Long-term climatic stability drives accumulation and maintenance of divergent lineages in a temperate biodiversity hotspot

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Abstract

Aim: Anthropogenic climate change is forecast to drive regional climate disruption and instability across the globe. This issue is likely to be exacerbated within biodiversity hotspots, both due to the greater potential for species loss but also to the possibility that endemic lineages might not have experienced significant climatic variation in the past, limiting their evolutionary potential to respond to rapid climate change. We assessed the role of climatic stability on the accumulation and persistence of lineages in an obligate freshwater fish group endemic to a biodiversity hotspot.

Location: Southwest Western Australia (SWWA).

Taxa: Western pygmy perch (*Nannoperca vittata*) and little pygmy perch (*Nannoperca pygmaea*).

Methods: We sampled 33 individuals from nine populations spanning the range of both study taxa to explore their phylogeographic history. Using a combination of genomic (ddRAD-seq) and environmental approaches, we investigated population divergence and phylogenetic relationships, delimited species and estimated changes in species distributions since the Pliocene.

Results: We identified two deep phylogroups comprising three divergent clusters, which showed no historical connectivity since the Pliocene. We conservatively suggest these represent three isolated species with additional intraspecific structure within one widespread species. All lineages showed long-term patterns of isolation and persistence owing to climatic stability.

Main conclusions: Our results highlighted the role of climatic stability in allowing the persistence of isolated lineages in the SWWA. This biodiversity hotspot is under compounding threat from ongoing climate change and habitat modification, which may further threaten previously undetected cryptic diversity across the region.

Keywords: Australia, cryptic species, ddRAD-seq, freshwater fish, phylogenomics, phylogeography, species delimitation, Percichthyidae

1. Introduction

Global biodiversity is increasingly threatened by anthropogenic climate change, with contemporary rates of extinction substantially higher than throughout much of geological history (Le Roux et al., 2019). These impacts are expected to be particularly exacerbated within regions that have experienced long-term climatic stability due to several convergent factors (Harrison & Noss, 2017). Firstly, species that have not been exposed to major climatic changes over their evolutionary histories are less likely to have evolved traits allowing adaptive responses to contemporary climate change (Sandoval-Castillo et al., 2020), and may have lower dispersal ability (Sandel et al., 2011), greater ecological specialisation (Dynesius & Jansson, 2000) and lower thermal tolerance (Addo-Bediako, Chown, & Gaston, 2000) than species from more historically variable regions. Secondly, variation in

long-term climatic stability is often suggested to be a primary mechanism driving the spatial heterogeneity of biodiversity (Harrison & Noss, 2017; Sandel et al., 2011; Mercier et al., 2023), particularly in the high diversity of equatorial regions (Mittelbach et al., 2007) and in hotspots of biodiversity (Habel et al., 2019). Over geological timescales, regional climatic stability is predicted to promote the persistence and speciation of endemic lineages (Carnaval, Hickerson, Haddad, Rodrigues & Moritz, 2009), driving higher cumulative diversity (Sundaram et al., 2019). However, several alternative (but not exclusive) biogeographic mechanisms may also drive regional diversification (Moritz, Patton, Schneider, & Smith, 2000). Thus, determining the relative role of climatic stability on biodiversity is important to any prediction of extinction risk under climate change.

The combination of conservation concerns within highly biodiverse regions is epitomised within global 'biodiversity hotspots'. These regions are delineated by high species diversity and endemism (>1,500 endemic species), and the degree of habitat loss (>70% of primary vegetation; Myers, Mittermeier, Mittermeier, da Fonseca, & Kent, 2000). The disproportionate risk of extinction in biodiversity hotspots is also exacerbated by the fact that a considerable proportion of global biodiversity remains undocumented (Joppa, Roberts, Myers, & Pimm, 2011). Additional surveys and genetic research routinely reveal species obviously new to science but also more subtle distinctions in the form of cryptic species (Adams, Raadik, Burridge & Georges, 2014; Beheregaray & Caccone, 2007; Struck et al., 2018). Thus, accurately delimiting species remains a critical component of conservation management at both the taxon-specific (through revision of species classifications

and associated legislation) and regional (through identifying hotspots of cryptic diversity) scales.

