Whole-species structure and connectivity

K-means clustering best grouped the species-wide dataset into two clusters ($K = 2$), representing the two known wild populations (Nangur and Oakview). This was identified by a clear drop in BIC prior to $K = 2$ (Online Resource 10a). In the plots generated by *netviewr*, Nangur and Oakview formed two distinct networks across all *k*-NN values appropriate to the dataset (i.e., until *k*-NN=47, at which point all individuals had made all other possible connections within their respective populations; Fig. 2). In the species-wide

AMOVA, with populations defined as being Nangur and Oakview, between-population variation explained approximately half of the genetic variation (47%, $p < 0.001$), while most of the remaining variation was explained by within-individual differences (52%, $p < 0.001$; Online Resource 13–14). The F_{ST} between Nangur and Oakview was 0.479 ($p = 0$) without the first-degree relationship filter, and 0.474 ($p = 0$) using the first-degree relationship filter. The AFD between Nangur and Oakview was 0.288 without the first-degree relationship filter, and 0.287 with the filter. The mean genomic distance between Nangur and Oakview was 0.301 without the first-degree filter, and 0.302 with the filter.

Within-population structure and connectivity

When K-means clustering was applied to the Nangur population, there was no distinct drop in the BIC that would indicate an optimum value of K (i.e., number of clusters) greater than 1. The BIC for K=2 clusters was lower than for K=1, but there was less than a two-point BIC difference between K=1, 2, or 3, meaning that there is no strong support for splitting Nangur into two or three clusters rather than just one (Raftery 1995; Online Resource 10b). In the *netviewr* plots, smaller sub-networks could be identified within Nangur indicating close relationships between individuals in the same geographical sampling areas (Fig. 2). These sub-networks were arranged roughly north-west to south-east, were identical to the clusters identified in K-means clustering at K=3, and were joined together at k-NN=7. By k-NN=21, the number of possible connections within the Nangur *netviewr* network became almost saturated and the sub-networks completely lost their distinctiveness (Fig. 2). In the Nangur-only AMOVA, over 90% ($p = 0.427$) of the genetic variation was accounted for by within-individual variance (Online Resources 15, 16, 17 and 18). The AMOVA's between-population variance component was at its greatest (~10%, $p < 0.001$) when two or three provisional 'populations' were defined using the within-population groupings also observed in the K-means clustering for K=3 and *netviewr* plots (Online Resources 15–18; Fig. 2). For all AMOVAs, filtering first-degree relationships from the dataset tended to reduce the between-population variance component and increase the between- and within-individual variance component. However, between-individual variance was a negligible component, ranging between -6% and 4% (Online Resources 13–22).

Oakview was best represented by a single cluster, with BIC not showing any distinct drop but increasing incrementally for values of K greater than 1 (Online Resource 10c). The *netviewr* plots distinguished Oakview's northern (purple; Fig. 2) and eastern (navy blue) sampling areas as clearly distinct sub-networks, while the central/southern sampling

areas (blue and pink) formed a looser sub-network, suggesting more connectivity and diversity throughout the densely populated area. All Oakview *netviewr* sub-networks showed some inter-connectivity at k-NN=4 and above (Fig. 2; Online Resource 12). The solitary western-most individual (light blue) connected most closely to the northern sub-network, even before many other geographically closer individuals had connected (Fig. 2). However, two genetically-distinct individuals remained unconnected to the Oakview network until k-NN=21 (Fig. 2). When first-degree relationships were removed, the previously identified *netviewr* sub-networks were less distinct, but still evident (Online Resource 12). In the Oakview-only AMOVA, over 90% ($p = 0.427$) of the genetic variation was accounted for by 'within-individual' variance (Online Resources 19, 20, 21 and 22). The AMOVA's between-population variance component (using within-population groupings as the provisional 'populations') was smaller than in Nangur, being approximately equal across different population definitions at approximately 5% ($p < 0.001$; Online Resources 15, 16, 17, 18, 19, 20, 21 and 22).

Between-individual relationships and dispersal

Colony inferred the presence of 11 pairs of individuals that were in first-degree relationships (full-siblings or parent-offspring pairs) in Nangur, comprising 13 individuals (i.e., many of the individuals belonged to multiple pairs), and four 'first-degree families' (groups of individuals connected directly or indirectly via first-degree relationships; Online Resource 23). These were: two families (of five and two individuals, respectively) making up all of the individuals in the south-east area, one family in the approximate centre of the population (three individuals), and one in the north-west (three individuals). *Colony* inferred 27 second-degree relationships, comprising 24 individuals. Most individuals were in one of five 'second-degree families' (groups of individuals connected directly or indirectly via both first- and second-degree relationships; Fig. 5a). These were: a single family making up all seven individuals of the south-eastern group (highlighting the high degree of relatedness in that semi-isolated area), a family group made up seven individuals spread throughout the central area, and three families inter-mixed in the north-western area (of five, three, and three individuals, respectively; Fig. 5a). Four individuals in Nangur did not belong to any second-degree family and likely possess unique genetic diversity (Fig. 5a).

Colony inferred the presence of 18 first-degree relationships in Oakview, comprising 25 individuals (including those now in the captive population). *Colony* also inferred a further 10 second-degree relationships in Oakview, comprising 32 individuals. These formed six identified first-degree

families in the wild population at Oakview, and six second-degree families (Online Resource 23; Fig. 5a). Three first-degree families were found in the northern cluster, two of which merged into two second-degree families. There were two first-degree families, which were each part of two larger second-degree families in the eastern cluster. And there was one first-degree family in the central cluster, and two second-degree families (Fig. 5a). All four first-degree families in the captive population—where the mother, offspring, and sometimes fathers were already known—were successfully identified by *Colony*.

In both populations, the relationship between pairwise genomic distance and geographic distance approximately follows a logarithmic curve that plateaus just prior to 500 m (Fig. 3). In terms of genomic distance, this plateau occurs in Nangur at approximately 0.12 and in Oakview at approximately 0.15 (Fig. 3).

Pairwise genomic distances between individuals increased in Nangur from a minimum of 0.053 between the two most closely-related individuals (which was a first-degree relationship) to a maximum of 0.136, with a mean genomic distance of 0.109 under the first-degree relationship filter and 0.106 without the filter (Fig. 3). The pairwise genomic distance between *Colony*-identified first-degree relationships in Nangur was 0.053–0.071 (median=0.067;

Fig. 3), and the geographic distance between first-degree relationships in Nangur was 0–398 m (median=18 m, with or without individuals found in the same burrow; Fig. 3). The pairwise genomic distance between individuals in a second-degree relationship in Nangur was 0.076–0.103 (median=0.088), with a geographic distance apart of 7–429 m (median=43 m; Fig. 3). Interestingly, the maximum pairwise genomic distance between any two individuals in Nangur was the same level as a *Colony*-identified second-degree relationship in Oakview (Fig. 3), regardless of their actual *Colony*-identified relationship.

