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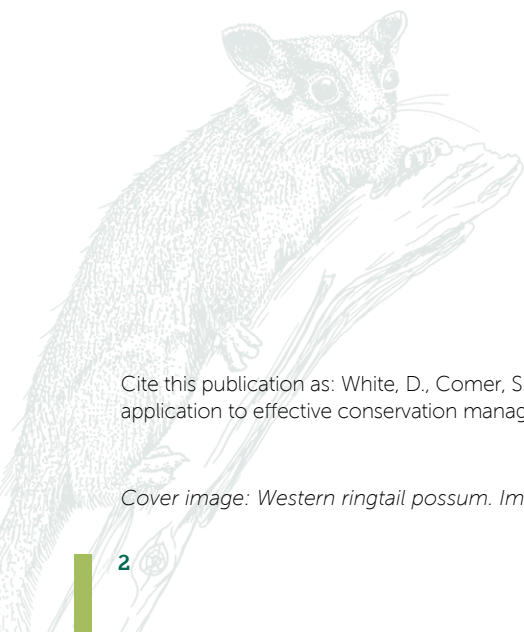


Understanding genomic variation in the western ringtail possum and its application to effective conservation management

Final Report

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Contents

Executive summary.....	4
Introduction	6
Current management	6
Context	9
Methodology	10
Samples	10
Sequencing	10
Data filtering	10
Population genetic analyses.....	11
Isolation by distance.....	11
Spatial clustering.....	12
Population viability analyses	12
Findings	14
Genetic Diversity	14
Structure analysis.....	17
Isolation by distance.....	20
Spatial clustering.....	21
Population viability analyses	23
Discussion	25
Genetic diversity	25
Genetic Clusters.....	25
Spatial clustering.....	26
Population viability analysis	26
Application of research	27
Impact of the research	27
Future research priorities.....	28
Data sets	28
Recommendations	29
Conclusion.....	29
Acknowledgements.....	29
Ethics statement.....	29
References	30

Executive summary

The western ringtail possum (*Pseudocheirus occidentalis* or ngwayir, pronounced n-wa-yer) is a folivorous, arboreal and nocturnal marsupial endemic to the south-west of Australia. Its preferred habitat provides high nutrient foliage and has canopy continuity for evading predation and dispersal (de Tores 2008). Across its range, ngwayir can be found in woodlands of peppermint (*Agonis flexuosa*), jarrah (*Eucalyptus marginata*), marri (*Corymbia calophylla*) as well as karri (*Eucalyptus diversicolor*) forests, Allocasuarina woodlands and Bullich (*Eucalyptus megacarpa*) dominated riparian zones.

Before European colonisation, the distribution of ngwayir stretched continuously from north of Perth in Australia's south west to east of Albany, and as far inland as the towns of Pingelly and Borden (de Tores 2008), with total numbers likely reaching into the hundreds of thousands. Since European arrival their range has contracted, and distribution has become increasingly patchy largely due to habitat reduction and fragmentation as a direct result of human development. It is likely that current total census size is around 20,000 animals (Teale and Potts 2020), which is several orders of magnitude lower than pre-colonisation times and contained in often isolated populations distributed between remnant patchy habitat. As a result of the recent large population declines, and the prediction that populations are continuing to decline because of habitat loss due to continued anthropogenic activity (Burbridge and Zichy-Woinarski 2017), the conservation status of ngwayir has recently been increased to Critically Endangered by the International Union for Conservation of Nature (IUCN) (March, 2014) and the Environment Protection and Biodiversity Conservation (EPBC) Act (May, 2018).

To help manage the species, three (geographic) management zones have been established: (1) the South Coast, ranging from as far west as Walpole and east to Mount Manypeaks east of Albany; (2) the Southern Forest, including the forests and woodlands from Pemberton north to Palgarup and the expansive forest blocks east of Manjimup in the Upper Warren; and (3) the Swan Coastal Plain, including the southern Swan Coastal Plain, from Dawesville to Dunsborough, and the Capes area from Dunsborough south to Augusta. Isolated scattered remnants also persist inland of the west coast between Harvey, Collie, Nannup and Bridgetown (Department of Parks and Wildlife 2017). Since 1991, many translocations of displaced or rehabilitated ngwayir to suitable sites have been attempted, mostly from areas in and around Busselton, however populations persist at just three locations – Karakamia Sanctuary, Perup Sanctuary and Yalgorup National Park. In response to the predicted trajectory of ngwayir, in 2017 the Western Australia Department of Parks and Wildlife released the Western Ringtail Possum Recovery Plan, designed to guide the recovery of ngwayir over the next 10 years. In it, knowledge gaps are identified, and five 10-year recovery objectives made. However, there is also a paucity of published information on remnant genetic diversity within the species. Genetic diversity is important for threatened species as high levels are associated with greater fitness and adaptive potential of populations (Frankham 2005), and its documentation is the first step to prevent irreversible loss. Small, isolated populations are particularly susceptible to loss of genetic variation by genetic drift, often suffer inbreeding depression and may need to be 'rescued' using genetically diverse populations (Akeasson et al. 2016; Frankham 1998; Frankham 2015; Weeks et al. 2017).

Here, we have recovered 23,146 single nucleotide polymorphisms (SNPs) from 305 samples and 52 locations, obtained from historical collections and opportunistic means, to describe genetic variation across the ngwayir range so the number of genetically distinct populations can be determined, diversity within populations and locations quantified and genetic distance between populations revealed. We go on to associate genetically distinct subregions with bioclimatic variables to increase our understanding of environmental conditions across the ngwayir range. Finally, to explore further potential associations and future trajectories of populations from the Southern Forest that have undergone dramatic population crashes, population viability analyses focused on the Upper Warren catchment in the Southern Forest management zone were developed. Compared to other marsupials whose genetic diversity has been assessed with comparable numbers of genome-wide SNPs and samples, the genetic diversity of ngwayir is low. The highest diversities were found in populations of the Southern Forest management zone, the lowest were found in the Swan Coastal Plain and two sampling locations, both urban, showed strong evidence of inbreeding – Manjimup and Albany. Novel allelic diversity was revealed in three previously undocumented locations – Molloy Island, Pemberton and Two People's Bay.

Genetic insights from this study are broadly consistent with the separation of western ringtail possums into three management zones – Swan Coastal Plain, Southern Forest and South Coast – in agreement with the limited genetic work to date (Wilson 2009). However, our comprehensive sampling has allowed us to extend our understanding suggesting there is a total of eight subregions within the three zones, and Pemberton may contribute to a ninth. The Two People's Bay and Lower Blackwood River Catchment subregions show relatively high drift and allelic divergence from other clusters as well as high numbers of private alleles, indicating that both these subregions have high genetic and conservation value. We were not able to identify more than one operational taxonomic unit (OTU) within ngwayir but results for two subregions with restrictively low sample sizes indicated separate OTU status which could be confirmed with more samples. It is likely that most clusters can be explained by non-continuous sampling across the landscape, either because of limitations of our sampling strategy or because of the fragmented nature of the WRP populations. Spatial clustering analysis showed that assignment to a genetic cluster can be predicted by environmental predictors and that the three most informative variables (in order) are mean summer rainfall, mean summer temperature and areal actual evapotranspiration rate in February. Population viability analysis (PVA) modelling shows that mortality rates in the Upper Warren need to be less than the only empirically derived estimate from that region for survival probabilities to remain above 0.9 (Wayne et al. 2005), and ongoing/increased fox and cat control is a prerequisite for long-term survival. Modelling indicates that population viability is particularly sensitive to disturbance events such as fire and drought as a one in ten-year mild event reduced population survival probability by 69% (1.00 to 0.31) over 100 years. It is therefore critical to quantify the demographic impact of catastrophic events such as drought and fire, which will require a concerted strategic monitoring effort.

In summary, we recommend that

- There is recognition of at least eight distinct genetic subregions within ngwayir and that they are considered as 'management units' in all conservation and recovery planning
- The Swan Coastal Plain management zone is re-named to West Coast
- There is an immediate species-wide concerted effort to gather empirical life history data, in particular mortality rates, population census sizes and the impact on population dynamics of environmental stochastic events including fire and drought
- Ongoing genetic monitoring be undertaken to track potential future declines in variation (e.g., Upper Warren) and signs of inbreeding depression (e.g., Manjimup and urban Albany)
- Efforts should be made to prevent substantial and irreversible loss of the high levels of genetic diversity observed in the Upper Warren in this study. This is highly likely given the indications of ongoing demographic decline and the PVA modelling which suggests that the population may be at high risk of extinction
- Efforts should be made to prevent further loss of genetic diversity in the Southern Swan Coastal Plain subregion by informed mixing between locations and supplementation from other genetic clusters
- Before translocation of possums between genetic clusters consideration should be made of cluster assignment, genetic diversity and associated environmental variable predictors
- Increased sampling and further OTU analysis are done on the Lower Blackwood River and Two People's Bay subregions, and the Pemberton locality



Introduction

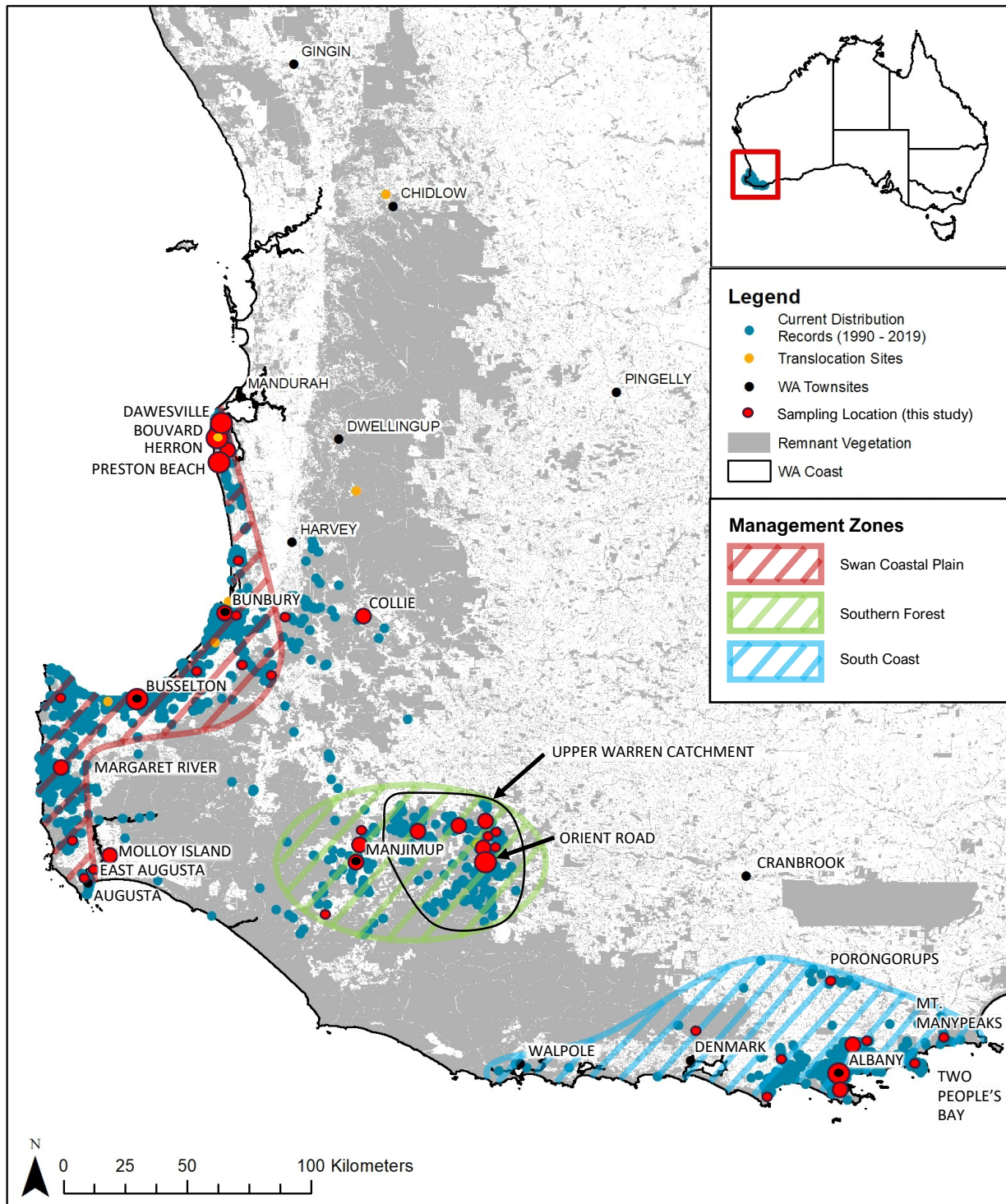
The western ringtail possum (*Pseudocheirus occidentalis* or ngwayir, pronounced n-wa-yer) is a folivorous, arboreal and nocturnal marsupial endemic to the south-west of Australia. Its preferred habitat provides high nutrient foliage and has canopy continuity for evading predation and dispersal (de Tores 2008). Across its range ngwayir can be found in woodlands of peppermint (*Agonis flexuosa*), jarrah (*Eucalyptus marginata*), marri (*Corymbia calophylla*) as well as karri (*Eucalyptus diversicolor*) forests, Allocasuarina woodlands and Bullich (*Eucalyptus megacarpa*) dominated riparian zones (Bader et al. 2019; Department of Parks and Wildlife 2017; Jones et al. 1994a).