The first (of only two) biodiversity hotspots to be declared for Australia was the temperate southwest, commonly referred to as the Southwest Western Australia (SWWA) hotspot or the Southwest Australia Floristic Region (Hopper & Gioia, 2004; Myers et al., 2000). This region features high floristic diversity and endemism, with >8,000 species of plants recorded and >4,000 of those endemic to the region (Gioia & Hopper, 2017). While species diversity appears lower for animal groups (~500 vertebrate species), relatively high rates of endemism (~23%) suggest that this pattern extends to non-plant taxa as well (Rix et al., 2015). The region's classification as a biodiversity hotspot is also driven by extensive habitat loss and deforestation, with ~70% of native land vegetation cleared primarily for agricultural purposes (Habel et al., 2019; Monks et al., 2019). Despite the breadth of biodiversity, the SWWA features a simplistic landscape with limited topographic variation (Funnekotter, Millar, Krauss, & Nevill, 2019), no major river drainage divides, stable geology (Hopper & Gioia, 2004) and little climatic variation since the Pliocene (Spooner, De Deckker, Barrows, & Fifield, 2011). Within the region, biogeographic subdivisions have been delineated primarily based on rainfall, including the High Rainfall Province (HRP, >600mm annual rainfall) and the Transitional Rainfall Province (TRP, 300 – 600mm annual rainfall; Hopper & Gioia, 2004; Rix et al., 2015).

Several biogeographic mechanisms have been proposed to explain the high biodiversity of the SWWA (detailed in Rix et al., 2015). These include ancient Gondwanan lineages that diverged throughout the Mesozoic until the late Eocene

~100 Ma (Hopper, Smith, Fay, Manning, & Chase, 2009); vicariantly-isolated mesic lineages that diverged from eastern Australian lineages during the Miocene (14–16 Ma; Buckley et al., 2018; Crisp & Cook, 2007; Rix & Harvey, 2012); as well as *in situ* diversification (Hopper & Gioia, 2004). Despite the apparent lack of topographic or environmental barriers (Cowling & Lombard, 2002), SWWA studies have demonstrated both interspecific (speciation) and intraspecific (phylogeographic structure) diversification primarily associated with late Miocene – early Pliocene aridification and contraction of mesic refugia (Byrne et al., 2011; Rix et al., 2015; Rix & Harvey, 2012). Intraspecific phylogeographic structure has been described in several plants (e.g. Dalmaris, Ramalho, Poot, Veneklaas, & Byrne, 2015; Nistelberger, Gibson, Macdonald, Tapper, & Byrne, 2014), narrow range terrestrial invertebrates (e.g. Cooper, Harvey, Saint, & Main, 2011; Harms, 2018), and freshwater fauna (e.g. Klunzinger et al., 2020; Unmack, Bagley, Adams, Hammer, & Johnson, 2012), suggesting that traits such as limited dispersal and ecological specialisation may play a role in driving phylogeographic patterns. Understanding how these factors may drive diversification within the region is key to predicting how biodiversity may respond to anthropogenic climate change.

Freshwater taxa are ideal models for investigating the role of climatic history on diversification as their limited dispersal capacity and reliance on constrained habitats make them effective indicators of historical environmental change (Thomaz, Malabarba, & Knowles, 2017). Freshwater species also show a high propensity for cryptic diversity, enabling thorough investigations of the interaction of Earth history and diversification (e.g. Buckley, Brauer, Unmack, Hammer, & Beheregaray, 2021; Waters, Burridge, & Craw, 2020). Within SWWA, the western pygmy perch

(*Nannoperca vittata*) and little pygmy perch (*Nannoperca pygmaea*) demonstrate these traits, given their late Miocene origins (Buckley et al., 2018), limited dispersal capacity (Beatty, Morgan, Rashnavadi, & Lymbery, 2011), and role as ecological specialists (Allen, Morgan, Close, & Beatty, 2020). *Nannoperca vittata* is significantly more widespread and occurs throughout the HRP, whereas *N. pygmaea* has a restricted occurrence within only three rivers and a lake in the south-eastern HRP (Allen et al., 2020). While both species show some similar ecological characteristics such as body plan and reproductive strategy, they show marked differences in body size, morphology, growth rate, salinity tolerance and reproductive timing (Allen et al., 2020). Additionally, clear-cut genetic differentiation in allozymes (Morgan, Beatty, & Adams, 2013), mitochondrial genes (Unmack, Hammer, Adams, & Dowling, 2011) and genome-wide markers (Buckley et al., 2018), and a lack of observed hybridisation with *N. vittata* (Morgan et al., 2013; Allen et al., 2020), corroborates the identity of the *N. pygmaea* as a distinct species. This is reflected by their strong evolutionary distinctiveness, with divergence between the two species estimated at ~4 million years ago (Buckley et al., 2018; Unmack et al., 2011). In addition, up to three distinct cryptic species have been suggested within *N. vittata* based on allozyme, mitochondrial and nuclear data (Unmack et al., 2011), and genome-wide data (Buckley et al., 2018). However, these studies were either lacking in genetic resolution (Unmack et al., 2011) or geographic sampling (Buckley et al., 2018), and as such a more thorough investigation of phylogeographic patterns across the region is required to refine hypotheses of species delineation.