Pairwise genomic distances in Oakview ranged from a minimum of 0.074 to a maximum 0.169, with a mean of 0.145 with or without the first-degree relationship filter (Fig. 3). The genomic distance between *Colony*-identified first-degree relationships was 0.074–0.095 (median=0.084; Fig. 3) in Oakview, and the geographic distance between first-degree relationships was 0–17.5 m (median=0 m, or 14 m when excluding individuals found in the same burrow) in Oakview. The pairwise genomic distance between individuals in a second-degree relationship was 0.096–0.136 (median=0.124), with a geographic distance apart of 18–278 m (median=95 m; Fig. 3).

The radius of an average genetic neighbourhood in both Nangur and Oakview was estimated to be approximately

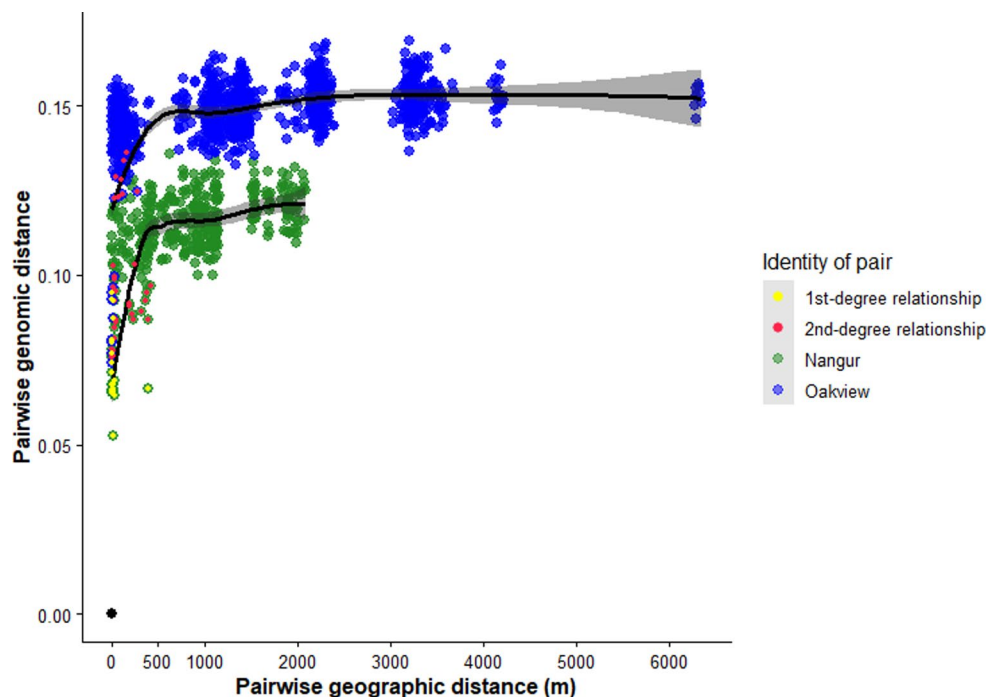


Fig. 3 Pairwise genomic and geographic distances between *Nangura spinosa* individuals within Nangur (green) and Oakview (blue) from Dataset 2. Pairwise genomic distances within Nangur are lower on average than in Oakview, and all Nangur pairs are at most equivalent to a *Colony* identified second-degree relationship from Oakview. All pairs with *Colony*-identified first- and second-degree relationships (yellow and pink, respectively) are within 500 m of one another, and

average genomic distance plateaus at approximately 500 m. Results reflect all possible pairs of individuals within either population, but none between each population. Trend line is a locally estimated scatterplot smoothing ('loess') curve with a span (i.e., degree of smoothing) of 0.75 and 95% confidence intervals. The black dot represents the pairwise genomic dissimilarity and geographic distance between an individual and itself

1000 m (Online Resources 24a and 25a). At this radius, the distribution of F_{IS} values for neighbourhoods in each population was closest to being centred on zero. Theoretically, this suggests that the standard deviation of the parent-offspring dispersal distance in both populations is approximately 500 m. Under the first-degree relationship filter, estimating the radius of an average genetic neighbourhood became unclear because the F_{IS} distribution never fully centred on zero (Online Resource 24b and 25b). In Nangur, with the first-degree relationship filter, the F_{IS} distribution was closest to centring on zero at 400 m (Online Resource 24b). However, no estimate could be made for Oakview with first-degree relationships removed, as this filter led to no F_{IS} values that were negative or equal to zero (Online Resource 25b).

Genetic health metrics

Population-wide genetic diversity

Genetic diversity in both populations was extremely low (Table 1). However, Nangur had notably lower diversity than Oakview across all metrics. Both observed and expected heterozygosity was approximately 20% lower in Nangur than in Oakview. Compared to Oakview, Nangur had 43% fewer polymorphic SNPs and 52% fewer SNPs with private alleles. F_{IS} was ~24 times higher in Oakview than in Nangur (Table 1). Subsampling of populations showed that Nangur has lower genetic diversity than Oakview regardless