Before European colonisation, the distribution of ngwayir stretched from north of Perth in Australia's south west to east of Albany, and as far inland as the towns of Pingelly and Borden (de Tores 2008), with total numbers likely reaching into the hundreds of thousands. Since European arrival their range has contracted and become increasingly patchy, largely due to habitat reduction and fragmentation as a direct result of human development (Department of Parks and Wildlife 2017; Jones 2004; Jones et al. 1994a, see Figure 1). This has contributed to large population reductions over the last two centuries. Ngwayir are also threatened by predation from introduced feral foxes (*Vulpes vulpes*) and cats (*Felis catus*), altered fire regimes, logging activity, and a climate that is becoming increasingly hotter and drier (Bureau of Meteorology 2021; Molloy et al. 2014; Wayne et al. 2006). It is believed that only around 10% of the former range is now occupied (Jones 2004; Molloy et al. 2014). While it has been challenging historically to obtain reliable population estimates for ngwayir, due in part to their elusiveness making monitoring estimates difficult, some regional estimates have been made. For example, most populations in the Bunbury to Dunsborough region have been in decline since the early 1990s, and have reduced in numbers by 20 to 80 % since 2005 (Woinarski et al. 2014). 2000 to 5000 animals are believed to currently exist in the region (Department of Parks and Wildlife 2017). A recent WA Roads commissioned monitoring project using line transect distance sampling estimated the abundance for the entire Swan Coastal Plain management zone to be approx. 9,300 individuals (Teale and Potts 2020). In the Kingston area of the Upper Warren there was a drastic reduction in sightings of around 99% in the early 2000s. While a slight increase in their numbers has been recorded since 2012, peaking in 2017 at 7% of their 1998 pre-decline numbers, they have further declined to < 2% of their pre-decline numbers by 2021 (A. Wayne unpublished data). The 2019 estimate for the Upper Warren region was around 8400 animals (Teale and Potts 2020). Approximately 3,300 animals have been estimated in a 30 km radius of Albany (Teale and Potts 2020). Taken together, it is likely that current total census size is around 20,000 animals (Teale and Potts 2020), which is several orders of magnitude lower than pre-colonisation times and contained in often isolated populations distributed between remnant patchy habitat.

Current management

As a result of the recent large population declines, and the prediction that populations are continuing to decline because of habitat loss due to continued anthropogenic activity (Burbridge and Zichy-Woinarski 2017), the conservation status of ngwayir has recently been increased to Critically Endangered by the IUCN (assessed in March, 2014) and the EPBC Act (effective in May, 2018). Three (geographic) main management zones have been established, to help manage the species (Figure 1). These are (1) the South Coast, ranging from as far west as Walpole and east to Mount Manypeaks east of Albany; (2) the Southern Forest, including the forests and woodlands from Pemberton north to Palgarup and the expansive forest blocks east of Manjimup in the Upper Warren; and (3) the Swan Coastal Plain, including the southern Swan Coastal Plain, from Dawesville to Dunsborough, and the Capes area from Dunsborough south to Augusta. Isolated scattered remnants also persist inland of the west coast between Harvey, Collie, Nannup and Bridgetown (Department of Parks and Wildlife 2017). Interestingly, across these management zones, there is variation in the predominant vegetation type associated with possum habitat. For example, Swan Coastal Plain populations are associated with stands of peppermint trees. In the Southern Forest, where peppermint trees are less common, ngwayir are found in jarrah and marri forests in the Upper Warren, wandoo (*Eucalyptus wandoo*) stands to the north-east of Manjimup and karri forest to the west and south of Manjimup. In the South Coast possums are associated with a range of habitats including jarrah, marri, karri, casuarina and peppermint woodlands, myrtaceous heaths and shrublands and Bullich and Taxandria riparian zones (Bader et al. 2019; Department of Parks and Wildlife 2017).

Figure 1. Contemporary distribution of western ringtail possums showing the three management zones, major landmarks, historical translocation sites and sampling locations for this study. Adapted from (Department of Parks and Wildlife 2017).



Since 1991, many translocations of displaced or rehabilitated ngwayir to suitable sites have been attempted, mostly from areas in and around Busselton, with the objective of re-establishing populations outside the current range (Department of Parks and Wildlife 2017). However, not all of these translocation attempts have been successful. Approved translocations include those to Leschenault Peninsula Conservation Park, Yalgorup National Park, Lane Poole Reserve and Keats State Forest Block in Dwellingup, Locke Nature Reserve in Busselton, Karakamia Sanctuary, Gelorup bushland south of Bunbury and Perup Sanctuary in the Tone Perup Nature Reserve east of Manjimup. Of these eight potential sites, populations are known to persist at just three locations - Karakamia Sanctuary, Perup Sanctuary and Yalgorup National Park.

In 2017, the Western Australia Department of Parks and Wildlife released the Western Ringtail Possum Recovery Plan, designed to guide the recovery of the western ringtail possum over the next 10 years (Department of Parks and Wildlife 2017). The 10-year goal is to 'slow the decline in population size, extent and area of occupancy through managing major threatening processes affecting the subpopulations and their habitats and allowing the persistence of the species in each of the identified key management zones: Swan Coastal Plain, southern forests and south coast'. The recovery plan highlights gaps in knowledge important to conservation which include: 1. A lack of information for small, isolated, or remote populations, 2. Robust and comparable survey methods for estimating abundance across various habitats, 3. Co-ordinated program to monitor population trends over time, 4. Causes of population decline, 5. The relative importance of threatening processes, 6. Factors important to persistence in urban environments, 7. Factors required to make translocations successful, and 8. Optimal parameters in habitat restoration. It is hoped addressing these knowledge gaps will help achieve the five 10-year recovery objectives which, in order of precedence, are:

1. Habitat critical for survival for western ringtail possums is identified and protected in each key management zone,
2. Threatening processes that are constraining the recovery of western ringtail possums are mitigated in each key management zone,
3. An evidence-based approach is applied to the management and recovery of western ringtail possums,
4. The management of displaced, orphaned, injured and rehabilitated western ringtail possums aids the conservation outcome for the species,
5. Increased awareness of the status of western ringtail possums and support behaviour change to mitigate anthropogenic threatening processes.

There is also a paucity of published information on remnant genetic diversity within the species. Genetic diversity is important for threatened species as high levels are associated with greater fitness and adaptive potential of populations (Frankham 2005). Where possible, it is important to document all genetic variation within threatened species so that efforts can be made to prevent irreversible loss. Small, isolated populations are particularly susceptible to loss of genetic variation by genetic drift, so their identification is required for appropriate management actions to be put in place, e.g., facilitating connectivity or supplementation programs. Further, small and isolated populations often suffer inbreeding depression and may need to be 'rescued' using genetically diverse populations (Akesson et al. 2016; Frankham 1998; Frankham 2015; Weeks et al. 2017).

In this study we generate genetic data for ngwayir from across their remnant range, including from locations for which little is known such as Collie, East Augusta, Molloy Island, Pemberton and Two People's Bay, as well as the focal management centres, e.g., Busselton, Bunbury, Albany and Manjimup. We assess levels of genetic diversity using thousands of genome-wide markers, identify genetic clusters and estimate the relationships between these clusters. We then associate our genetic clusters with environmental variables so that environmentally distinct regions that sustain unique genetic populations can be identified. In addition, we develop population viability analysis models focused on the populations of the Upper Warren that suffered the largest recently recorded population crashes of 99%, with the objective of predicting future population trajectories as well as identifying key threatening factors. This work will help address knowledge gaps 1, 4, 5 and 7 (see above) and contribute to recovery objectives 2, 3 and 4 by increasing the number of locations for which we have genetic data, identifying populations of high conservation value or priority, and improving our understanding of factors important to survival – all important components of future conservation programs.

Context

Genetics, translocations and population modelling are important aspects to the conservation and recovery of species such as the ngwayir. The value of including genetics in conservation management is increasingly being acknowledged (Ottewell et al. 2014; Pacioni et al. 2019; Tracy et al. 2011; Weeks et al. 2011; White et al. 2020). Maintaining genetically heterozygous populations is important for avoiding any detrimental impacts of inbreeding depression, and populations that have high levels of standing genetic variation may be better equipped to deal with changing environmental conditions (Frankham et al. 2017; Weeks et al. 2011). Small and isolated populations are at particular risk of genetic diversity loss as the impacts of genetic drift are greater, and if immigration is restricted the probability of inbreeding depression is higher – meaning populations can more easily fall into irreversible population decline (Frankham 1998; Frankham 2005). While mixing isolated populations is one approach for increasing genetic variation and heterozygosity, it does not come without risks, not least outbreeding depression. Outbreeding depression can be defined as the progeny from crosses between distinct populations having lower fitness than the parents, which may occur if the parental populations have adapted to distinct local habitats (Edmunds 1999; Marr et al. 2002; Tymchuk et al. 2007).

One of the few genetic studies that have been conducted on ngwayir thus far is a 2009 study that used 14 microsatellite markers and mtDNA cytb sequence to assess population structure and diversity, as well as phylogeographic structure (Wilson 2009). Although sampling was limited to part of the Swan Coastal Plain management zone (no further south than Locke Nature Reserve) and only 21 animals from the Southern Forest management zone, this study revealed two genetically distinct populations in the southern Swan Coastal Plain (Gelorup and Busseton), both separate from the Upper Warren population. Phylogenetic analyses did not detect any structuring, suggesting that phylogenetic divergence has not yet occurred within ngwayir and all variation can be described as one evolutionary significant unit (ESU) (Moritz 1994), although this observation was based on just 391 bp of cytb sequence and a sample set that excluded important parts of the range. Other published work has had a more local focus and has revealed the importance of waterways for maintaining fine-scale structure in ngwayir (Yokochi et al. 2016), and shown the impact that urbanisation has on gene flow and inbreeding, and ultimately genetic diversity (Van Helden 2020). A further project currently being finalised expands on the microsatellite work of Wilson (2009) to include a wider sampling catchment, with a focus on revealing the extent of admixture within the Swan Coastal Plain management zone as well as estimating times of major demographic events (Spencer et al. *in prep.*).

To help understand patterns of genetic variation and drivers of phenotypic variation across landscapes several approaches have been developed. Amongst these are Genotype Environment Association studies, which aim to associate environmental variables with genetic variation (Forester et al. 2018; Schweizer et al. 2016). For example, in Canadian wolves six genetically distinct ecotypes were defined that had strong environmental predictors, particularly daily temperature range and annual rainfall (Schweizer et al. 2016). Considering ngwayir live in regions that vary greatly in rainfall, temperature, and vegetation types (Bureau of Meteorology 2021), and are highly susceptible to temperature extremes especially when leaves have low moisture content (Jones et al. 2004; Yin 2006), such analyses could be particularly relevant for understanding the importance of geographically distinct genetic clusters.

Conservation translocations are increasingly being used in Australia to reintroduce species back to regions from which they have become locally extinct (reintroductions), reinforce at-risk populations (reinforcements), or introduce species to suitable habitat outside indigenous range (introductions or assisted colonisations), all with the common aim of increasing numbers and/or range (Armstrong et al. 2015). However, the success of translocations is not guaranteed, can require substantial logistical support and resources, and they can put further extinction pressure on already at-risk source populations (Fischer and Lindenmayer 2000; Germano and Bishop 2009; Morris et al. 2015; Perez et al. 2012; Easton et al. 2019). Many factors can contribute to the failure of translocations. For example, a study investigating translocation failure of ngwayir to sites in the Swan Coastal Plain showed that non-native predation, inter-species competition with the sympatric common brushtail possum (*Trichosurus vulpecula*) and limited carrying capacity of the target site all contribute to translocated populations failing to establish (Clarke 2011). As conservation resources are finite and many source populations have constricted drastically since pre-European colonisation, it is important to maximise the chances of success of future translocations of ngwayir, which can be achieved by greater understanding of threatening processes.

Population modelling can be a useful tool for conservation management. Modelling allows researchers to change the values of life history parameters and abiotic factors to assess their impact on population trajectories, all *in silico*. Individual-based population viability analysis (PVA) modelling, as run in Vortex software (Lacy 1993; Lacy and Pollak 2017), estimates how factors intrinsic to individuals within populations alter growth rates, birth rates and extinction probabilities. They can be particularly useful when planning translocations as key threatening processes to successful establishment can be identified and managed accordingly, and performance of competing translocation scenarios can be compared (Pacioni et al. 2019; White et al. 2020). While few PVA analyses have been done for ngwayir, an important earlier study focused on the Locke Nature Reserve predicted that fox predation and road mortality have profound impact on persistence of that population, reducing survival by 92% and 68%, respectively (Yokochi 2015).

Methodology

Samples

The target of this study was to sample from across the remnant range spanning a distance of around 650 km. Considering various ngwayir sample collections already existed in the labs and offices of researchers and conservation managers, and the limited amount of project time available, sampling was done according to the following strategy: 1. Contact managers, researchers and museum collections who have or were suspected of having historical sample collections to confirm whether samples exist and are accessible, 2. Assess how well the range is represented by available samples, 3. Approach local DBCA offices, Natural Resource Management groups (NRMs), local conservation groups and rehabilitators to assist with the opportunistic collection of samples to fill 'gaps' in the sampling landscape or augment current representation. Opportunistically attained samples were acquired often through roadkill or by chance encounters with dead animals. In each case the collector would either take a tissue sample at location if resources permitted or freeze bodies for collection and tissue sampling at UWA. Tissue sampling of newly acquired samples involved removing a 5mm² piece of ear tissue and placing into a 1.5ml screw cap vial filled with 1 ml of 100% ethanol. Samples were assigned a project ID number and entered, along with their metadata, into a database. At project completion the database contained records for 515 samples from 57 locations.