Nannoperca pygmaea is currently listed as Endangered under both the national Environment Protection and Biodiversity Conservation (EPBC) Act 1999 and state

legislation, whilst *N. vittata* is unlisted under the EPBC Act with no local legislative protections (Allen et al., 2020). Freshwater fishes within the SWWA are currently impacted by secondary salinization of rivers (Beatty et al., 2011), land clearing leading to habitat alteration (Andrich & Imberger, 2013) and introduced freshwater fishes (Beatty & Morgan, 2013). These threats are exacerbated by reduced rainfall and increased temperatures over the last few decades (Smith & Power, 2014), which are predicted to worsen under anthropogenic climate change (Pittock, Hansen, & Abell, 2008). Thus, freshwater taxa within the SWWA appear to be ubiquitously threatened and may be more susceptible to extinction than their current conservation status suggests.

Here, we investigate the role of long-term climatic stability in the SWWA biodiversity hotspot on the diversification and accumulation of lineages in a group of freshwater fishes. We used genomic (ddRAD-seq) data to characterise divergence across the clade based on genetic differentiation, phylogenetic patterns and species delimitation. Additionally, we used environmental data and modelling analyses to reconstruct species and lineage distributions since the Pliocene. We hypothesised that long-term climatic stability across the biodiversity hotspot would lead to both the maintenance of divergent and isolated genetic lineages, including cryptic species, and long-term stable species distributions. Alternatively, if localized biogeographic events resulted in the diversification of pygmy perches in the SWWA, we expect that divergence events would coincide with ancient environmental changes and fluctuating lineage and species distributions over time.

2. Methods

2.1 Sample Collection and Library Preparation

Sampling sites were selected to capture the full distribution of the lineages spanning the HRP, as well as a potentially relictual population found in the northernmost extreme of the distribution in the TRP (Figure 1; Buckley et al., 2018; Unmack et al., 2011). This sampling design covers all described and previously suggested cryptic species within a broader *N. vittata* species complex (Buckley et al., 2018; Unmack et al. 2011), with a total of 25 *N. vittata* from seven sites and eight *N. pygmaea* from two sites sampled (Table S1). An additional four Yarra pygmy perch (*Nannoperca obscura*) were included as an outgroup for phylogenetic analyses (Buckley et al., 2018). Specimens were collected using electrofishing, dip-, fyke- or seine-netting (see Allen et al., 2020). Either the caudal fin or the entire specimen was stored at −80°C at the South Australian Museum, or in 99% ethanol at the Molecular Ecology Lab at Flinders University.

DNA was extracted from muscle tissue or fin clips using a combination of a modified salting-out method (Sunnucks & Hales, 1996) and a Qiagen DNeasy kit (Qiagen Inc., Valencia, CA, USA). Genomic DNA was checked for quality using a spectrophotometer (NanoDrop, Thermo Scientific), integrity using 2% agarose gels, and quantity using a fluorometer (Qubit, Life Technologies). The ddRAD genomic libraries were prepared in house following Brauer, Hammer, & Beheregaray (2016). Of the 33 samples, eight were paired-end sequenced on an Illumina HiSeq 2000 at Genome Quebec (Montreal, Canada) as part of a previous phylogenomic study (Buckley et al., 2018). The remaining 25 samples were single-end sequenced on a single lane of Illumina HiSeq 2500 at the South Australia Health and Medical Research Institute in Adelaide.

2.2 Filtering and Alignment

Sequences were demultiplexed using the ‘process_radtags’ module of Stacks 1.29 (Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013), allowing up to 2 mismatches in the 6 bp barcodes. Barcodes were removed and sequences trimmed to 80 bp to remove low-quality bases from the end of the reads. Cut reads (forward only for paired-end) were aligned using PyRAD 3.0.6 (Eaton, 2014), and further cleaned by removing reads with >5 bp with a Phred score < 20. Loci were retained if they occurred in at least ~80% of samples (30) within the dataset. For SNP-based analyses, a single SNP per ddRAD locus was subsampled to reduce the impact of linkage disequilibrium.

2.3 Population Divergence and Population Clustering

We first assessed population divergence by estimating mean pairwise uncorrected genetic distances (*p*-distances) between populations using PAUP* 4 (Swofford, 2002) based on the full sequences of all ddRAD loci. Population clustering was assessed using a PCoA of all SNPs in dartR (Gruber, Unmack, Berry, & Georges, 2018). We also calculated the number of fixed differences between pairwise population comparisons (Unmack et al., 2022), with SNPs considered fixed differences at a threshold of 0.05 (i.e. >95% frequency in one population and <5% in the other) to account for sequencing errors or ‘near fixation’ (Gruber et al., 2018; Johnson & Slatkin, 2007).