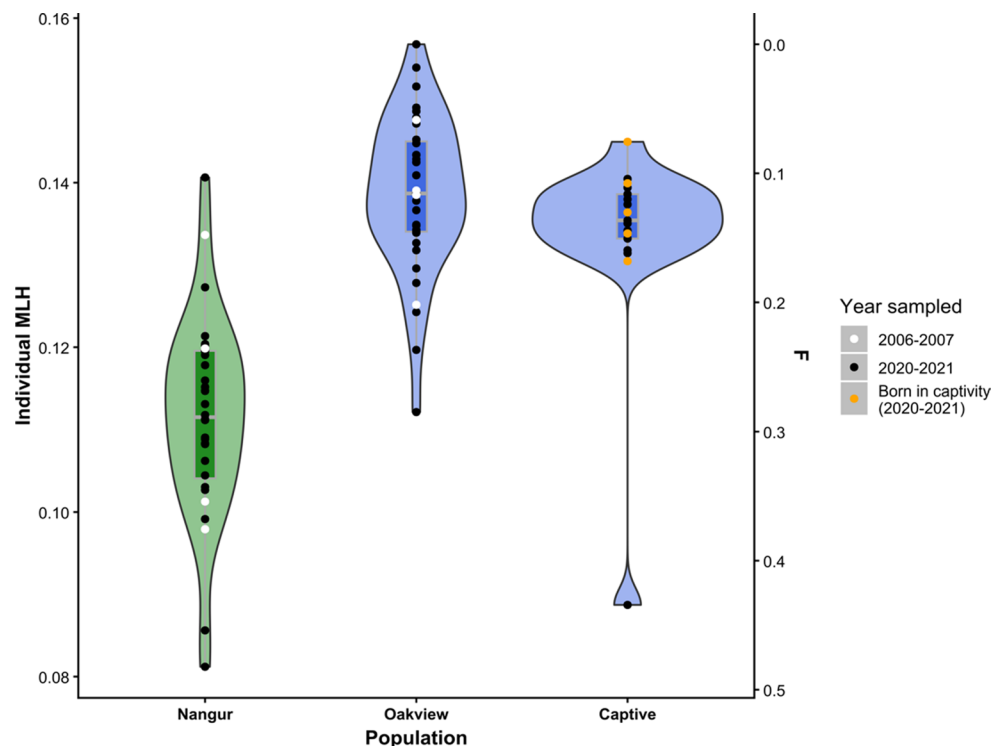
of the sample size (Online Resources 26–27). The captive population also had lower diversity than Oakview, although not as low as Nangur. Observed heterozygosity was 3% less in the captive population compared to Oakview, expected heterozygosity was 6% less, and there were 29% fewer polymorphic loci. F_{IS} was ~3.7 times higher in the captive population compared to Oakview. The first-degree relationship filter had a small effect on genetic diversity (Online Resource 30). Heterozygosity increased by ≤5% when first-degree relationships were removed, while there was only a negligible change in the percentage of SNPs that were polymorphic, unique, or fixed in each population. The first-degree relationship filter decreased F_{IS} to below zero for Nangur, but increased F_{IS} for Oakview and the captive population (Online Resource 30).

Individual genetic diversity and inbreeding

Low multiple locus heterozygosity (MLH) indicated high inbreeding across all sampled *N. spinosa* individuals. Values (as calculated in Dataset 3) ranged from 0.081 to 0.141 (Q_1 : 0.104, Q_3 : 0.120) in Nangur and 0.089–0.157 (Q_1 : 0.134, Q_3 : 0.143) in Oakview (Fig. 4; Table 1). The mean and median MLH for Nangur were both 0.112, approximately 20% lower than for Oakview (0.137 and 0.138, respectively).

There was no obvious geographic pattern for individual inbreeding in either population, except that the most inbred individuals in Oakview tended to be found in the eastern

Fig. 4 Individual multiple locus heterozygosity (MLH) and inbreeding coefficients (F) in Nangur, Oakview, and the captive population, calculated from Dataset 3. Individuals with a lower MLH and a higher F are more inbred, with some individuals reaching extreme levels of inbreeding equivalent to matings between a parent and its offspring ($F=0.25$) or between clones ($F=0.5$). Individuals from the captive population were originally born in Oakview unless marked otherwise in orange. Individuals marked in white were sampled in 2006–2007 as part of an earlier project, showing the state of the population over a decade before the main sampling event



group (Fig. 5). The captive population mostly represented the middle range of Oakview MLH values (0.130–0.145, Q_1 : 0.133, Q_3 : 0.139), except for one individual that was born in the central/southern area of Oakview which had an extremely low MLH value of 0.089, lower than any other

individual in Oakview and all but two individuals in Nangur. The samples from 2006 to 2007 indicate that inbreeding has been high in the two populations of *N. spinosa* for over a decade (Fig. 4). The mean MLH was 0.113 for the 2006–2007 Nangur individuals and 0.138 for the 2006–2007