Sequencing

A total of 325 samples were selected for processing, including 15 sourced from the Western Australian Museum. DNA was extracted from the majority of tissue samples by Diversity Arrays Technology Ltd., according to company protocol (DArT Pty Ltd, Canberra, Australia). Due to their elevated value, DNA was extracted for 96 samples in the zoology genetics lab at UWA by Helix Molecular Solutions Pty Ltd. These samples were part of historical collections that could not be easily replaced. Extractions were done according to Helix Molecular Solutions Ltd. company protocol using the QIAGEN DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. Samples were eluted into volumes of 30µl and 50µl and quantified using 5µL of extracted DNA on the Qubit 2.0 Fluorometer (Life Technologies, ThermoFisher Scientific, USA) using the Qubit dsDNA Assay Kit as per manufacturer's instruction. Samples were also visualised on 1.5% agarose gels stained with GelRed (Biotium Inc).

Sequencing and SNP genotyping was done on all DNA extractions using DArTseq™ (DArT Pty Ltd, Canberra, Australia). DArTseq™ is a genome complexity reduction technique that relies on restriction enzymes to fragment genomes for sequencing and SNP calling (Kilian et al. 2012; Sansaloni et al. 2011). Restriction enzymes are chosen dependent on the fraction of the genome represented, number of polymorphic loci and average read depth of fragments. The enzymes deemed most appropriate for our species were *PstI* and *NlaIII*. To call SNPs, sequences generated from the genomic fragments were processed using analytical pipelines proprietary to DArT Pty Ltd (but for details see Georges et al. 2018). In total, the DArTseq™ assay developed here, which is now an at-cost service available to the scientific community, generated 23,146 SNP markers from our samples.

Data filtering

Of the 325 samples, 16 failed to generate genotypic data, which included 12 of the 15 museum-sourced samples. A further four samples were duplicates as they were part of assay development. This left 305 samples from 52 locations for this study. Genotype data along with locus and individual metadata were loaded into the dartR package (Gruber et al. 2018) and converted into a genlight data object, run in R version 4.0.3 (R Core Team 2020). Two samples (SCP129_DAWES and SCP126_HERR) were known to be pouch young and were removed from downstream analyses as the inclusion of relatives impacts population genetic analyses, such as overestimates of inbreeding coefficients and extent of genetic clustering. Filtering of data is required to ensure the dataset is of high quality and robust to potential biases. While different analyses require specific filtering parameters, a preliminary set of filtering steps was i. remove all loci that did not provide 100% reproducibility, ii. remove monomorphic loci, iii. remove loci that have more than 10% missing data, iv. keep one locus per sequence tag, v. remove individuals with greater than 20% missing data, vi. re-check that all loci are polymorphic and have no more than 10% missing data, and vii. prune loci that are in linkage disequilibrium in R using SNPRelate v1.24 (Zheng et al. 2012). The number of markers and individuals are dependent on filtering parameters and vary between datasets.

Population genetic analyses

Descriptive genetic diversity statistics were estimated on sample locations that had at least two individuals, and to avoid biases that could result from overrepresentation 20 samples were randomly selected from the 59 Orient Road samples. Unless otherwise stated, the reduced number of Orient Road samples were used in all population genetic analyses. Observed and expected heterozygosities, the number of private alleles per sample and inbreeding coefficients were estimated for all locations, using GenAlEx v 6.51 (Peakall and Smouse 2012; Peakall and Smouse 2006).

To assess structure across all ngwayir, a principal coordinates analysis (PCoA) ordination was done on all locations using the R package adegenet v2.1.3 (Jombart 2008; Jombart and Ahmed 2011). The program Structure v2.3.4 (Pritchard et al. 2000) was also used on all locations after removal of loci whose minor allele frequency was less than 0.01, as rare alleles can lead to biases in structure analysis (Linck and Battey 2019). 'Structure' uses a Bayesian clustering method to assign individuals to one of k populations and to estimate the degree of inter-population admixture. As our system includes samples from regions known to have had admixture in the past, we tested performance of all combinations of demographic parameters and allele models. After preliminary assessment of convergence times for the Monte Carlo Markov chain, a burn-in period of 5,000 steps was chosen, followed by 10,000 steps of the chain (15,000 steps in total). To estimate k , four replicate runs at each value of k from 1 to 10 were performed, and the most likely value was estimated from the plot of $\ln \Pr(X|k)$ vs. k , as well as from Evanno's method (Evanno et al. 2005) which plots Δk (a second order rate of change of $\ln \Pr(X|k)$) versus k . 'Structure' figures were generated using Distruct v1.1 (Rosenberg 2004) in Clumpak (Kopelman et al. 2015). To confirm assignment of locations to genetic clusters, and visualise genetic distance between locations, two neighbor-joining (NJ) distance phenetic trees were created using Euclidean distance calculated from allele frequencies at each locus, one for all locations and one for locations with $n \geq 2$. Based on results from the full dataset which supported definition of $\geq$ one genetic cluster in each of the 3 management zones, a result supported by the PCoA and NJ tree analyses, further structure analysis was conducted on the management zones independently. PCoA analyses were run as before, and 'Structure' was run on all three management zones using a burn-in period of 5000 steps followed by 10,000 steps of the chain, and we again tested performance of all combinations of demographic parameters and allele models.

Combining results from structure analyses and principal coordinates analysis, genetic clusters were defined. The level of differentiation between genetic clusters was determined in R from pairwise F_{ST} s using the StAMPP R package (Pembleton et al. 2013), and Jost's D_{est} in mmod (Winter 2012). Statistical significance of the pairwise F_{ST} s was calculated from 100 bootstraps across all loci. Finally, we ran fixed difference analysis on the genetic clusters to determine the number of operational taxonomic units (OTUs). This type of analysis attempts to amalgamate populations that do not have fixed differences in alleles, so that individuals in an OTU share alleles that are absent in others. This is a strong indication of an absence of gene flow. We ran this analysis and estimated statistical significance of the number of estimated fixed differences between populations in dartR using the `gl.collapse.recursive` and `gl.fixed.difference` functions (Gruber et al. 2018).

Isolation by distance

To determine whether there is association between genetic and geographic distance all samples that had GPS coordinates were included in an isolation-by-distance analysis, using locations that had two or more individuals. Between-population geographic distance was expressed as Euclidian distance, calculated from GPS coordinates using the `dismo` v1.3.3 R package (Hijmans et al. 2020). Between-population genetic distance was calculated as $F_{ST}/1-F_{ST}$ using the StAMPP R package (Pembleton et al. 2013). Statistical significance of the correlation between geographic and genetic distance matrices was calculated by running a mantel test with 999 permutations, using the `vegan` R package (Oksanen et al. 2020).

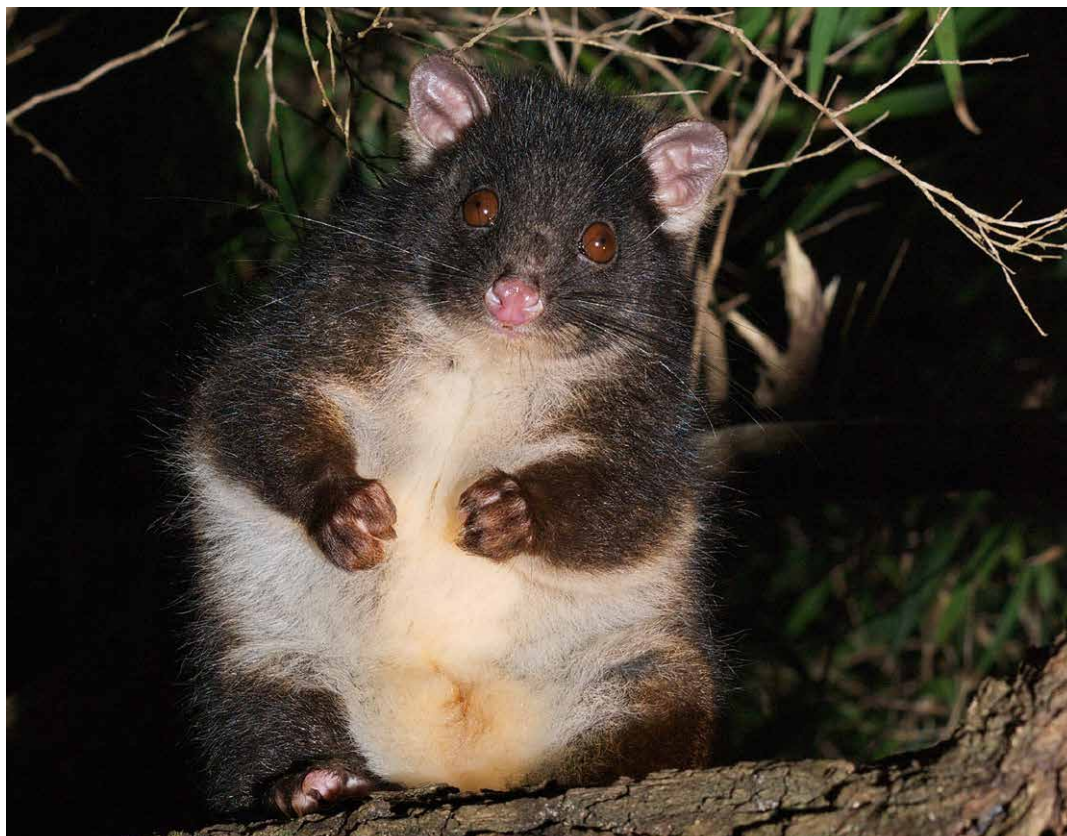


Spatial clustering

We were interested in describing environmental variation across the landscape of the ngwayir range and exploring whether there is any association between environmental variables and genetic clusters. To do this, geospatial data was collected for 34 environmental variables. Only samples with GPS coordinates were used in analysis (n=196). Samples for which GPS coordinates were not consistent with their genetic cluster assignment were removed from analysis as these samples were likely translocated individuals and their inclusion would confound analyses (SCP155 and SCP119). Initially, each of the 34 environmental geospatial datasets were converted to a projected coordinate system (UTM Zone 50S) and generalised into layers of the same extent and resolution as the SRTM derived digital elevation layer (30m resolution). At each coordinate where a possum sample was collected, the value of each environmental layer was extracted and assigned to the corresponding sample. We assessed the collinearity of our geospatial layers using Pearson correlation coefficients and subsequently removed any layers that had a coefficient above 0.90 from the pool. The genetic clusters that were assigned to each individual then formed the response variable while the remaining layers of environmental data formed the predictor variables for supervised classification. The response and predictor data were randomly sampled to generate a training set with 90% of the data and a validation set of 10%. We used the randomForest package (Liaw and Wiener 2002) to fit a classification tree to the training data. We did not limit the node depth of the trees while a total of 500 trees were generated. Lastly, the random forest model was used to predict the expected genetic clusters for the region based on the environmental layers.

Population viability analyses

To explore further potential associations and future trajectories of populations from the Southern Forest that have undergone dramatic population crashes, population viability analyses focused on the Upper Warren catchment in the Southern Forest management zone were developed in Vortex v10.5.0 (Lacy 1993; Lacy and Pollak 2017). Vortex is an individual-based model that uses Monte Carlo simulations to estimate how factors intrinsic to individuals within populations alter growth rates, birth rates and extinction probabilities (Lacy and Pollak 2017). As there is a paucity of empirical monitoring data for ngwayir, we wanted to explore the impact of changing various parameters on population trajectories and survival. With the high level of uncertainty for some parameters it is not possible to achieve robust absolute estimates, but rather our aim is to assess relative importance of factors that have the potential to be managed. To do this we kept baseline parameters constant while changing each test parameter separately, including adult mortality (ranging from 10% to 50%) and the frequency of drought (0%, 10% and 20%). Baseline model parameters are provided in Table 1.



Western ringtail possum in an Australian peppermint tree (*Agonis flexuosa*). Image: Alan Danks

Table 1. Demographic parameters for the Upper Warren catchment of the Southern Forest management zone. Parameter values were obtained from Wayne et al (2005) and Department of Parks and Wildlife (2017) and refined by Adrian Wayne and Barbara Jones.

Parameter	<i>Pseudocheirus occidentalis</i>
Scenario Settings	
Number of iterations	500
Number of years (timesteps)	100
Duration of each year in days	365
Extinction definition	Only 1 sex remains
Modelled population	Upper Warren
Species Description	
Inbreeding depression	n/a
EV correlation between reproduction and survival	0.5
Reproductive Systems	
Reproductive system	Polygynous
Age of first offspring	F:1, M: 1
Maximum age of reproduction	F:5, M:5
Maximum lifespan	6
Broods per year	1
Progeny per brood	1
Sex ratio at birth (% males)	50
Reproductive Rates	
% adult females breeding	85
SD in % breeding due to EV	8.5
No. of broods per year	1
No. of offspring per female per brood	1
Mortality rates	
Mortality of females and males as %	
Mortality from age 0-1 ($\pm$ SD)	30 ($\pm$ 3)
Annual mortality after age 1 ($\pm$ SD)	23 ($\pm$ 2.3)
Catastrophes	
Types of catastrophes	Fire
Frequency and extent of occurrence	
Frequency %	1 in 10 years
Severity (proportion of normal values)	
Reproduction	0.5
Survival	0.7
Mate Monopolisation	
% Males in breeding pool	85
Population Size	
Initial Population Size	8400
Age Distribution	Stable age distribution
Carrying Capacity (K)	150000
SD in K due to EV	15000
Genetics	
Number of neutral loci to be modelled	1000
Loci included in summary statistics	Additional loci only

EV: environmental variation; F: female; M: male.