2.4 Phylogenetic Analysis

A ML phylogeny was estimated using RAxML 8.2.11 (Stamatakis 2014) and the concatenated sequence alignment to determine evolutionary relationships and guide species delimitation analyses. The ML phylogeny was estimated under the GTR+Γ model of evolution and 1,000 resampling estimated log-likelihood bootstraps. A ML phylogeny with the alignment partitioned by ddRAD locus was also estimated, as well as estimating individual gene trees per locus, using IQ-TREE2 (Minh et al., 2020b). This was done to account for the potential impact of genome-wide heterogeneity in evolutionary rates (Liu, Xi, & Davis, 2015). Concordance between gene trees and the partitioned phylogeny was estimated using site and gene concordance factors (Minh, Hahn, & Lanfear, 2020a), with a summary species tree estimated using ASTRAL-III (Zhang, Rabiee, Sayyari, & Mirarab, 2018) and assuming each population as an individual 'species' (Rabiee, Sayyari, & Mirarab, 2019).

2.5 Species Delimitation and Divergence Time Estimates

The species tree and delimitation were estimated using SNAPP 1.5.0 (Bryant, Bouckaert, Felsenstein, Rosenberg, & RoyChoudhury, 2012) within BEAST 2.6.1 (Bouckaert et al., 2019). We iteratively tested nine different scenarios of species composition, ranging from two to nine species (i.e. each population as a separate species) based on phylogenetic patterns (see Figure 2d). Given that SNAPP can resolve species identities with only a few thousand SNPs (Leaché, Fujita, Minin, & Bouckaert, 2014), and to reduce computational time, we subsampled the alignment down to the two individuals with the lowest missing data per population and 5,000 randomly selected SNPs. We used broad priors with gamma distributions for speciation rate (λ; $\alpha = 3$, $\beta = 2.5$) and population sizes (θ; $\alpha = 2.85$, $\beta = 955.27$)

based on sequence divergence for all scenarios to cover possible parameter ranges. Mutation rate priors were left at their default settings. Two separate chains were run per model to assess convergence of parameter estimates. Models were run for at least 10 million generations and/or until ESS > 200 was reliably achieved. Model traces were visualised using Tracer 1.5 (Rambaut & Drummond, 2009) with the first two million generations discarded as burn-in. Species composition likelihoods were estimated using an AIC through Markov chain Monte Carlo analysis (AICM; Raftery, Newton, Satagopan, & Krivitsky, 2006). Although other methods such as reverse-jump MCMC via path-sampling or stepping-stone analysis are more widely used (Grummer, Bryson, & Reeder, 2013), given the large size of the dataset we opted to use AICM based on its reasonable performance and reduced computational demand (Baele et al., 2012).

Divergence times across the complex were inferred by using an extension of SNAPP described in Stange, Sanchez-Villagra, Salzburger, & Matschiner (2018). We calibrated the oldest divergence in the tree using a normal distribution with mean of 9.27 Ma and standard deviation of 0.51 (95% CI = mean ± 1 Ma) based on previous divergence estimates for all pygmy perches (Buckley et al., 2018). We applied broader ranges around calibration nodes than previously suggested to accommodate potential variation in calibration age not captured by the methods in that study. The topology of the tree was fixed using the ML tree and the model run for one million generations. Confidence intervals of divergence times were inferred using TreeAnnotator 2.6 (Drummond & Rambaut, 2019) and 95% posterior probabilities.

2.6 Historical Admixture and Introgression

We tested whether populations and lineages were historically isolated using two different allele frequency-based approaches. First, we determined historical connectivity across populations based on the SNP dataset using TreeMix (Pickrell & Pritchard, 2012). The number of migrations within the model were iteratively increased from none to nine, with the fit of each model estimated using the covariance matrices and overall tree likelihood. Additionally, we calculated the percentage of variation explained per migration model (https://github.com/wlz0726/Population_Genomics_Scripts/tree/master/03.treemix), with the best supported number of migrations determined by the asymptote of likelihood. We also determined whether substantial introgression occurred between putative species (see Results) suggested by species delimitation approaches using an ABBA-BABA test (Martin, Davey, & Jiggins, 2014) in D-Suite (Malinsky, Matschiner & Svardal, 2021). Introgression was determined assuming a pattern of divergence following the phylogenetic tree and summarised based on Patterson's D (Patterson et al., 2012) and the fraction of introgressed alleles (f_4-ratio).