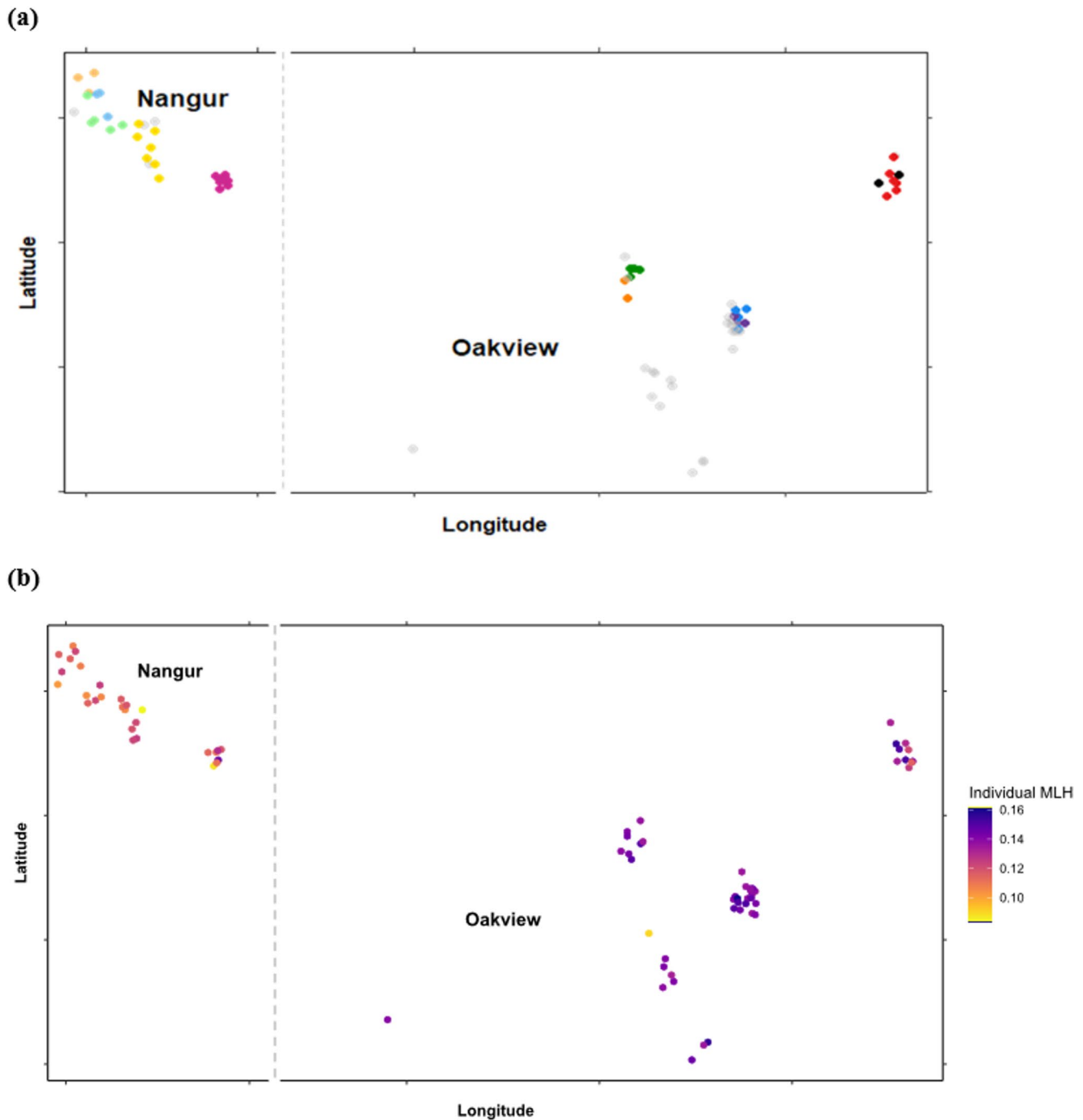


Fig. 5 Mapped second-degree families of sampled individuals **(a)** and individual multiple-locus heterozygosity **(b)** of all *Nangura spinosa* sampled at Nangur and Oakview. **(a)** Individuals colour-coded to reflect membership to a second-degree family (i.e., groups of individuals that were connected directly or indirectly via first- or second-degree relationships, as inferred using *Colony version 2.0.7.1*; Jones et

al. (2016). Transparent grey individuals do not belong to any identified second-degree family of sampled individuals. **(b)** Individuals colour-coded by individual multiple-locus heterozygosity. Lower (orange–yellow) individual MLH values represent a higher level of individual inbreeding

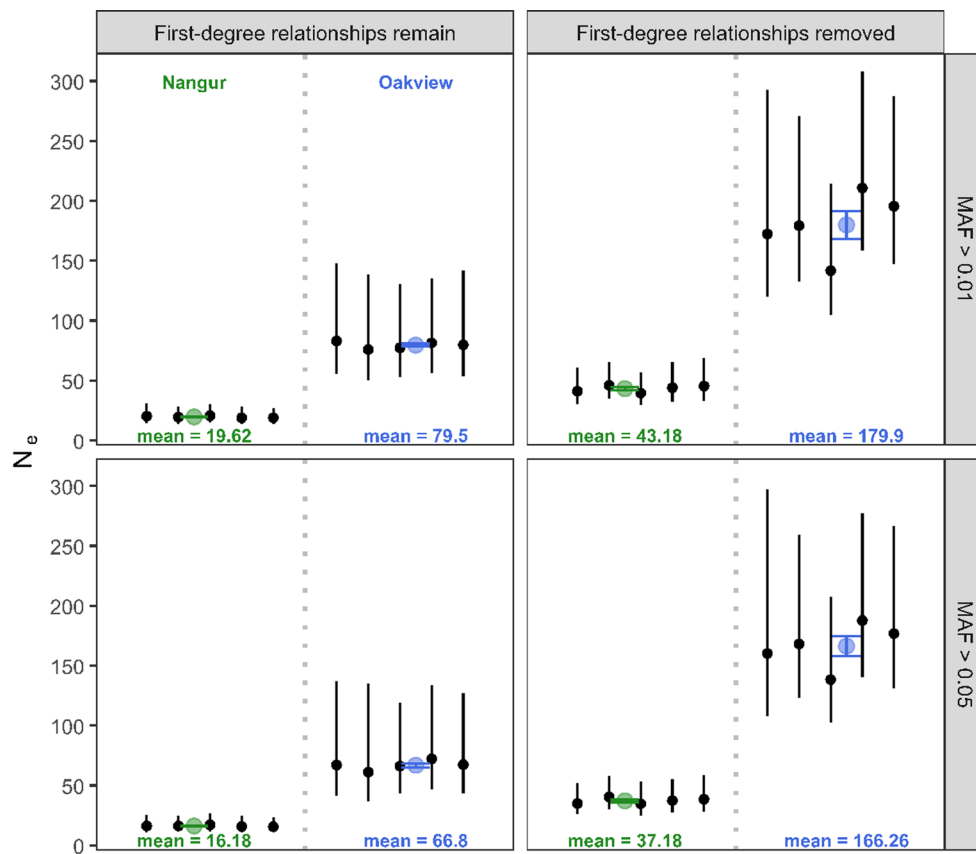


Fig. 6 Effective population size (Linkage Disequilibrium [LD] N_e) estimates for Nangur (green, left of dotted line) and Oakview (blue, right of dotted line) across minor allele frequency (MAF) and first-degree relationship filters, calculated from Dataset 1. Nangur has consistently lower N_e than Oakview, and low enough N_e to cause harmful inbreeding and loss of genetic diversity within a few generations ($N_e < 100$; Frankham et al. 2014). Filtering out first-degree relationships doubled mean N_e in all cases, while increasing the MAF filter lowered the mean N_e estimate by 20%. Each of the black points represents one

of five random subsets of 1000 SNPs from Dataset 1. Black whiskers represent Jackknife 95% Confidence Intervals for each subset (Jones et al. 2016). The blue (Oakview) and green (Nangur) circles are the means of the five subsets with whiskers representing standard error. Sample sizes for each population are: Nangur, first-degree relationships remain, $n=28$; Nangur, first-degree relationships removed, $n=20$; Oakview, first-degree relationships remain, $n=47$; Oakview, first-degree relationships removed, $n=40$

Oakview individuals (Fig. 4). Variation in individual MLH was not explained by individual read depth ($R^2=0.0077$, $p=0.42$; Online Resource 29). Individual inbreeding coefficients (F ; calculated with Dataset 3) in Nangur were 0.103–0.482 (Q_1 : 0.238, Q_3 : 0.0336), with a mean and median of 0.289. In Oakview, F was 0.000–0.285 (Q_1 : 0.075, Q_3 : 0.145), with a mean and median of 0.116. The F of the captive population ranged from 0.076 to 0.434 (Q_1 : 0.116, Q_3 : 0.151). The mean F was 0.278 for the 2006–2007 Nangur individuals and 0.123 for the 2006–2007 Oakview individuals (Fig. 4; Table 1).