Findings

Genetic diversity

After quality filtering, removal of duplicated samples, and sub-sampling of the Orient Road (refer to Figure 1) cohort, the dataset consisted of 247 individuals and 5894 loci. This dataset was further reduced depending on the type of analysis. To estimate genetic diversities only locations with a sample size of two or more were used, using a dataset consisting of 222 individuals from 31 locations and 5704 loci. Expected heterozygosities ($\pm$ s.e.) ranged from 0.037 ($\pm$ 0.002) at Albany King River to 0.077 ($\pm$ 0.002) at Orient Road, with a mean across 31 locations ($\pm$ s.d.) of 0.055 ($\pm$ 0.010). Observed heterozygosities ($\pm$ s.e.) ranged from 0.039 ($\pm$ 0.002) at the Porongorups to 0.078 ($\pm$ 0.002) at Yackelup, with a mean of 0.056 ($\pm$ 0.010). Considering only locations that had 5 or more samples, mean expected heterozygosity was 0.061 ($\pm$ 0.010) and mean observed heterozygosity was 0.057 ($\pm$ 0.010), and this difference was found to be statistically significant using a Wilcoxon signed rank test ($V = 118.5$, $p < 0.001$). The eight most genetically diverse locations are all in the Southern Forest management zone. Inbreeding coefficients, F_{IS} ($\pm$ s.e.), for locations that have a sample size of five or greater ranged from -0.037 ($\pm$ 0.004) in Camelar to 0.162 ($\pm$ 0.005) in Manjimup. F_{IS} values can range from -1 to 1 where negative values indicate greater heterozygosity than expected and positive values indicate less than expected, indicative of inbreeding. See Table 2 and Figure 2 for a summary. The number of private alleles per sample is the number of alleles per individual from a given population that are not shared with any other population and reflects connectivity of a location. High values could reflect low connectivity and unique allelic diversity. Values ranged from 0.5 in Albany King River to 18.7 in Two People's Bay (Figure 2b), and there was a step increase to the three highest values in Molloy Island (17.0), Pemberton (17.5) and Two People's Bay. Low values in greater Albany locations are expected due to their geographic proximity and are presumably freely sharing genetic material.



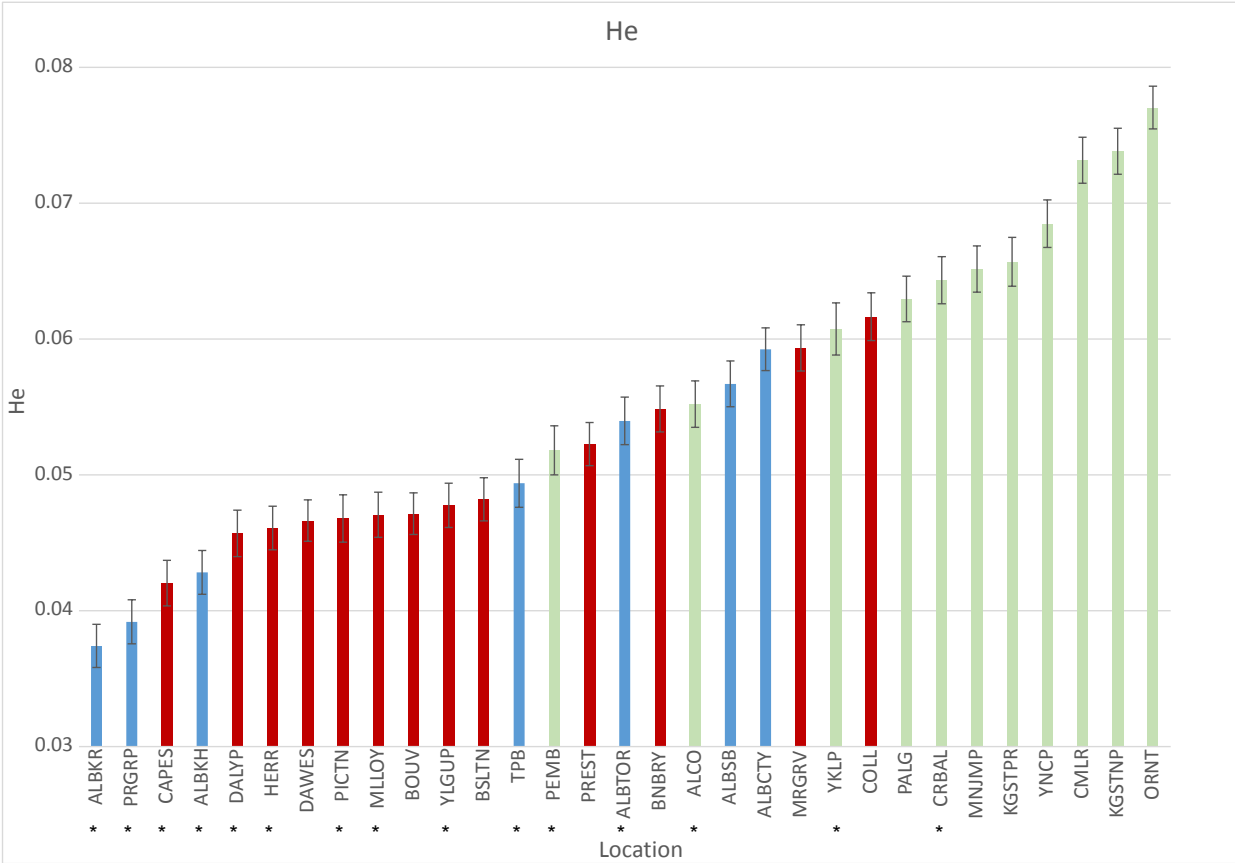
Western ringtail possum in a jarrah tree (*Eucalyptus marginata*). Image: Adrian Wayne

Table 2. Genetic summary statistics based on 5704 polymorphic SNPs.

Site	Abbrev.	Management Zone	N	Sampling Year	Ho ($\pm$ se)	He ($\pm$ se)	F _{IS} ($\pm$ se)
Albany City	ALBCTY	SC	20	2017-2020	0.050 ($\pm$ 0.001)	0.059 ($\pm$ 0.002)	0.118 ($\pm$ 0.004)
Albany Kalgan Heights	ALBKH	SC	3	2017	0.044* ($\pm$ 0.002)	0.043* ($\pm$ 0.002)	-0.072* ($\pm$ 0.006)
Albany King River	ALBKR	SC	2	2017-2018	0.048* ($\pm$ 0.002)	0.037* ($\pm$ 0.002)	-0.287* ($\pm$ 0.007)
Albany Shoal Bay	ALBSB	SC	7	2017-2018	0.054 ($\pm$ 0.002)	0.057 ($\pm$ 0.002)	0.007 ($\pm$ 0.005)
Albany Torbay	ALBTOR	SC	3	2018	0.057* ($\pm$ 0.002)	0.054* ($\pm$ 0.002)	-0.080* ($\pm$ 0.007)
Alco Nature Reserve	ALCO	SF	4	2016	0.055* ($\pm$ 0.002)	0.055* ($\pm$ 0.002)	-0.029* ($\pm$ 0.006)
Bunbury	BNBRY	SCP	5	2020	0.052 ($\pm$ 0.002)	0.055 ($\pm$ 0.002)	0.011 ($\pm$ 0.006)
Bouvard	BOUV	SCP	22	2014-2020	0.044 ($\pm$ 0.002)	0.047 ($\pm$ 0.002)	0.041 ($\pm$ 0.003)
Busselton	BSLTN	SCP	20	2018-2020	0.046 ($\pm$ 0.002)	0.048 ($\pm$ 0.002)	0.040 ($\pm$ 0.003)
Capes nonspecific	CAPEs	SCP	2	2020	0.047* ($\pm$ 0.002)	0.042* ($\pm$ 0.002)	-0.155* ($\pm$ 0.008)
Camelar	CMLR	SF	6	2003	0.074 ($\pm$ 0.002)	0.073 ($\pm$ 0.002)	-0.037 ($\pm$ 0.004)
Collie	COLL	SCP ¹	6	2018-2020	0.060 ($\pm$ 0.002)	0.062 ($\pm$ 0.002)	0.004 ($\pm$ 0.005)
Corbal	CRBAL	SF	4	2003	0.068* ($\pm$ 0.002)	0.064* ($\pm$ 0.002)	-0.075* ($\pm$ 0.005)
Dalyellup	DALYP	SCP	2	2020	0.057* ($\pm$ 0.002)	0.046* ($\pm$ 0.002)	-0.256* ($\pm$ 0.007)
Dawesville	DAWES	SCP	23	2014-2017	0.044 ($\pm$ 0.002)	0.047 ($\pm$ 0.002)	0.041 ($\pm$ 0.003)
Herron	HERR	SCP	4	2014-2017	0.050* ($\pm$ 0.002)	0.046* ($\pm$ 0.002)	-0.097* ($\pm$ 0.005)
Kingston National Park	KGSTNP	SF	7	2012-2017	0.073 ($\pm$ 0.002)	0.074 ($\pm$ 0.002)	-0.007 ($\pm$ 0.004)
Kingston Project	KGSTPR	SF	5	1997	0.062 ($\pm$ 0.002)	0.066 ($\pm$ 0.002)	0.017 ($\pm$ 0.005)
Molloy Island	MLLOY	SCP	4	2020	0.050* ($\pm$ 0.002)	0.047* ($\pm$ 0.002)	-0.076* ($\pm$ 0.006)
Manjimup	MNJMP	SF	8	2009-2020	0.051 ($\pm$ 0.001)	0.065 ($\pm$ 0.002)	0.162 ($\pm$ 0.005)
Margaret River	MRGRV	SCP	7	2019	0.056 ($\pm$ 0.002)	0.059 ($\pm$ 0.002)	0.023 ($\pm$ 0.005)
Orient Road	ORNT	SF	20	2002-2003	0.073 ($\pm$ 0.002)	0.077 ($\pm$ 0.002)	0.035 ($\pm$ 0.003)
Palgarup	PALG	SF	6	2011-2020	0.060 ($\pm$ 0.002)	0.063 ($\pm$ 0.002)	0.017 ($\pm$ 0.005)
Pemberton	PEMB	SF	2	2012	0.063* ($\pm$ 0.002)	0.052* ($\pm$ 0.002)	-0.243* ($\pm$ 0.007)
Picton	PICTN	SCP	2	2018-2020	0.058* ($\pm$ 0.002)	0.047* ($\pm$ 0.002)	-0.259* ($\pm$ 0.007)
Preston Beach	PREST	SCP	15	2016	0.052 ($\pm$ 0.002)	0.052 ($\pm$ 0.002)	0.003 ($\pm$ 0.004)
Porongorups	PRGRP	SC	2	2019-2020	0.038* ($\pm$ 0.002)	0.039* ($\pm$ 0.002)	-0.035* ($\pm$ 0.008)
Two People's Bay	TPB	SC	3	2014-2019	0.045* ($\pm$ 0.002)	0.049* ($\pm$ 0.002)	0.038* ($\pm$ 0.008)
Yackelup	YKLP	SF	2	2003	0.078* ($\pm$ 0.003)	0.061* ($\pm$ 0.002)	-0.296* ($\pm$ 0.006)
Yallingup	YLGUP	SCP	4	2019	0.050* ($\pm$ 0.002)	0.048* ($\pm$ 0.002)	-0.069* ($\pm$ 0.006)
Yandicup	YNCP	SF	5	2003	0.065 ($\pm$ 0.002)	0.068 ($\pm$ 0.002)	0.007 ($\pm$ 0.005)
Σ	31		222	Mean (for n $\geq$ 5 sites)	0.057	0.061	0.030
				sd	0.010	0.010	0.048