2.7 Species and Lineage Distribution modelling

Species distribution models (SDMs) for *N. vittata* were estimated using an ensemble modelling approach within biomod2 (Thuiller, Lafourcade, Engler, & Araújo, 2009). SDMs were estimated across eleven time periods ranging from contemporary conditions to the Pliocene using the PaleoClim database (Brown, Hill, Dolan, Carnaval, & Haywood, 2018). These time periods included the Late Holocene (4.2–0.3 Kya), Mid Holocene (8.326–4.2 Kya), Early Holocene (11.7–8.326 Kya), Younger Dryas Stadial (12.9–11.7 Kya), Bølling-Allerød (14.7–12.9 Kya), Heinrich Stadial 1 (17.0–14.7 Kya), Last Interglacial (~130 Kya), MIS19 (~787 Kya), mid Pliocene warm

period (3.205 Ma), and M2 (~3.3 Ma) phases. Occurrence records for all *N. vittata* were obtained from a combination of sampled sites within this and past studies (Allen et al., 2020; Buckley et al., 2018; Morgan, Gill, & Potter, 1998; Morgan et al., 2013; Unmack et al., 2011), as well as from the Atlas of Living Australia (http://www.ala.org.au). We further filtered the occurrence data to reduce the impact of spatial autocorrelation (Elith et al., 2011) by removing occurrences outside the known distribution of species (P. Unmack, pers. Comm.; Allen *et al.* 2020) and sampling a single occurrence per environmental raster cell. This resulted in a final dataset of 114 observations used within the SDMs.

Highly correlated climatic variables ($r < |0.8|$) were pruned from the dataset based on a Pearson's correlation test within SDMToolbox (Brown, Bennett, & French, 2017), resulting in eight climatic layers used across the models (Table S2). The final input variables were annual mean temperature (bio1), isothermality (bio3), mean temperature of the wettest quarter (bio8), mean temperature of the driest quarter (bio9), mean temperature of coldest quarter (bio11), annual precipitation (bio12), precipitation of the driest month (bio14), precipitation seasonality (bio15) and sea-level corrected elevation. For the three oldest time periods (Late Pleistocene-Pliocene), bio3 was unavailable and thus not included within the projections. We generated three replicates of 1,000 pseudoabsences randomly from the background >30 km from occurrences to reduce the likelihood of generating false absences within habitable areas. Each dataset was replicated three times, with 80% of sites independently and randomly subset to train the model (n = nine datasets).

The SDMs were estimated using five separate models: classification tree analysis (CTA), a generalised boosting model (GBM), multiple adaptive regression splines (MARS), maximum entropy (MaxEnt) and random forest (RF) (n = 45 models total). These approaches cover a range of different statistical approaches (Hao, Elith, Guillera-Arroita, & Lahoz-Monfort, 2019), and model averaging in an ensemble framework is expected to reduce bias associated with any single method (Marmion, Parviainen, Luoto, Heikkinen, & Thuiller, 2009). Each model was evaluated using both the relative operating characteristic (ROC) and the true skill statistic (TSS). Individual methods were checked for consistency by averaging models per method under contemporary conditions. Ensemble SDMs amalgamating the results of all SDMs per time period were generated, excluding models with TSS < 0.7. The mean and standard deviation of suitability were calculated across ensemble models to assess long-term habitat suitability trends.

The ensemble species distribution model was then subdivided into individual lineage-specific distributions following the cost-distance approach described in Rosauer, Catullo, VanDerWal, Moussalli, & Moritz (2015). Lineage distribution models (LDMs) were determined by assigning the seven *N. vittata* localities to one of two putative cryptic species based on the species delimitation results. *Nannoperca pygmaea* was not included as a lineage given its narrow current range and sympatry with *N. vittata* (Morgan et al., 2013).

3. Results

3.1 Bioinformatics

The combined sequencing runs returned a total of 71.44 million reads, with an average of 2.16M reads per sample. After quality control and alignment of sequences, a dataset of 19,426 ddRAD loci was obtained across all putative species. This alignment contained 18,177 putatively unlinked biallelic SNPs and an average of 4.93 (±1.76)% missing data per sample (Figure S1.1).

3.2 Population Divergence

Pairwise genetic distances and fixed differences inferred highly divergent structure across the species complex. Pairwise population averages of genetic differences ranged from 0.03% to 1.55% whist the number of pairwise fixed differences ranged from 8 to 5,947 SNPs (Figure 2a). A PCoA demonstrated three major and highly divergent clusters, with the two primary axes explaining a combined total of 77.6% of the variation (Figure 2b). The three clusters are herein referred to as *N. pygmaea, N. vittata* [A] (widespread across SWWA; 5 sites) and *N. vittata* [B] (restricted to south-central HRP; 2 sites). Similar clusters could be observed within the pairwise *p*-distances (>0.5% between clusters) and by the number of fixed differences (>2000 between clusters).