Effective population size

Effective population size (Linkage Disequilibrium [LD] N_e) estimates were extremely small for both populations (Fig. 6). Mean LD N_e estimates for Nangur ranged from

16.18 to 43.18, although Jackknife 95% Confidence Intervals for the five random SNP subsets ranged from a minimum of 10.7 to a maximum of 68.6. Mean LD N_e estimates for Oakview ranged from 66.8 to 179.9, but Jackknife 95% Confidence Intervals for the five random subsets ranged from a minimum of 36.5 to a maximum of 308.2. Nangur estimates were on average 24% smaller than for Oakview across the four combinations of filters that were applied. Removing first-degree relationships from the dataset (to remove a source of non-random sampling) had a large effect, doubling mean LD N_e in all cases (Fig. 6). Changing the minor allele frequency filter from 0.01 to 0.05 had a smaller but still consistent effect on LD N_e estimates, decreasing the mean by <20% in all cases (Fig. 6). Relative differences between random subsets were approximately conserved between the two minor allele frequency filters (Fig. 6).

Discussion

Whole-species structure and connectivity

The current genetic analysis supports the Nangur Spiny Skink (*Nangura spinosa*) being recognised as two populations—Nangur National Park (Nangur) and Oakview National Park (Oakview)—with no further subdivisions. The SNP divergence detected between Nangur and Oakview was substantial, supporting the 3.77% ND4 mtDNA and 1.26% 16S mtDNA divergence previously found (Borsboom et al. 2010). The between-population divergence accounted for nearly half of the variation in the species-wide AMOVA (Online Resources 13–14) and contributed to a substantial drop in the Bayesian Information Criterion (Online Resource 10a). Similarly, F_{ST} , Allele Frequency Difference, and genomic distance were all high between Nangur and Oakview. The evidence of strong differentiation between the two populations of *N. spinosa* is not entirely surprising given the populations of *N. spinosa* are currently about 36 km apart and the two patches of semi-evergreen vine thicket were separated by unsuitably dry habitat prior for thousands of years prior to land clearing (Borsboom et al. 2010). Small population sizes, either those recently observed or in earlier bottlenecks, would also likely contribute to present-day genetic differentiation through rapid genetic drift (Weeks et al. 2016).

Much of the genetic differentiation observed in *N. spinosa* was driven by the 86% (28% in Nangur; 58% in Oakview) of SNPs that had private alleles in one of the two populations (Table 1). A similar pattern was seen in a SNP-based study of Northern Bettongs (*Bettongia tropica*): ~40% of SNPs had private alleles in three relatively connected southern populations, compared to only ~10% in an isolated and inbred northern population. This differentiation contributed to an F_{ST} of >0.4 between populations from the two regions which were >80 km apart (Todd et al. 2023). In contrast, in populations of the Western Tiger Snake (*Notechis scutatus occidentalis*) that were recently (<200 years) and partially isolated, only 16% of SNPs had private alleles in only one population (Lettoof et al. 2021). The number of fixed differences between *N. spinosa* populations (1%; Table 1) is quite large relative to other species. For instance, <0.20% of SNPs exhibited fixed differences between the aforementioned bettong populations (Todd et al. 2023).

Within-population structuring and connectivity

Despite extensive sampling in each of Nangur and Oakview, and the ability of SNPs to detect fine-scale patterns of population structure, there was no evidence for sub-units within

either population, suggesting near-panmixia within each until very recently. K-means clustering provided no evidence to support any more than one genetic cluster in either Nangur or Oakview (Online Resource 10). Population sub-structure accounted for negligible genetic variance in the Oakview-only AMOVA regardless of the sub-units defined (Online Resources 19–22). For the Nangur population, sub-structure accounted for up to only 10% of the genetic variation (Online Resources 15–18), and this was likely due to the eastern-most cluster consisting entirely of a single closely-related family group. Despite clear sub-networks observed in *netviewr* plots, these sub-networks became connected to each other at very low k-NN values (i.e., low compared to the number of individuals in those sub-networks; Fig. 2). This suggests that each of the populations have been able to maintain within-population gene flow throughout their range at least to the current generation.

Connectivity within each population is likely driven by the small area occupied by each population, particularly at Nangur, and by regular small movements and occasional longer distance dispersal of individuals. Most individuals in first-degree relationships were typically found <40 m away from one another. This included closely related adults living in close proximity, but also several instances of females with juvenile offspring in the same burrow or in adjacent burrows (with unrelated adults sometimes in the burrow with the juveniles). Despite this, some first-degree relationship pairs were up to 200–300 m apart in each population (Fig. 3). The average radius of a genetic neighbourhood for *N. spinosa* was estimated to be ~1000 m, which implies that the standard deviation of parent-offspring dispersal distance is only ~500 m. It is likely that only a few individuals move longer distances in each generation, but this would be enough to maintain evidence of connectivity. Low dispersal distances are also seen in other Australian skinks. Prickly Forest Skinks (*Gnypetoscincus queenslandiae*), related to *N. spinosa*, were estimated to disperse 843 m²/generation using a mark-recapture method, or 404 m²/generation when estimated from genetic neighbourhood size data (Sumner et al. 2001). Similarly, in other skink studies, White's Skinks were found to have positive spatial genetic structure only up to 100 m in juveniles and 50 m in adults (Chapple and Keogh 2005) and Guthega Skinks were not found to move between colonies, even those that were 300 m away (Atkins et al. 2020). Limited dispersal is likely to be an increasing problem for the Nangur population, where the density of individuals is low, and there are few unrelated individuals to breed with. For example, the eastern-most individuals in Nangur consisted of only two adults and their offspring but were situated >800 m away from all other individuals (Fig. 5).

Genetic health metrics

Effective population size

The genetic health of *N. spinosa* is extremely poor owing to low genetic diversity, high levels of inbreeding, and small effective population sizes (N_e) in both populations. The estimates of N_e are particularly concerning. The very low N_e estimates for Nangur (range in means across analyses: 16–43; Fig. 6) reflect the very low census estimates of 90 adults in 2015 and 44 adults in 2021 (D. J. Ferguson et al., unpub. data). However, the N_e estimates for Oakview (range in means across analyses: 67–180; Fig. 6) are much lower than expected from census estimates of ‘hundreds, potentially up to 1000’ from surveys in 2000–2010 (Borsboom et al. 2010) and 2020–2021 (D. J. Ferguson et al., unpub. data). In Nangur, all N_e estimates and associated confidence intervals (Fig. 6) are below the recommended threshold of $N_e = 100$, an N_e at which an isolated population will experience severe genetic erosion in just a few generations (Frankham et al. 2014). Oakview is not much better, with N_e estimates generally being close to 100 and no confidence intervals reaching higher than 310 (Fig. 6). A small N_e contributes to a faster loss of genetic health (Waples et al. 2013; Waples 2022).