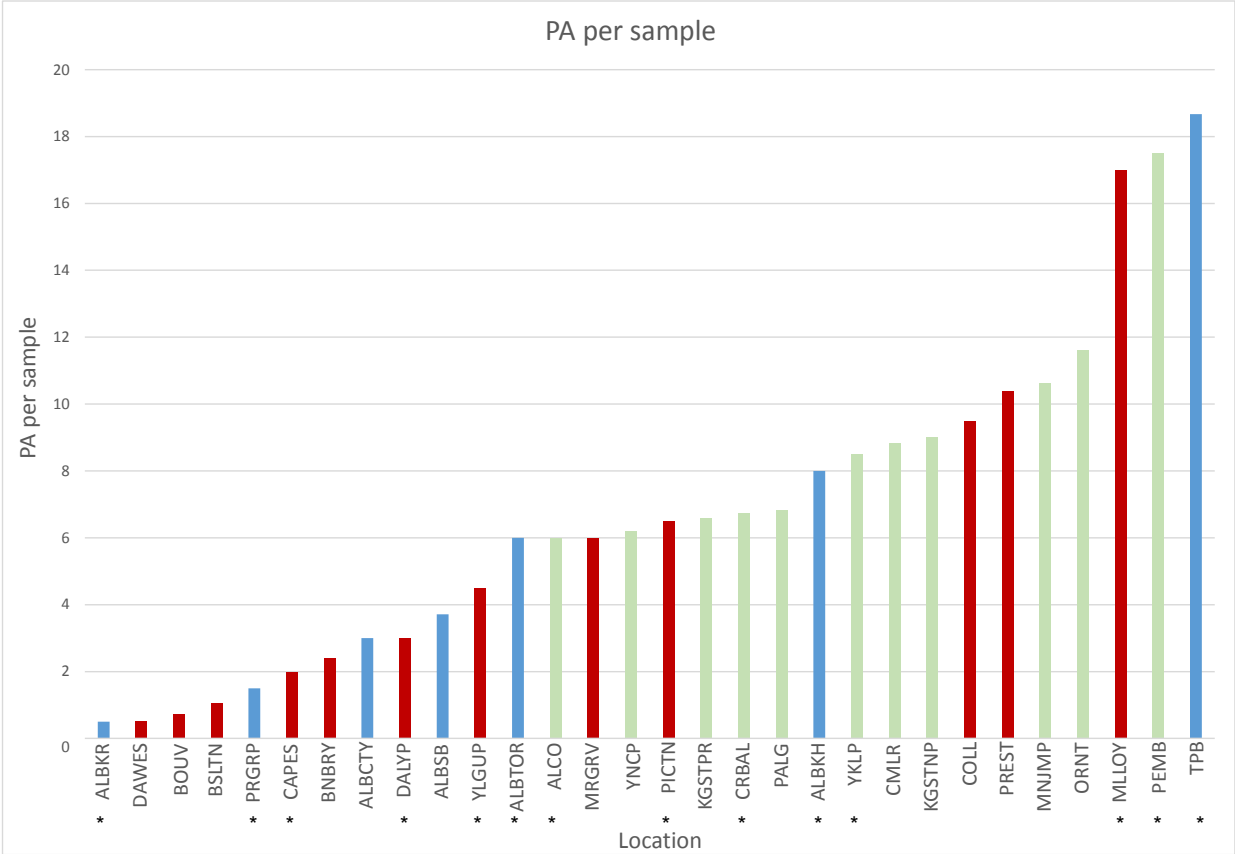
Ho: observed heterozygosity; He: expected heterozygosity; F_{IS}: inbreeding coefficient; se: standard error. Management Zones - SC: South Coast, SF: Southern Forest, SCP: Swan Coastal Plain. * indicates values based on sample sizes < 5. ¹Collie is included in the SCP management zone but is not a coastal population.

Figure 2a. Expected heterozygosity calculated from 5704 polymorphic SNPs. Colour of bar represents management zone – blue (South Coast), light green (Southern Forest) and red (Swan Coastal Plain).



* locations with sample size $2 \leq n < 5$.

Figure 2b. Private alleles per sample based on 222 individuals from 31 locations. Colour of bar represents management zone – blue (South Coast), light green (Southern Forest) and red (Swan Coastal Plain).



* locations with sample size $2 \leq n < 5$.

Structure analysis

Structure within ringtail possums was investigated using principal coordinate analysis and with the program Structure. PCoA on all locations suggests that, in general, the three management zones as currently described explain genetic diversity relatively well, with some important exceptions (Figure 3). When using the first two axes, which explain 15% of total genetic variation, the true Swan Coastal Plain locations (Busselton, Dawesville, Herron, Bouvard, Preston Beach) cluster tightly and are clearly separate from Southern Forest and South Coast locations on axis 1, while axis 2 separates Southern Forest from South Coast. Along axis 1, however, intermediate Swan Coastal Plain clusters are apparent, one including samples from Collie, Picton, and Donnybrook (i.e., Leschenault Catchment) and another focused around samples from Bunbury. Samples from Capes Region locations including Margaret River, Yallingup and Augusta do not cluster well with any of the Swan Coastal Plain clusters; similarly, for Molloy and East Augusta samples cluster with each other and appear to be genetically more similar to Southern Forest locations than Swan Coastal Plain. Within the Southern Forest, locations from Upper Warren cluster tightly together, while sites within the upper catchment area of the Donnelly River (Manjimup, Palgarup and Alco) cluster out separately. All South Coast Albany locations cluster tightly, however the few available samples from outside Albany including Two People's Bay, Mount Manypeaks and Mount Lindesay lie intermediate between Albany and the Southern Forest.

Results from global Structure analyses were not definitive but best convergence between runs was achieved with a parameter set that allowed for admixture between populations, assumed allele frequencies were correlated and used sample location as a prior probability. There was not good agreement on the most likely K, and estimates differed depending on parameter combination and the estimation method. However, when looking at plots for each of the tested Ks, there was good agreement on cluster assignment. For example, the structure plot for K = 5 shows clear distinction between each of the current management zones and suggests further structure within each of the management zones (Figure 4). Results from PCoA analyses and NJ trees agreed well with the global Structure analysis (Figure 4, Supplementary Figure 1). The hierarchical structure analyses were able to resolve population assignment within each management zone (Supplementary Figures 2a-c). There is evidence for four clusters in the Swan Coastal Plain (Supplementary Figure 2a), two contemporary clusters in the Southern Forest, although the Kingston Project samples show evidence of drift and there is some evidence for admixture or drift in the Orient Road samples (Supplementary Figure 2b). In the South Coast there were two clearly defined clusters although possible drift or admixture in greater Albany regions (Supplementary Figure 2c).

Figure 3. Principal coordinates analysis of all samples. Shapes of symbols refer to the management zone to which an individual is assigned – triangle (South Coast), square (Southern Forest) and circle (Swan Coastal Plain). Colours of symbols refer to location, and location names are grouped adjacent to the corresponding data points. For definitions of location abbreviations see Table 1. Additional abbreviated locations (with definition and Management Zone in brackets) are PERUP (Perup Sanctuary ex. Busselton, SF); MYLUP (Myalup, SCP); MCLD (McCleod River, SCP); GOODE (Goode Beach, SC); MTMP (Mt. Manypeaks, SC).

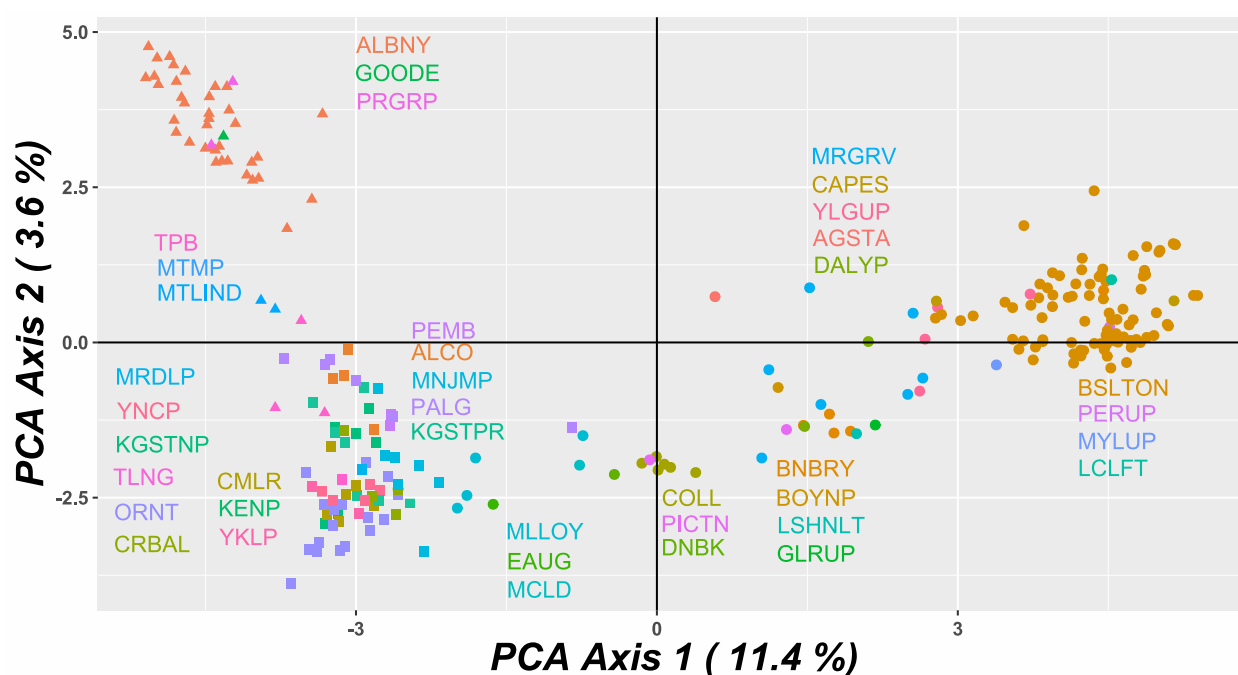
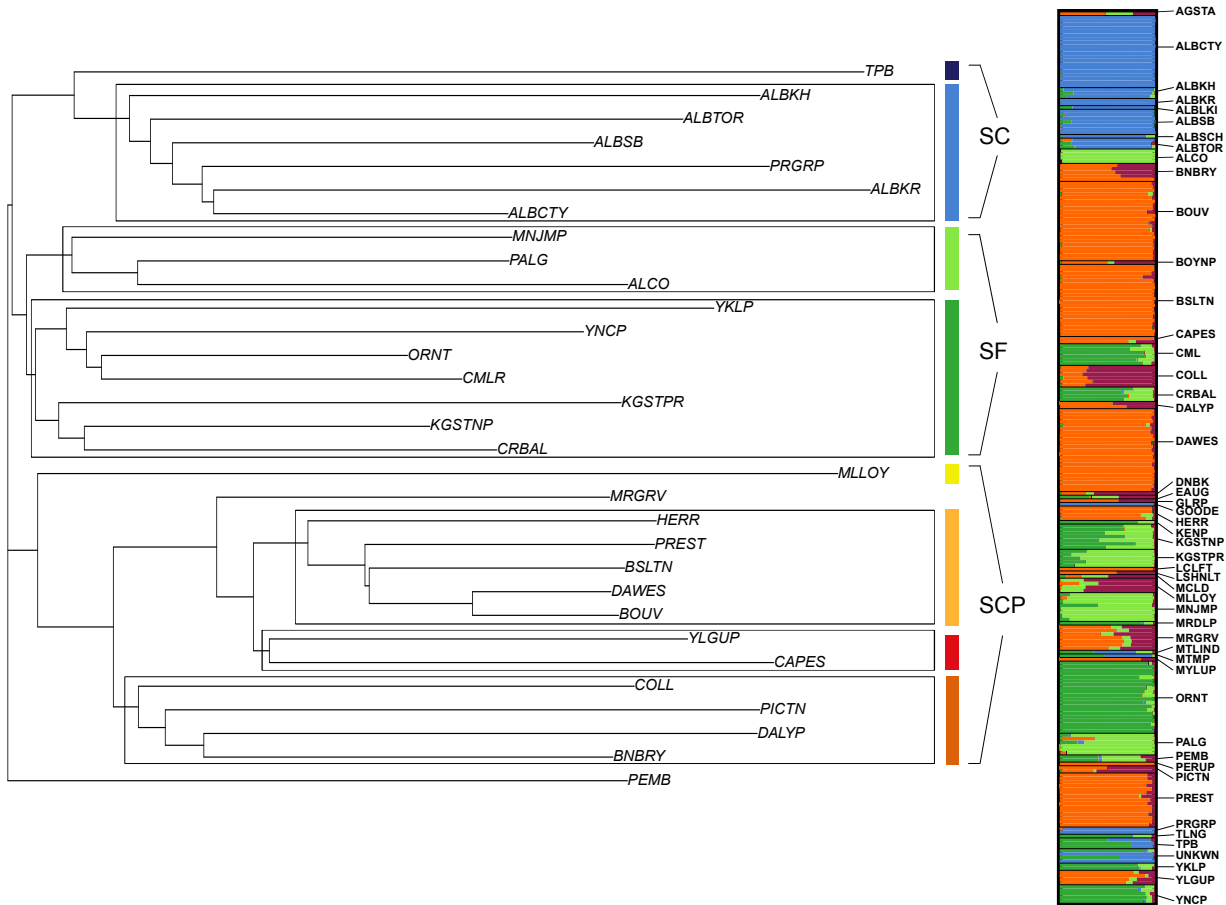


Figure 4. A summary of genetic structure within western ringtail possums. Left: neighbor-joining tree based on Euclidian distance on populations with $n \geq 2$; centre: proposed genetic clusters; right: Structure plot for all western ringtail possums at $k = 5$ and using a parameter set that allows for admixture between populations, presumes correlated allele frequencies between populations and uses a sample's population as a prior probability for assignment.



Eight genetic clusters were defined based on a combination of all structure and distance analyses, which we refer to as subregions (Table 3a, Figure 4). We have placed the two individuals from Pemberton into the Upper Warren cluster based on results from hierarchical 'Structure' and PCoA analyses (Supplementary Figure 2b), however private allele and Neighbor-Joining distance analyses suggest it may form a separate cluster (Figures 2b, 4). A greater sample size may help resolve Pemberton's assignment. Based on the identification of four genetic clusters in the Swan Coastal Plain management zone, three of which are not in the Swan Coastal Plain, we propose this management zone be renamed to West Coast (Table 3b). Basic genetic diversity statistics for each cluster, summarised in Table 4a, show Upper Warren to have greatest expected heterozygosity and allelic diversity and Southern Swan Coastal Plain to have lowest expected heterozygosity. Genetic distance analysis (Table 4b) shows that the Two People's Bay cluster generates some of the highest genetic distances using pairwise F_{ST} and Jost's D_{est} , which is likely compounded by the small sample size ($n=4$). Nonetheless, Two People's Bay shows less genetic distance with Upper Warren than Greater Albany using both measures of genetic distance. The Lower Blackwood River cluster is genetically less distant from the Upper Donnelly River Catchment and Upper Warren than to its geographically closest cluster, the Capes Region.