3.3 Phylogenetic Analysis

Partitioning the alignment by ddRAD loci did not affect the topology or branch lengths of the tree, and site concordance factors supported this tree (Figure S1.2a–S1.2c). The summary species tree likewise showed strong support for the separation of lineages, with posterior probabilities of one for almost all nodes (Figure S1.2d). The highly divergent nature of lineages across the group was supported by the concatenated phylogeny, with all population-level and above nodes supported by

100% bootstrap values (Figure 2c). The phylogeny demonstrated three divergent lineages matching the clusters defined earlier. Despite their co-occurrence, the sympatric populations of *N. vittata* [A] and *N. pygmaea* (NviHay and NpyMR) were not closely associated within the phylogenetic tree; instead, *N. vittata* [B] was the sister lineage to *N. pygmaea*.

3.4 Species Delimitation and Divergence Time

Species delimitation models increased in likelihood with an increasing number of species, with the highest likelihood and lowest AICM for a delimitation scenario considering all populations as separate species (Figure 2d; scenario S10). However, there was a noticeable plateau in AICM values after S5, with greater numbers of species conferring relatively small increases in model likelihood beyond this point (Figure S1.3). Divergence time estimates suggested that most lineages diverged from one another between 1 and 5 Ma, with most populations diverging more recently, within the last 300 Kya (Figure 3). For all nodes, there was minimal overlap in 95% confidence intervals.

3.5 Gene Flow and Introgression

No signal of historical population connectivity was inferred across the clade, with TreeMix best supporting a tree that contained no migrations over models with any (Figure 4). This non-migratory model explained 99.99% of the variation in allele frequencies (Figure 4; Figure S1.4). Similarly, D-Suite results showed negligible evidence of introgression across putative species, with a Patterson's *D* of 0.20 and f_4-ratio of 0.028.

3.6 Species Distribution Modelling

All models demonstrated high fit to the data, accurately capturing the full distribution of the species complex (Figure S1.5a). Ensemble models built separately for each method demonstrated highly similar areas of suitability, suggesting that variation among methods was minimal (Figure S1.5c). Annual precipitation (bio12) was the strongest driving variable across all models, with approximately double the variable importance of all others (Figure S1.5b). The ensemble projections of the SDMs over time showed little fluctuation in distribution extent since the Pliocene (Figure 5a). Across all time periods, the most significant portion of suitable habitat tended towards the coastal edges of the region, with some expansion across the continental shelf during lower sea levels but not inland. This long-term distribution stability was similarly reflected by high mean suitability (Figure 5b) and low standard deviation (Figure 5c) over time. Similar to the SDMs, LDMs appeared stable over time, excluding during the mid-Pliocene (3.2 Ma) where there was more significant overlap and a northward shift in southern lineages (Figure S1.6).

4. Discussion

Several divergent lineages were identified across a species complex of freshwater fish endemic to a temperate biodiversity hotspot, including at least one cryptic species of pygmy perch. We demonstrated that these lineages probably persisted in isolation since the late Miocene (9–3.5 Ma) over a climatically stable region. Current and projected climatic conditions disproportionately threaten this region because its high lineage diversity appears to have evolved in the absence of major climatic and geological changes.

4.1 Maintenance of Highly Divergent Lineages Through Climatic Stability

The highly divergent and ancient nature of lineages within the clade was likely facilitated by the long-term climatic stability of the region, with divergence events spanning the Miocene and Pliocene. Although major environmental changes associated with aridification during the Pliocene impacted the biota of the SWWA (Hopper & Gioia, 2004), temperature and rainfall remained relatively constant throughout the glacial cycles of the Pleistocene, especially within the HRP (Rix et al., 2015). The stable species distribution reflected these relatively constant climatic conditions since the Pliocene (Spooner et al., 2011), with a coastward expansion when sea levels were lower during glacial maxima. Similar patterns of limited variation in inland regions have been shown for several coastal plant species in the SWWA (Nevill, Bradbury, Williams, Tomlinson, & Krauss, 2014; Nistelberger et al., 2014), suggesting that distribution extent was consistently limited by aridity and temperature in inland habitats (Brouwers et al., 2012). These spatial patterns reflect the intensity of aridification further inland compared to the relatively benign HRP (Rix et al., 2015), and likely placed significant limitations on inland range expansions of *N. vittata*.