Interestingly, based on these estimates, the ratio of N_e/N_c is quite high at Nangur (i.e., between ~ 0.37 and ~ 0.98), compared to the expected 0.11 (Frankham et al. 2014). The ratio for Oakview is harder to assess because the census estimates are less accurate. Using a census estimate of 1000 gives a ratio between ~ 0.067 and ~ 0.180 . These ratios are closer to the expected 0.11. This ratio represents the relationship between the actual number of potentially breeding adults in the population (N_c) and the rate of genetic diversity loss (N_e). The ratio is influenced by the distribution of reproductive success across individuals, which is linked to demographic factors such as fluctuations in population size and life history traits such as relative age at maturity rather than solely representing the raw number of adults that contribute to reproduction (Lee et al. 2011; Waples et al. 2013, 2022). In addition, genetic N_e estimates represent a generation previous to the one being sampled (Waples 2022). In the case of Nangur, the estimates are reflecting a previously larger population size. Nangur has experienced recent declines, and as recently as 2015 had at least 90 individuals (more than double the population sizes in 2021; D. J. Ferguson et al., unpub. data). At the 2015 census size, the N_e/N_c ratio is closer to expected (0.18–0.48). Alternatively, high N_e/N_c ratios have also been seen in other species with small population sizes including in other threatened reptiles (e.g., Bradke et al. 2018) and amphibians (e.g., Bertola et al. 2023), where

the high ratio has been attributed to ‘genetic compensation’: a process whereby a small population size leads to more equal reproductive success between individuals (i.e., a reduction in variance; Ardren & Kupiscinski, 2003).

Population-wide genetic diversity

The levels of genetic diversity seen in *N. spinosa* are very low and comparable to other small, isolated populations of threatened Australian skinks (Table 1). For instance, the range of expected and observed heterozygosities reported in this study overlaps with that of the Endangered Alpine She-oak Skink (*Cyclodomorphus praelatus*; $H_{obs} = 0.118$ – 0.134 , $H_{exp} = 0.132$ – 0.159 , $N_e = 11.5$ – 203.3 ; Hartley et al. 2023) and Endangered Swamp Skink (*Lissolepis coventryi*; $H_{obs} = 0.10$ – 0.11 , $H_{exp} = 0.12$, $N_e = 10.6$ – 53.5 ; Senior et al. 2022), but is lower than that of the Endangered Guthega Skink (*Lio-pholis guthega*; $H_{obs} = 0.205$ – 0.255 , $H_{exp} = 0.174$ – 0.276 , $N_e = 9.4$ – 93.9 ; Atkins et al. 2020). Further, as a comparison, the estimates for both *N. spinosa* populations are substantially lower than for most populations of the non-threatened Cunningham’s Skink (*Egernia cunninhami*; $H_{obs} = 0.042$ – 0.192 , $H_{exp} = 0.056$ – 0.201 , N_e unknown; Ofori et al. 2017).

Including invariant sites in the calculation of heterozygosity to produce a ‘genome-wide’ (vs. ‘SNP’) estimate has been suggested to make inter-study comparisons of heterozygosity more robust (Schmidt et al. 2021; Sopniewski and Catullo 2024). Although few reptile studies are recent enough to have considered this recommendation, Hartley et al. (2023) reports ranges of observed (0.00055–0.00095) and expected (0.00060–0.00121) genome-wide heterozygosities in Alpine She-oak Skinks, which are much higher than seen in *N. spinosa* (Table 1). The low genetic diversity in *N. spinosa* is likely to reduce the adaptability and resilience of both populations, but particularly in Nangur. There was a measured decrease in heterozygosity (–20%), polymorphic SNPs (–43%), and private alleles (–53%) in Nangur compared to Oakview (Table 1). This difference is much worse than the suggested 10% decrease threshold for ‘tolerable genetic erosion’ outlined by Frankham et al. (2017).

Individual genetic diversity and inbreeding

Across *N. spinosa*, individual multiple locus heterozygosity (MLH) ranged from 0.157 to almost half of that (0.081), suggesting extreme levels of inbreeding (Fig. 4). As a measure of inbreeding, individual MLH has been shown to predict both true genomic inbreeding (Kardos et al. 2015) and differences in individual total fitness (Huisman et al. 2016; Harrison et al. 2019). In Helmeted Honeyeaters (*Lichenostomus melanops cassidix*), a 28% decrease in individual MLH resulted in up to a 90% reduction in individual total

fitness (i.e., a reduction in the total number of reproductive offspring; Harrison et al., 2019), while in Red Deer (*Cervus elephus*) a 22% decrease in MLH resulted in a 72% reduction of female total fitness and a 95% reduction in males (Huisman et al. 2016). Furthermore, the largest absolute value of MLH reported for *N. spinosa* is approximately 10% lower than the lowest value seen in the Helmeted Honeyeater (Fig. 4). This, along with high values of individual inbreeding coefficient (F), is suggestive of extreme levels of inbreeding that is likely accompanied by inbreeding depression.

Almost three-quarters of the Nangur samples have an estimated F greater than expected for offspring of non-inbred individuals in a first-degree relationship ($F=0.25$; Fig. 4; Table 1). This amount of inbreeding was enough for inbreeding depression to reduce total fitness (i.e., number of mature offspring produced over an individual's lifetime) by over 90% in a mammal (Huisman et al. 2016), a bird (Szulkin et al. 2007), and a plant (Newman and Pilson 1997). Some individuals in Nangur are even approaching $F=0.5$, equivalent to that of the offspring of mating clones (Fig. 4). When F is estimated based on an already-inbred reference individual, it is likely to be biased downwards from the true estimate (Crow and Kimura 1970; Wang 2014). In this case, the reference population used for calculating F (i.e., Oakview) is already small, has experienced several recent reductions in population size (logging, invasive plants, retractions in range; Chapple 2019), and as reported here, has low genetic diversity with small-moderate effective population sizes (Table 1; Fig. 6). Hence, the true level of inbreeding for *N. spinosa* in both populations is likely to be much higher than is estimated.