Fixed difference analysis considering a fixed allele to have a frequency of 1 was able to only identify one OTU that encompassed all clusters using default parameters. However, when the frequency threshold of a fixed allele difference was relaxed so that a major allele could have a frequency as low as 0.9 (rather than 1) and a minor allele frequency as high as 0.1 (rather than 0), three OTUs were identified – Two People's Bay, Lower Blackwood River Catchment and a larger OTU that amalgamated all other genetic clusters.

Table 3a. Definition of genetic clusters

Cluster number	No. Individuals	Location	Subregion name
1	12	Augusta, Margaret River, Yallingup	Capes Region
2	18	Bunbury, Boyanup, Collie, Dalyellup, Donybrook, Gelorup, Leschenault, Picton	Leschenault Catchment
3	88	Bouvard, Busselton, Dawesville, Herron, Lake Clifton, Myalup, Preston Beach	Southern Swan Coastal Plain
4	5	East Augusta, Molloy Island	Lower Blackwood River Catchment
5	18	Alco, Manjimup, Palgarup	Upper Donnelly River Catchment
6	55	Camelar, Corbal, Keninup, Kingston National Park, Mordalup, Orient Road, Pemberton, Talling, Yackelup, Yendicup	Upper Warren
7	41	Albany and suburbs, Goode Beach, Mount Lindesay	Greater Albany
8	4	Mount Many Peaks, Two People's Bay	Two People's Bay

Table 3b. Management zone classification and subregion assignment

Old Management Zone	New Management Zone	Subregion Assignment
Swan Coastal Plain	West Coast	Capes Region, Leschenault Catchment, Southern Swan Coastal Plain, Lower Blackwood River Catchment
Southern Forest	Southern Forest	Upper Donnelly River Catchment, Upper Warren
South Coast	South Coast	Greater Albany, Two People's Bay

Table 4. Genetic diversity in the eight defined genetic clusters. a. Descriptive diversity statistics; b. Pairwise genetic distance between genetic clusters. Pairwise F_{ST} s lower diagonal, Jost's D_{est} ($\times 10^4$) upper diagonal. All pairwise F_{ST} s were statistically significant at $p < 0.001$.**a.**

Subregion	N	Ho ($\pm$ se)	He ($\pm$ se)	Na ($\pm$ se)
Capes Region	12	0.052 ($\pm$ 0.002)	0.059 ($\pm$ 0.002)	1.26 ($\pm$ 0.006)
Leschenault Catchment	18	0.055 ($\pm$ 0.001)	0.066 ($\pm$ 0.002)	1.33 ($\pm$ 0.006)
Southern Swan Coastal Plain	87	0.045 ($\pm$ 0.001)	0.049 ($\pm$ 0.002)	1.38 ($\pm$ 0.006)
Lower Blackwood River Catchment	5	0.050 ($\pm$ 0.002)	0.055 ($\pm$ 0.002)	1.18 ($\pm$ 0.005)
Upper Donnelly River Catchment	18	0.054 ($\pm$ 0.001)	0.068 ($\pm$ 0.002)	1.38 ($\pm$ 0.006)
Upper Warren	55	0.069 ($\pm$ 0.001)	0.078 ($\pm$ 0.001)	1.70 ($\pm$ 0.006)
Greater Albany	41	0.050 ($\pm$ 0.001)	0.062 ($\pm$ 0.002)	1.41 ($\pm$ 0.007)
Two People's Bay	4	0.048 ($\pm$ 0.002)	0.058 ($\pm$ 0.002)	1.17 ($\pm$ 0.005)

N: sample size; Ho: observed heterozygosity; He: expected heterozygosity; Na: mean number of alleles per marker; se: standard error.



b.

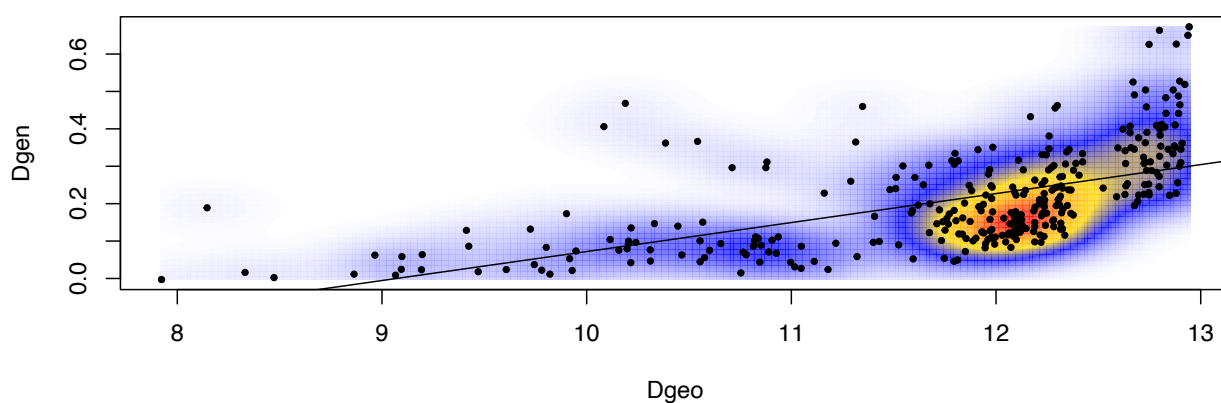
	Southern Swan Coastal Plain	Leschenault Catchment	Capes Region	Lower Blackwood River Catchment	Upper Donnelly River Catchment	Upper Warren	Greater Albany	Two People's Bay
Southern Swan Coastal Plain	0	0.06	0.05	0.19	0.13	0.11	0.15	0.23
Leschenault Catchment	0.09	0	0.07	0.15	0.09	0.06	0.12	0.17
Capes Region	0.08	0.08	0	0.16	0.10	0.09	0.13	0.20
Lower Blackwood River Catchment	0.27	0.17	0.19	0	0.15	0.12	0.18	0.25
Upper Donnelly River Catchment	0.18	0.10	0.11	0.15	0	0.04	0.08	0.15
Upper Warren	0.15	0.07	0.09	0.12	0.04	0	0.07	0.11
Greater Albany	0.21	0.14	0.16	0.21	0.10	0.08	0	0.13
Two People's Bay	0.30	0.17	0.22	0.24	0.14	0.10	0.15	0

Isolation by distance

In total 191 samples from 26 locations were used in isolation by distance analysis. These samples revealed a positive Pearson's product-moment correlation coefficient of 0.591 between geographic and genetic distance, which was statistically significant at $p < 0.001$ (Figure 5).

Figure 5. Isolation by distance plot using 191 samples from 26 locations, plotting geographic distance (x axis) vs. genetic distance (y axis).

Isolation by distance



Spatial clustering

After collinearity analysis the 34 geospatial datasets were reduced to 14, which formed the set of predictor variables used for supervised classification. Definition of the 14 environmental predictor variables is provided in Table 5. A cluster prediction tree shows how combinations of the variables can predict genetic cluster inclusion (Supplementary Figure 4), and a summary of environmental layer predictions of genetic clusters is provided in Figure 6.

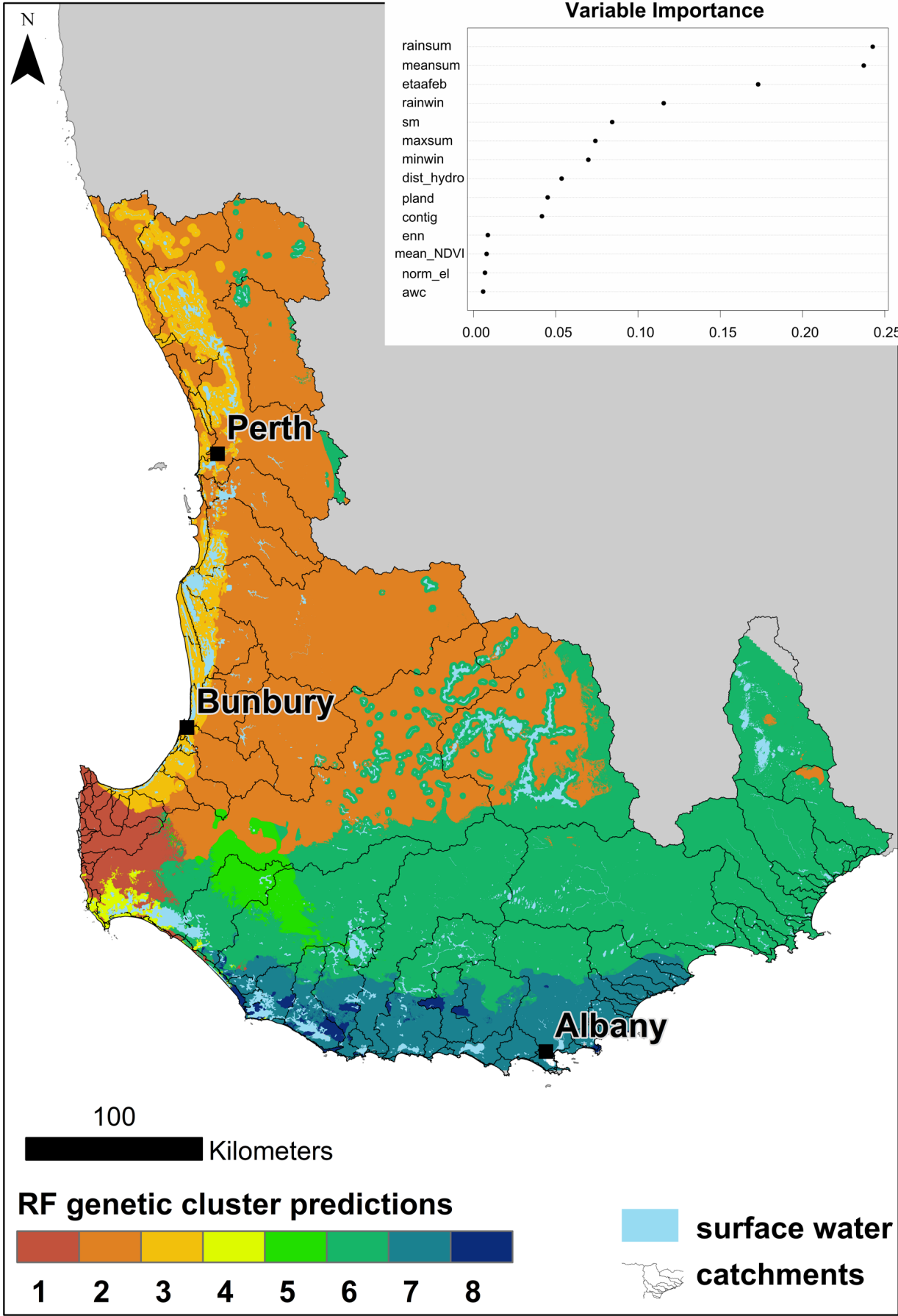
Table 5. A summary of the 14 environmental variables used for predicting genetic clusters.

Environmental variable	Definition
rainsum	mean summer rainfall
meansum	mean summer temperature
etaafeb	areal actual evapotranspiration rate in February
rainwin	mean winter rainfall
sm	soil moisture
maxsum	maximum summer temperature
minwin	minimum winter temperature
dist_hydro	distance to non-natural hydrological features
pland	percentage of landscape belonging to each class of landscape/habitat, equal to 100 when only one patch is present
contig	habitat contiguity
enn	Euclidean nearest-neighbor distance, measures patch isolation
mean_NDVI	mean normalised difference vegetation index
awc	actual water content of soil
norm_el	normalised elevation



Western ringtail possum in a heartleaf tree (*Gastrolbium bilobum*), a toxin-producing native. Image: Adrian Wayne

Figure 6. Environmental variable predictors of genetic cluster membership, showing relative importance of variables (mean summer rainfall is most important).



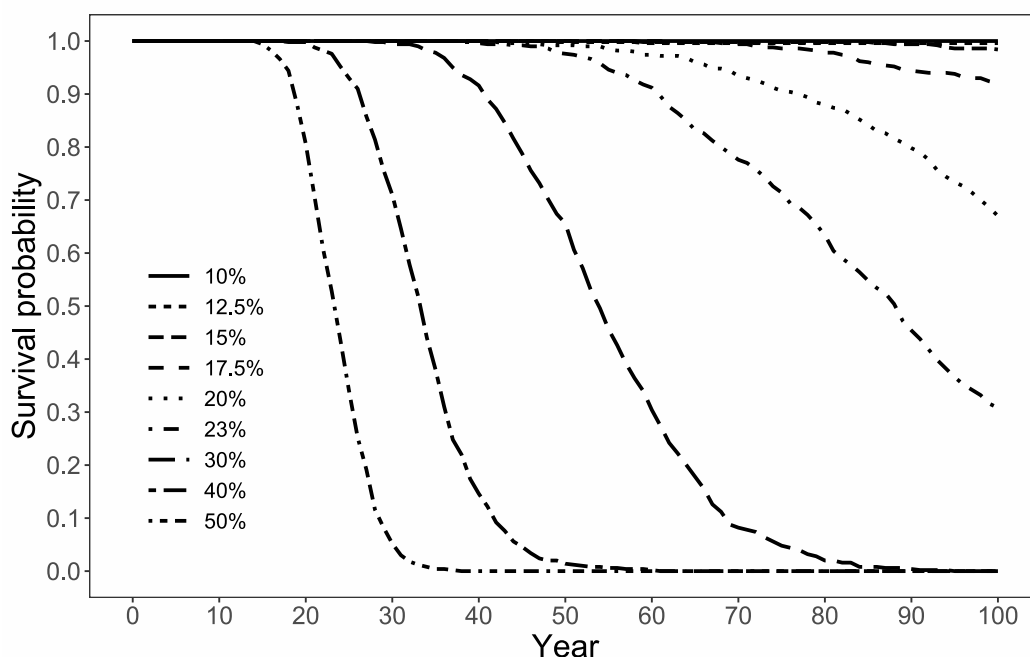
Population viability analyses

Using the baseline scenario which includes a 1 in 10-year modest catastrophic event, adult annual mortality of 30% or greater led to zero chance of survival in the Upper Warren catchment of the Southern Forest management zone after 100 years. At the baseline adult annual mortality rate (23%) survival probability dropped to 0.3. Adult annual mortality rates at 17.5% or less, resulted in survival probability estimates above 0.9 (Figure 7a). Population growth was only achieved with an adult mortality rate of 12.5% or less, although at 15% population size remained relatively stable (Figure 7b).