While causation of divergences within the clade was not inferred here, the historical demographic isolation of lineages, the overall low genetic diversity and low dispersal capacity of pygmy perches (Brauer et al., 2016; Buckley et al., 2018) together suggest that their persistence must owe, in part, to an enduring stable habitat. Overall, these results add to a growing body of evidence that broad-scale and long-term climatic stability in the SWWA has driven the accumulation of regional biodiversity (Appendix 2). These endemic species encompass an array of lineages

ranging from Gondwanan relicts originating as early as the Cretaceous to late Miocene – early Pliocene diversification events (Rix et al., 2015), reflecting both ancient and recent lineages (Sundaram et al., 2019). These concerted patterns thus demonstrate how biodiversity in SWWA has persisted and accumulated over millions of years due to the region's climatic stability.

4.2 Diversification of Western Australian Pygmy Perches

Our results highlight a hierarchy of divergence across Western Australia's *Nannoperca* clade, ranging from morphologically differentiated and cryptic species to divergent populations. No historical migration nor introgression was detected across the clade, indicating that the inferred evolutionary distinctiveness is a product of long-term isolation. Summaries of population divergence largely identified three major clusters, including two separate *N. vittata* lineages, one of which was sister to *pygmaea*. This was supported with phylogenetic analysis, which highlighted the paraphyletic nature of the species name *N. vittata* (although *N. pymgaea* was also recently split from the *N. vittata* complex).

Species delimitation results implied the presence of a higher number of putative species within the complex, with greatest support for a model considering each population a separate species, and an approximate plateau in likelihood at five species. However, multispecies coalescent approaches such as SNAPP have been demonstrated to over-split lineages (Chambers & Hillis, 2020), particularly when populations are highly structured and divergent (Derkarabetian, Castillo, Koo, Ovchinnikov, & Hedin, 2019). Thus, we instead suggest that the genomic data unequivocally indicate that at least three species are present in the clade

corresponding to the lineages *N. pygmaea*, *N. vittata* [A] and *N. vittata* [B], following similar nomenclature in Unmack et al., 2011. This conservative interpretation of three species both resolves the current paraphyly of *N. vittata* and avoids the risk of over-splitting (Coates, Byrne, & Moritz, 2018). A morphological revision is required to diagnose the three lineages (i.e. especially *N. vittata* [B]), and then match these to the morphotypes of available names of which two are potentially available; *N. vittata* [non-specific type locality] and *Nannoperca viridis* [King George Sound near Albany]. It is more likely that type material of *N. vittata* s.s. was collected from within the wide range of *N. vittata* [A] compared to the more inaccessible south-central HRP (habitat of *N. vittata* [B]), and if so *N. viridis* occurring well to the east is likely to remain in synonymy with *N. vittata* s.s., and a new description required for *N. vittata* [B].

Taxonomic revision of the species complex is critical given that species are the most common operational unit of conservation management (Stanton et al., 2019), and particularly, are the focus of protective legislation (Coates et al., 2018). For example, the identification of the narrowly distributed *N. vittata* [B] lineage implies that it may be of greater conservation concern than the current status of *N. vittata* would denote (Buckley et al., 2018), with its situation being more parallel to *N. pygmaea* considered as one of the most imperilled freshwater fishes in the country (Lintermans et al., 2020). Given that *N. vittata* [B] and *N. pygmaea* occupy narrow distributions under threat from salinisation and aridification (Allen et al., 2020), and that pygmy perches are highly vulnerable to human-induced habitat loss (Brauer & Beheregaray, 2020), *ex situ* conservation efforts such as translocation and the formation of insurance populations may be required in the immediate future (as done for other pygmy perches; Hammer et al., 2013; Marshall et al., 2022). These

programs will need to maximise the maintenance of genetic diversity (Attard et al. 2016) and may rely on genetic rescue across populations of putative species (Bell et al., 2019).

A previous phylogenomic study of all pygmy perch species, including species delimitation using a multi-species coalescent approach, suggested that *N. vittata* was a complex of three cryptic species (Buckley et al., 2018). However, this was based on few populations within SWWA, all of which were included in this study. Two of these species correspond to the most divergent populations within *N. vittata* [A] (NviCBP and NviAR), suggesting that the higher number of species proposed in the previous study reflected a lack of intermediate sampling within the lineage. However, given that NviCBP was the most divergent population within the *N. vittata* [A] lineage despite being located in the centre of its geographic range, the possibility remains that it represents a fourth candidate species, as predicted by an allozyme dataset for over 40 *N. vittata* [A] sites (M. Adams, unpublished).