There was no decrease in individual heterozygosity apparent when comparing individuals between the two sampled time periods (2006–2007 vs. 2020–2021) in each population (Fig. 4). This suggests that low genetic diversity and high inbreeding have been affecting the species for at least a decade. Generation time could be as low as two years (the youngest age at which captive *N. spinosa* have been observed to reach sexual maturity) but is more likely three to four years in the wild (D. J. Ferguson, unpub. data.). However, *N. spinosa* individuals can live longer than a decade, and juvenile recruitment at Nangur has been limited during this time period (Borsboom et al. 2010; D. J. Ferguson, unpub. data), so this result may reflect the difference across only a few generations. The small N_e observed in both populations (discussed below; Fig. 6) indicates that both populations were experiencing increased genetic drift, leading to continued losses of genetic diversity and increases in inbreeding, at least one generation prior to sampling (Waples 2022). It is important to note that even if a population has been small with low genetic health for several generations, it is

still likely to be affected by inbreeding depression and have a reduced capacity for adaptation, both of which increase extinction risk (Ralls et al. 2020).

Genomic distances between individuals also suggest a high level of inbreeding. The maximum genomic distance reported between any two individuals in Nangur was no greater than the maximum genomic distance between individuals that *Colony* identified as having a second-degree relationship in Oakview (Fig. 3). This indicates 'pedigree collapse' where individuals have fewer ancestors than is normally expected due to inbreeding (e.g., they should have four grandparents, but they only have two or three). The mean genomic distance in Nangur is also lower than the lowest genomic distance between mates reported in an inbred population of Helmeted Honeyeaters, the pairing of which experienced severe fitness losses compared to less genetically similar pairings (Harrison et al., 2019). Inbreeding depression and low genetic diversity, driven by small effective population sizes and matings between genetically similar individuals, are likely to be major contributors to the declining population in at least Nangur, and possibly also Oakview. The advanced state of genetic issues in both populations means that fitness impacts should be assumed and management actions should be based around slowing and reversing these issues (Bail et al. 2026).

Genetic health of the captive population

A trial captive population has been in place since 2020, founded from 14 individuals from Oakview, and 9 wild-conceived juveniles that were subsequently born in captivity. The captive population had moderate success in capturing the diversity present in the Oakview population sample, with 71% of the polymorphic loci found in Oakview, and mid-range inbreeding across individuals and their offspring. One individual in the captive population is extremely inbred (Fig. 4) and should only be bred with careful consideration of partner. If the trial is to continue as an insurance population, then 95% of the genetic diversity of Oakview will need to be captured, requiring the addition of at least another 26 individuals (to reach 40 individuals) from the wild based on the current sample (Online Resource 31; Weeks et al. 2011). Furthermore, the effective population size will need to be increased as rapidly as possible so that this diversity is maintained (Weeks et al. 2011), which may be difficult considering the low reproductive rates of *N. spinosa* (EPBC Act 1999). Obtaining individuals from different parts of the Oakview population, targeting the sampling clusters evident in Fig. 2, and then using genetic data to strategically select individuals to pair for breeding, will make it possible to optimise the maintenance of diversity in the captive population. However, removing individuals from the wild needs to

be balanced against the potential impact on the small wild population.

Management implications and recommendations for the wild populations

The genetic health assessment of *N. spinosa* found low genetic diversity (Table 1; Fig. 4), high inbreeding (Fig. 4), small effective population sizes (Fig. 6), and high relatedness between individuals (Figs. 3 and 5). In Nangur, these issues are at a critical point and are likely having a severe and direct impact on individual fitness, population growth, resilience, and adaptability. Without immediate interventions, it is likely that the Nangur population will go extinct. The Oakview population has relatively better genetic health than Nangur but the metrics are still poor, and no doubt declining towards the Nangur situation.

Based on the genetic health assessment presented here, and with the goal of avoiding extinction of the species as a whole, the main recommendations for the management of the Critically Endangered *N. spinosa* are:

Recommendation 1: recognise that poor genetic health makes the species more vulnerable to all other threats. This is the case for both populations, and critical for the Nangur population. Alongside the genetic management outlined in recommendations 2 and 3 (below), the traditional conservation measures to slow, stabilise, or reverse population decline must remain a priority. Protected area managers in the Queensland Parks and Wildlife Service have already been implementing key actions, including continued habitat management to mitigate the impact of invasive plants and animals, to exclude fire, and to minimise human disturbance (e.g., interference by wildlife enthusiasts or poaching). Efforts to reduce the impacts from predators (e.g., cats, foxes, pigs, and cane toads) and other invasive species (e.g., cattle, deer, and ecosystem-altering weeds) are essential. Incorporating genetic metrics into these management strategies can enhance their effectiveness by informing priorities and ensuring that neither conservation actions nor existing threats impede skink gene flow. Regular census and threat monitoring via low-impact visual counts of occupied burrows is essential for both populations for tracking population trends, assessing the success of threat management, and enabling timely adaptive management (Chapple 2019; EPBC Act 1999). Regular monitoring efforts also help identify critical points for urgent management interventions.

Recommendation 2: regularly move some individuals between clusters within each population to manage inbreeding and slow genetic health decline. In situations of poor genetic health, traditional management strategies are unlikely to prevent extinction unless genetic management strategies are also implemented (Hufbauer et al. 2015;

Trask et al. 2019). In the short term, inbreeding in both populations needs to be actively managed, which is best done through minimising mean kinship between breeding pairs (Frankham et al. 2019). Kinship is not an advisable metric to use for *N. spinosa*, because the populations are so inbred (Wang 2014), but genomic distance has been shown to be a suitable proxy (e.g., Harrisson et al. 2019). If individuals can be identified and matched to their genetic data, and matings can be controlled (e.g., in a captive setting), then it is possible to optimise matings to slow the loss of heterozygosity. But in situations where it is not possible to match wild individuals to genetic data, as is the case here, the best approach is to move individuals away from close relatives into other clusters of individuals.