Assuming baseline adult annual mortality to be 23% survival probability is projected to be 1.0 after 100 years with no catastrophic events, drops substantially with a 1 in 10-year event (0.3) and more so with a 1 in 5-year event (0.0) (Figure 8a). Populations decline irrespective of catastrophic event frequency (Figure 8b).

Figure 7. Impact of different adult mortality rates over 100 years on a. population survival probability and b. population size.

a.



b.

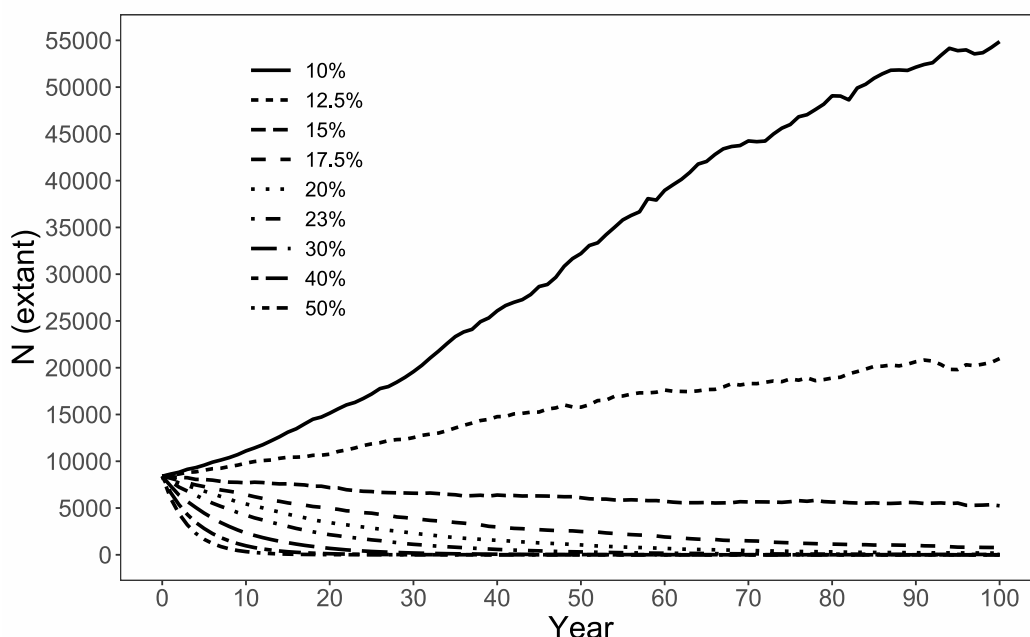
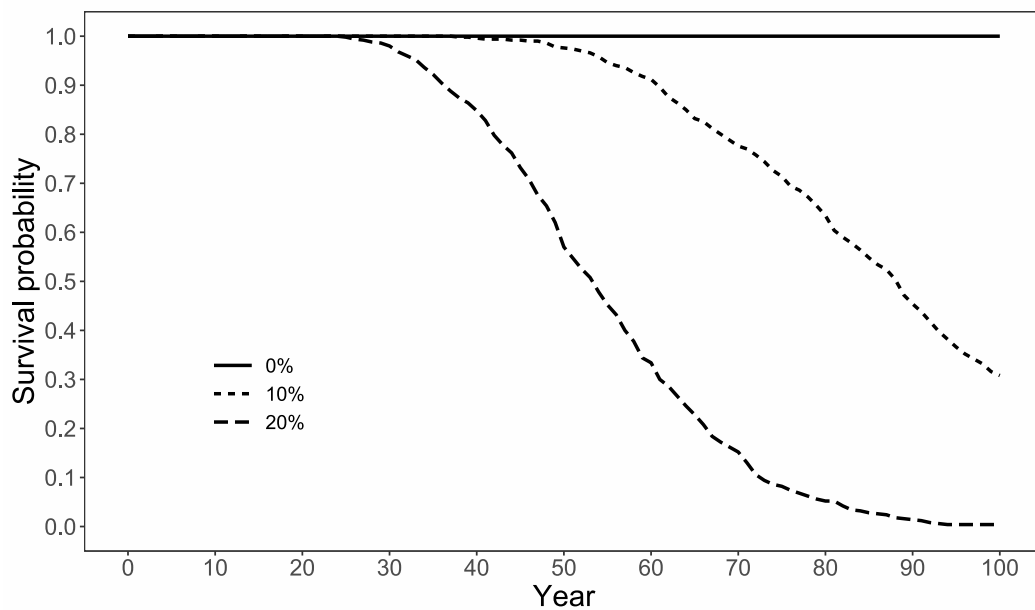
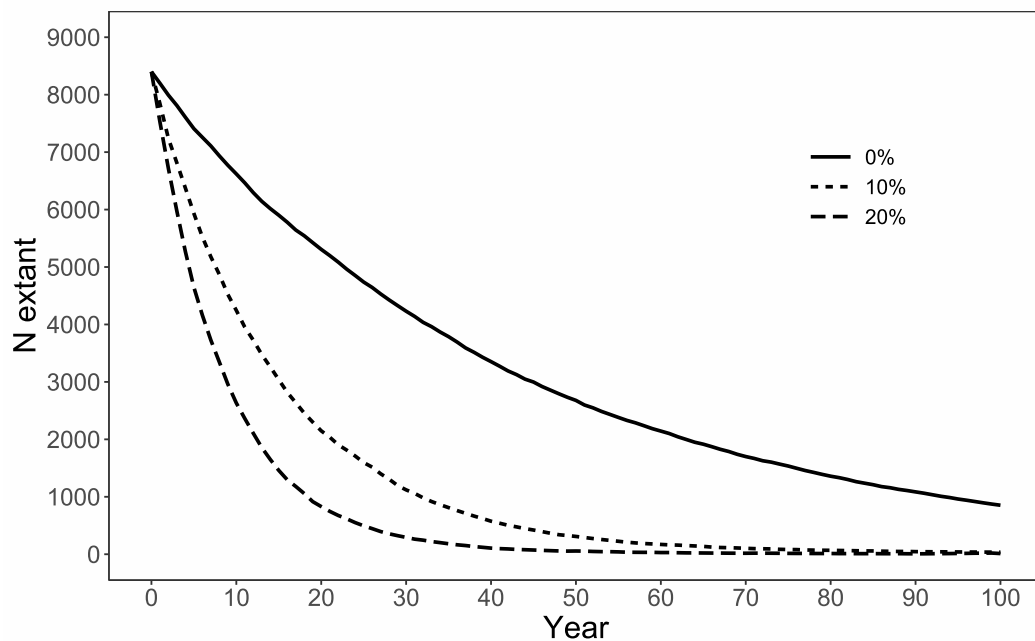


Figure 8. Impact of different frequencies of catastrophes (none (0%), approximately every decade (10%) and approximately every 5 years (20%)) on a. Upper Warren population survival probability and b. Upper Warren population size, over 100 years, based on a baseline adult annual mortality of 23%.

a.



b.



Discussion

Genetic diversity

Compared to other marsupials whose genetic diversity has been assessed with comparable numbers of genome-wide SNPs, the genetic diversity of ngwayir is low. For example, White et al (2018) showed that expected heterozygosity ranged from 0.13 to 0.24 in the Shark Bay bandicoot (using 1752 SNPs), from 0.21 to 0.34 in the Burrowing bettong (using 3775 SNPs), 0.31 in the greater bilby (using 6880 SNPs) and from 0.30 to 0.33 in the greater stick-nest rat (using 8703 SNPs). Here, expected heterozygosities for locations with 5 or more samples ranged from 0.05 to 0.08 using 5704 SNPs, around four times lower than these comparable examples. Interestingly, mean observed heterozygosity was significantly lower than mean expected heterozygosity, with 14 out of 16 locations with more than 5 samples having H_o lower than H_e and corresponding positive F_{IS} estimates. This is a concerning trend as it suggests that inbreeding is already underway in the majority of ngwayir localities (and all management zones), suggesting a lack of mixing between locations and increasingly isolated populations. While small samples sizes, such as some of those from this study, may lead to downward biases in observed heterozygosity estimates (Schmidt et al. 2021), the trend of lower-than-expected observed heterozygosities is upheld for locations with a sample size of ≥ 20 (Table 2), suggesting that these trends may be real in at least some of these sites.

Southern Forest locations have highest heterozygosities, whereas the lowest estimates came from locations from the Swan Coastal Plain (i.e., Busselton and localities north). High heterozygosity has been associated with high fitness in many species (DeWoody et al. 2021; Hansson and Westerberg 2002), suggesting the possums of the Upper Warren may have a fitness advantage. When assessing the number of private alleles three locations had substantially greater number per sample than others – Molloy Island, Pemberton and Two People's Bay. These three locations could therefore harbour unique allelic diversity, important for maintaining the adaptive potential of a species to cope with changing environments (Weiser et al. 2013).

Genetic Clusters

Genetic insights from this study are broadly consistent with the separation of ngwayir into three management zones – Swan Coastal Plain, Southern Forest and South Coast – in agreement with the limited genetic work to date (Wilson 2009). However, our comprehensive sampling has allowed us to extend our understanding suggesting there may be subregions within the three zones (Figures 1 and 4). In addition to the two genetically distinct populations previously described within the Swan Coastal Plain management zone, Busselton and Gelorup (which would be included in our Southern Swan Coastal and Leschenault Catchment subregions, respectively (Wilson 2009)), we show there are likely two further subregions – Capes Region including Margaret River, Yallingup and Augusta, and the Lower Blackwood River catchment comprised of Molloy Island and East Augusta – making four subregions in total for this management zone. As well as confirming that Southern Forest is genetically distinct from the other management zones, we show that it is likely comprised of two subregions – Upper Warren, consisting of the forest blocks to the east of Manjimup, and the Donnelly River catchment. Finally, the South Coast management zone is likely split into two subregions, greater Albany and a more remote subregion inclusive of Two People's Bay. In total we propose eight genetic subregions within ngwayir and indicate that a ninth possibly in the Lower Warren River catchment, inclusive of the Pemberton locality, could be confirmed with further sampling and genetic analyses.

From the strong isolation by distance signal, it is likely that most clusters can be explained by non-continuous sampling across the landscape, either because of limitations of our sampling strategy or because of the fragmented nature of ngwayir populations. Of the eight genetic subregions, Upper Warren shows the highest expected heterozygosity and the highest number of alleles per marker (Table 4a) suggesting this is, or until recently was, a relatively large outbreeding population. This agrees with the location-level analysis and supports the finding that there is no obvious consequence on genetic diversity of the large population crashes seen in the late 1990s, based on comparison of diversity in the Kingston locality samples collected in 1997 to samples collected in 2017.

However, declines reportedly continue in the region including an 80% decline in the four years to 2021 in the Great Kingston area (western half of Upper Warren, Adrian Wayne pers. comm.), resulting in numbers approaching undetectable levels. This is of major conservation concern for the species given the high genetic diversity of this population and a significant part of the genetic diversity of this species is likely to be irreversibly lost as the magnitude, extent and duration of the continued declines grows.

The Two People's Bay and Lower Blackwood River clusters, both previously undocumented, show high drift and allelic divergence from other clusters as well as high numbers of private alleles, indicating that both these subregions may have high genetic and conservation value. Interestingly, the Two People's Bay cluster is more genetically divergent from Albany than from Upper Warren, and the Lower Blackwood River catchment is more genetically divergent from the Capes Region than from both the Upper Donnelly River Catchment and Upper Warren (Table 4b). Both observations are contrary to expectations based on geographic distance between clusters and our isolation by distance analysis, warranting further investigation. For example, there might be factors other than geographic distance that act as barriers to gene flow at a landscape-scale in ngwayir (e.g., the Blackwood River separates the Blackwood River catchment from the Capes Region, and the Kalgan and King Rivers separate Lower King east of Albany from Two People's Bay). Alternatively, these trends could be indicative of recent population bottlenecks accelerating the impacts of genetic drift (a possibility for the Albany population). In addition, all distance estimates that include Two People's Bay and Lower Blackwood River clusters are likely overestimates based on their small sample sizes of four and five, respectively.