Based on the dispersal patterns reported here, it is recommended that individuals within each population are moved from one geographic cluster to another cluster approximately 1000 m away (where possible), to avoid breeding with closely related individuals (which are typically found <500 m away from one another; Fig. 3), and to escape the local genetic neighbourhood. The obvious question is ‘how many and how often?’ and a coarse answer is small numbers each year (Frankham et al. 2017). Additional factors that will impact this strategy include the sex ratio at each site, available habitat, and a long-term plan for avoiding inbreeding with future movements. An ideal method for this is a rotational scheme (Princée, 1955, as cited in Frankham et al. 2017), where three or four geographic ‘clusters’ are identified in each of Nangur and Oakview and a few individuals are moved in rotation among these clusters each generation. *Nangura spinosa* lives in burrows and this movement could be burrow-to-burrow because the skinks will use vacated burrows (D. J. Ferguson et al., unpub. data). The large range of inbreeding values in both populations mean that this strategy could make a substantial improvement to the severity of inbreeding (Fig. 4). However, even if breeding was optimised, further genetic erosion is inevitable, particularly as inbreeding is high, number of potential pairs is very small (especially at Nangur), and the number of genuinely unrelated individuals is zero. For this reason, within-population genetic management is an important, but short-term, solution.

Recommendation 3: genetic rescue is essential for the short- and long-term improvement of genetic health in both populations. Here, genetic rescue refers to the benefits to fitness, population growth, and adaptability resulting from translocation of genetic diversity between Nangur and Oakview. This could be achieved by moving individuals between the populations (i.e., ‘augmented gene flow’), or by crossing individuals in captivity and then introducing the progeny from these to both populations. We recommend the latter here (following trials, see below) because you can

ensure mating occurs, you can genetically test the progeny to ensure they are crosses (as opposed to females using stored sperm), and the viability of crosses can be assessed in terms of survival and growth to adulthood and subsequent backcrossing in captivity. Genetic rescues have been shown to have large and reliable benefits to inbred populations, across a variety of animal and plant taxa including reptiles such as the Swedish Adder (Madsen et al. 2004) and Swedish Sand Lizards (Lindsay et al. 2020; Frankham 2015). In typical conservation settings where populations have been isolated for <500 years, outbreeding depression is not likely to be an important risk to genetic rescue (Frankham et al. 2011; Ralls et al. 2018). However, outbreeding depression remains a consideration in the risk assessment for *N. spinosa* because the length of isolation between the two populations was estimated to be at least hundreds of thousands of years based on mtDNA divergence (Borsboom et al. 2010). This divergence time is likely to be an overestimate because measures of genetic differentiation between small populations can be inflated by loss of genetic diversity, as opposed to genuine divergent evolution over long periods of time, particularly when based on mtDNA (Rubinoff et al. 2006; Weeks et al. 2016). The current divergence estimates should not be used as a reason to avoid trialling crosses between the two populations. Genetic rescues have resulted from successful crosses between lineages that diverged 10,000 years ago (Wells et al. 2019), 20,000 years ago (Weeks et al. 2017), and even 50,000 years ago (Pavlova et al. 2023), and the amount of mtDNA divergence between Nangur and Oakview remains within the range where there is likely no or limited postzygotic isolation (Malone & Fontenot, 2008; Singhal et al. 2018; Coughlan and Matute 2020).

Balancing the very poor genetic health at Nangur (and hence the urgency of action) against the risk of outbreeding depression, we recommend trialling crosses of a small number of individuals, in an experimental way that best quantifies fitness outcomes (e.g., Osborn and Norman 1991; Hoskin et al. 2005; Pavlova et al. 2023). These initial trials should take place at a new site separate to the existing populations or in the established captive facilities so that they can be closely monitored. The trials would involve multiple reciprocal crosses (and controls) and collecting phenotypic and genetic data for offspring for multiple generations. Importantly, if between-population crosses are not considered at all then the risk of doing nothing is likely to be extinction—almost certainly for the Nangur population and possibly for the species as a whole. Hence, risk assessments need to weigh both the risks of outbreeding depression and the benefits of genetic rescue (Ralls et al. 2018; Liddell et al. 2021). Even in the presence of outbreeding depression, the benefits of improved genetic health may ultimately be worth

a temporary loss of fitness in the first-generation crosses (Hwang et al. 2011; Frankham et al. 2017). If crosses were to be successful, the benefits of genetic rescue to both populations could be large (Grueber et al., 2017), and likely in proportion to the amount of inbreeding that is reversed (Frankham et al. 2017). The major advantage of having two remaining populations, each with unique genetic variation, needs to be leveraged urgently to improve the prospects for the species.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s10592-026-01826-2>.

Acknowledgements We acknowledge the First Nations and traditional custodians of the lands on which this species lives: the Kabi Kabi (Oakview National Park) and Wakka Wakka peoples (Nangur National Park); as well as the First Nations and traditional custodians of where this work took place in Thul Garrie Waja and Gurambilbarra (Townsville): the Bindal and Wulgurukaba peoples, and in Meanjin (Brisbane): the Jagera and Turrbal peoples. We thank Lorenzo Bertola for assistance with laboratory methods and analysis, including running Stacks. We thank the following people for assistance with field surveys, data collection, and obtaining samples in the field: DETSI staff Lisa Owen, Adrian Borsboom, Melanie Venz, Michael Mathieson, Kristie Dejong, Stephanie Shaw, and Queensland Museum staff: Patrick Couper and Andrew Amey. We thank Manda Page, John Hodgson, and Luke Geelen, Threatened Species Operations (Queensland Government) for organising funding for sequencing. We would like to acknowledge the Gympie and Kingaroy Queensland Parks and Wildlife Service for their ongoing support of the Nangur Spiny Skink recovery program and in-situ threat management. We thank the team at the Australian Genomics Research Facility.

Author contributions Conceptualisation, CJH & NJB; Study Design, all authors; Field Data Collection, DF; Laboratory Methods, NJB; Analysis, NJB, MH, CJH; Original Draft, NJB; Review & Editing, all authors; Funding Acquisition, CJH & DF; Supervision, CJH & MH.

Funding Open Access funding enabled and organized by CAUL and its Member Institutions. This research was funded by Wildlife & Threatened Species Operations, Queensland Department of Environment, Tourism, Science, and Innovation, and The Foundation for Australia's Most Endangered Species. Support for NJB during the analysis and writing of this work was provided by the Commonwealth through an Australian Government Research Training Program Scholarship [DOI: <https://doi.org/10.82133/C42F-K220>] at James Cook University.

Data availability The demultiplexed raw sequence data, with meta-data, are available on the NCBI sequence read archive (BioProject Accession: PRJNA1470287) and the filtered SNP datasets are available on Fig-Share (<https://doi.org/10.6084/m9.figshare.32312667>).

Code availability A summary of all scripts (Online Resource 32 and 33) that were used for the analysis is provided as supplementary information. The scripts and all data supporting the analysis is available through Fig-Share (<https://doi.org/10.6084/m9.figshare.32312667>).

Declarations

Competing interests The authors declare no competing interests.

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