Our inability to detect more than one OTU suggests insufficient time has passed for fixed allele differences to establish between populations and that ngwayir were one large, interconnected meta population until relatively recently. This observation agrees with earlier work that was unable to define more than one evolutionary significant unit (Wilson 2009). With the more relaxed definition of a fixed difference (i.e., with a MAF of ≤ 0.1 rather than 0) it is of interest to note that Two People's Bay and the Lower Blackwood River Catchment form separate OTUs. However, our analysis suffers from small sample sizes in the Lower Blackwood River catchment ($n = 5$) and Two People's Bay subregions ($n = 4$), which will reduce the power to detect rare alleles and could lead to falsely claiming an allele is absent from these clusters (Gruber et al. 2018). However, this effect should not lead to an overestimate of private alleles in these subregions, both of which showed high levels of unique allelic diversity. Repeating analyses with greater sample sizes (ideally with $n \sim 20$) for these subregions is needed to confirm genetic distance and OTU findings. Nonetheless, as barriers to gene flow continue due to habitat fragmentation and further reduction is predicted (Molloy et al. 2014), the formation of distinct OTUs for these populations is the likely trajectory.

Spatial clustering

Spatial clustering analysis showed that assignment to a genetic cluster can be predicted by environmental predictors and that the three most informative variables (in order) are mean summer rainfall, mean summer temperature and areal actual evapotranspiration rate in February (Figure 6). This result suggests that genetically defined clusters can be partitioned from each other based on shared environmental variables. For example, the Two People's Bay and Greater Albany subregions enjoy the highest levels of summer rainfall (Supplementary Figures 3 and 4). This could be important information for conservation management as consideration may need to be given to possible outcomes of moving animals from these regions, for the purposes of bolstering genetic diversity elsewhere for example, to regions with low summer rainfall.

Population viability analysis

Our results show that mortality rates in the Upper Warren need to be less than the only empirically derived estimate from that region (Chariup) for survival probabilities to remain above 0.9 (Wayne et al. 2005). It is known that at least half of the deaths recorded at Chariup were due to predation by invasive predators, in particular foxes and cats. It is unlikely that the populations of Upper Warren are able to withstand this level of predation and it would appear that ongoing/increased fox and cat control is a prerequisite for long-term survival. This is in agreement with a previous PVA study on western ringtail possums in the Locke Nature Reserve that predicted fox predation and road mortality have profound impact on persistence of that population, reducing survival by 92% and 68%, respectively (Yokochi 2015).

Continued population decline in the Upper Warren is predicted and population growth only seems plausible if mortality rates are much lower (12.5% or lower), which suggests that further understanding of the poor recruitment and actual mortality rates in the absence of introduced predators (e.g. Perup Sanctuary) in the Upper Warren is required. For example, in other regions it is known that possums can have more than one young per brood, and more than one brood per year (Jones et al. 1994b). It would be useful to investigate why multiple births do not occur in the Upper Warren, e.g., stress due to increased predation by invasive predators or a drying climate, or a combination of both. For example, it appears that an area that endures low annual rainfall in the Australian south-west has already expanded to cover much of the Upper Warren subregion (Supplementary Figure 3), and considering the sensitivity of ngwayir to moisture levels such environmental conditions could provide sufficient stress to prevent multiple births (Jones et al. 1994a; Jones et al. 2004; Molloy et al. 2014; Yin 2006). Modelling indicates that population viability is particularly sensitive to disturbance events such as fire and drought. For example, the impacts of a potential disturbance were simulated here to be mild, and even a one in ten-year event reduced population survival probability by 69% (1.00 to 0.31) over 100 years. It is therefore critical to quantify the demographic impact of disturbances such as drought and fire, which will require a concerted strategic monitoring effort.

Application of research

It is crucial to understand all genetic diversity remaining in ngwayir so that it can be included in future conservation planning and recovery. Until now we have not had a species-wide assessment of genetic diversity. Our study has the most comprehensive sampling done to date and includes locations with well-known but previously unstudied possum populations, such as those in the Capes Region (Margaret River, Yallingup), populations in the central forest area in the Leschenault Catchment (Collie, Donnybrook) and locations on the South Coast outside of greater Albany (Two People's Bay, Mount Manypeaks). This has led to our identification of previously undocumented locations with potentially high conservation and genetic value, in particular Two People's Bay, Molloy Island/East Augusta, and Pemberton.

Genetically diverse, outbreeding populations are believed to have higher mean fitness and be better equipped to deal with a changing environment (DeWoody et al. 2021; Hansson and Westerberg 2002). Here we have shown that the locations within the Swan Coastal Plain (i.e., Bouvard, Dawesville and Busselton) have the lowest expected heterozygosities and around 1.6 times less than the most heterozygous locations (all in the Southern Forest management zone). These populations would benefit from ongoing genetic monitoring to assess whether decreasing genetic diversity is a continuing trend. Only two locations had mean inbreeding coefficients greater than 0.1, and these are Albany City (0.118) and Manjimup (0.162), both urban sites (Table 2). This suggests there may be non-random mating in these locations and higher breeding between related individuals than expected. We therefore recommend ongoing genetic monitoring to test for inbreeding depression (esp. Albany and Manjimup), as well as for continual declines in genetic variation in the Swan Coastal Plain locations, and in the Upper Warren subregion where numbers continue to decline since the large decimations of the late 1990s (Wayne 2012).

The identification of eight distinct genetic clusters could have important management implications. Foremost, how should these genetic 'units' be managed? We propose that each be considered a separate management unit. While we are not suggesting an overhaul of current conservation management, this information may help partition resources within each management zone so that the four subregions in the Swan Coastal Plain management zone, two Southern Forest subregions and two South Coast subregions are all considered in planning. Knowledge of these eight genetically defined subregions, the diversity within them and distance between them will be useful information for managers. For example, future decisions will need to be made on how to source founders for future conservation translocations, population supplementation and/or rescue. For example, if maximising genetic diversity is the requirement then individuals from more divergent subregions could be used, whereas if improving genetic diversity is not a priority then geographically closer animals could be used, reducing logistic cost. What's more, although mixing founders from genetically distinct populations can improve genetic health, there can be negative consequences if local adaptation has occurred and outbreeding depression is a risk, although this is unlikely in ngwayir considering all animals are part of one OTU and most structure appears to be driven by more recent habitat fragmentation (Frankham et al. 2011). However, while we have not here shown evidence for local adaptation, to help with founder selection we have been able to associate specific combinations of environmental variables with the eight genetically defined subregions, and we indicate the most informative variables for prediction of cluster assignment. For example, mean summer rainfall, mean summer temperature and areal evapotranspiration rates have the greatest predictive power, and ensuring movements are made between regions with similar or less severe values using our cluster prediction tree (Supplementary Figure 4) may maximise success of translocations.

Impact of the research

The results from this study provide a useful reference and guide for ngwayir management and conservation. End-users include state conservation and land management agencies, the Western Ringtail Possum Recovery Team and community groups. The information provided here provides a detailed overview of the diversity that has been retained across the species and identifies which populations have low genetic diversity as well as those that show high genetic and conservation value – indicating which populations should receive priority in future conservation efforts. Importantly we have revealed previously undocumented genetic variation that could otherwise have been overlooked in conservation programs. The number of genetically distinct clusters and relationships between them, and the bioclimatic variables associated to each, provide important guidance on how to bolster genetic diversity and increase the chances of successful translocations. The PVA models we have developed indicate that the most genetically diverse population may be at high risk of extinction in the near future. The model can be modified for this or any other population or locality that has the appropriate life history data. As the models are improved with additional empirical data, they will become increasingly more important and useful for conservation and management. All models created in this project will be made publicly available.

Broader implications

Species in regions that are exposed to changing environments face a compounding array of survival threats. It is important to document as comprehensively as possible all remnant genetic variation and environmental conditions across the landscape, so that important diversity is documented and conservation measures put in place before it is lost, and the resilience within species to various climatic scenarios can be understood. Here we have provided an accessible approach that can be applied to other at-risk species.

Future research priorities

The accuracy of population modelling in this study was hindered by the amount of available empirical life-history data. It would be useful to get mortality estimates for priority populations across the species' range that are at risk and/or exposed to different environmental conditions. This would give more reliable estimates and improve understanding of how mortality is impacted by environmental and other external factors. In particular, we show that accurate modelling of the impact of stochastic events such as fire and drought on mortality and fecundity is particularly relevant for ngwayir, and even more-so given future predictions of climate change and its impact in the south-west of Western Australia (Molloy et al. 2014). Similarly, estimates of census size over time for multiple locations would allow the PVA models developed here to be tailored to specific populations and increase understanding of how population sizes change temporally. For example, it has been recorded that numbers may fluctuate between years in response to periodic changes in climate (e.g., rainfall, temperature) (Wayne pers. comm.), which is a trend seen in other marsupials in Western Australia (Chapman et al. 2015). An accurate understanding of population dynamics is crucial to predicting census size trajectories and identifying thresholds below which population decline becomes irreversible.

One approach that may improve monitoring capacity of otherwise elusive species is the use of non-invasive sampling. Non-invasive sampling refers to the acquisition of tissue samples from individual animals through passive or opportunistic means and examples include shed feathers, scales, moulted fur and in this case scats (Broquet et al 2006, Ramon-Laca 2017 et al). The benefits of this approach include a profound simplification of the sampling process - from gaining ethics approval to handling of animals - greatly reducing logistics involved in sampling threatened or rare species, as well as reducing stress to the animals as no handling takes place. Negatives of the approach include the discovery of the non-invasive samples may be non-trivial (e.g., trying to find shed feathers in a forest), and for samples to be used in genetic analysis the quantity and integrity of the DNA that can be retrieved from such samples may be inadequate, and low DNA yield and allelic drop-out can be problematic (Ramon-Laca 2017 et al). For ngwayir we feel the use of scats as a source of non-invasive samples deserves further investigation due to i. the relative reported ease of finding these scats, ii. reported high abundances of scats in known habitat, iii. the use of scats for presence-absence analysis is already under way by DBCA, iv. the size and integrity of ngwayir scat, v. monitoring programs of ngwayir populations using established methods (distance-sampling) are currently underway facilitating validation of the approach. Integrity and expected yield of DNA should be quantified under controlled settings, and scat integrity after exposure to different environments and time periods should be further investigated.

While we have provided evidence for eight genetic clusters in this study and shown that inclusion in each cluster can be predicted by a specific set of environmental variables, we have not demonstrated whether genetic variation specific to each cluster is a response to the environmental variables, an indication of local adaptation. Local adaptation has been shown for other species (Schweizer et al. 2016; Thomas et al. 2017) and indicates that populations or 'ecotypes' within a species have functional variants that allow adaptation to differences in habitats across their range. It is possible this has occurred in ngwayir as they exist across a range of environmental conditions and, if so, it would be important information to include in conservation programs, i.e., moving locally adapted animals to other regions they are less adapted to, or mixing between locally adapted populations, could result in the opposite of the desired outcome.

Finally, ongoing monitoring of locations that have high inbreeding coefficients for signs of inbreeding depression would be important for understanding the sustainability of those locations, as well as the impact of inbreeding depression in ngwayir in general.

Data sets

The dartseq SNP dataset used in this study is available from Figshare, in the 'Population genomics of the western ringtail possum' project, <https://doi.org/10.6084/m9.figshare.16698244>. The PVA models have been deposited into the Data Catalogue repository at DBCA and are available upon request.

Recommendations

We recommend that

- There is recognition of at least eight genetically distinct subregions within ngwayir and that they are considered as 'management units' in all conservation and recovery planning
- The Swan Coastal Plain management zone is re-named to West Coast
- There is an immediate species-wide concerted effort to gather empirical life history data, in particular mortality rates, population census sizes and the impact on population dynamics of environmental stochastic events including fire and drought
- Ongoing genetic monitoring be undertaken to track potential future declines in variation (e.g., Upper Warren) and signs of inbreeding depression (e.g., Manjimup and urban Albany)
- Efforts should be made to prevent substantial and irreversible loss of the high levels of genetic diversity observed in the Upper Warren in this study. This is highly likely given the indications of ongoing demographic decline and the PVA modelling which suggests that the population may be at high risk of extinction
- Efforts should be made to prevent further loss of genetic diversity in the Southern Swan Coastal Plain subregion by informed mixing between locations and supplementation from other genetic clusters
- Before translocation of possums between genetic clusters consideration should be made of cluster assignment, genetic diversity and associated environmental variable predictors
- Increased sampling and further OTU analysis are done on the Lower Blackwood River and Two People's Bay subregions, and the Pemberton locality

Conclusion

In conclusion, we have used historical collections and opportunistically collected samples to get sampling coverage from across the range of ngwayir in one genetic study. Using thousands of genome-wide marker loci we have been able to document the genetic diversity within the species and confirm unique genetic identity of established groups. We have expanded on this by describing novel, genetic variation of potentially high conservation value, specifically in two genetically distinct clusters that may be on a trajectory to becoming operational taxonomic units. Importantly, we have mapped genetic variation on to environmental layers and have revealed that inclusion within distinct genetic clusters can be predicted by combinations of environmental variables. In a climate that is becoming hotter and drier, this will help understand environmental resilience within ngwayir and undoubtedly contribute to informed management decision-making. PVA modelling indicates that at least some populations may be at high risk of extinction but that greater accuracy of fecundity and mortality rates and the impact of factors such as fire and drought on these rates, are needed to improve the accuracy of these models.

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Ethics statement:

Samples were collected opportunistically from deceased animals or originated from previous studies and monitoring activities for which appropriate ethics approvals had been granted from state government conservation agencies. No live animals were handled as part of this study.

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Further information:

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