4.3 Phylogeographic Structure Within the SWWA

Intraspecific phylogeographic structure was also detected in the most widespread putative species (*N. vittata* [A]), with three groups possibly representing evolutionarily-significant units (ESU) following Funk, McKay, Hohenlohe, & Allendorf (2012). This is based on their relatively ancient divergence (>1 Myr) and long-term demographic and spatial isolation. Within this delineation, the Margaret River population (NviCBP) represented a unique ESU: this is corroborated by other aquatic taxa in the region, with a unique ESU of a freshwater mussel (*Westralunio carteri;* Benson, Stewart, Close, & Lymbery, 2022; Klunzinger et al., 2020) and two

morphologically distinct species of freshwater crayfish (the hairy marron (*Cherax tenuimanus;* Vercoe, Lawrence, & de Graaf, 2009) and the Margaret River burrowing crayfish (*Engaewa pseudoreducta*; Allen, Beatty & Morgan, 2017)) endemic to Margaret River. The remaining two ESUs captured disjunct pairs of populations at opposing extremes of the species distribution (in the north and the southeast).

4.4 Implications for Conservation of Biodiversity Hotspots

Freshwater biodiversity is considered one of the most threatened groups globally (Collen et al., 2014; Lintermans et al., 2020). Within the SWWA, freshwater species are currently threatened by several convergent issues, including ongoing aridification since the mid-last century (Smith & Power, 2014), habitat clearing primarily for agricultural development (Andrich & Imberger, 2013), secondary salinization of rivers (Morgan, Thorburn & Gill, 2003; Allen et al., 2020) and invasive species (Morgan, Gill, Maddern & Beatty, 2004; Beatty & Morgan, 2013). These threats will likely be exacerbated by anthropogenic climate change and their own interactive effects (Dinnage, Skeels, & Cardillo, 2020; Beatty, Morgan, & Lymbery, 2014; Ogston, Beatty, Morgan, Pusey & Lymbery, 2016; Stewart, Ford, & Benson, 2022): projections of climate change alterations alone predict 40–50% loss of habitat and 10–44% loss of endemic plants (294–1,293 species) in the SWWA (Habel et al., 2019). Additionally, spatial heterogeneity in the effects of climate change may affect some lineages more than others (Hansen et al., 2019; Stewart et al., 2022), such as due to a northern contraction of suitable mesic habitat (Klausmeyer & Shaw, 2009) or loss of permanent refuge pools (Allen et al., 2020). Relatedly, a reduction in fitness associated with heterogeneous climate change has already been observed in some SWWA plant species (Brouwers et al., 2012; Dalmaris et al., 2015; Monks et

al., 2019). Conservation programs must therefore consider both the spatial heterogeneity of these impacts, and how they may interact in increasing extinction risk, especially for freshwater fauna.

Disruption of climatic regimes across the globe, in conjunction with broadly increased temperatures (Jeremias et al., 2018), aridity (Davis, Pavlova, Thompson, & Sunnucks, 2013) and sea levels (Rotzoll & Fletcher, 2012) is already impacting species persistence globally. These effects will be exacerbated within regions of low historical climate variability due to both low species resilience and high species diversity, with compounding effects for coastal temperate ecosystems (Buckley et al., 2021). Species that evolved in benign climates are not expected to have the same adaptive resilience as those in more variable environments (Gutiérrez-Pesquera et al., 2016). Additionally, the high biodiversity of historically stable regions may mean they become hotspots of extinction as swathes of taxa struggle to respond to the selective challenges of anthropogenic climate change (Waldvogel et al., 2020). Given that biodiversity hotspots may also have an excess of undiscovered and cryptic biodiversity (Joppa et al., 2011), current estimates of extinction rates may be underestimated (Le Roux et al., 2019). Together, these components highlight why biodiversity hotspots are regions of high conservation concern and should be priorities in conservation management to maximise conservation of at-risk species and ecological communities. Two of the three putative species delineated here have small ranges, a pattern shared by other narrow-range endemics in biodiversity hotspots (Goldberg, Roy, Lande, & Jablonski, 2005).

Long-term climatic stability has driven the accumulation of divergent and isolated lineages of freshwater fishes in SWWA, providing a mechanism for its status as a biodiversity hotspot. This included multiple diversifications within the *N. vittata* complex. Given that regional climates are being disrupted globally by climate change, and that species that evolved in historically stable regions may lack the adaptive mechanisms to respond, biodiversity hotspots like SWWA may be exceptionally threatened. These findings only further solidify the conservation need for these regions, with preservation of threatened habitat such as rivers and refugial pools crucial for the persistence of a suite of endemic species. For those species most at risk, adaptive conservation management strategies such as the formation of insurance populations and captive breeding programs may become necessary to protect lineages with lower adaptive resilience to rapidly changing environments.

Conflict of Interest

The authors declare that there is no conflict of interest.

Data Availability Statement

Data to be made available via Figshare upon acceptance.

Figure Legends

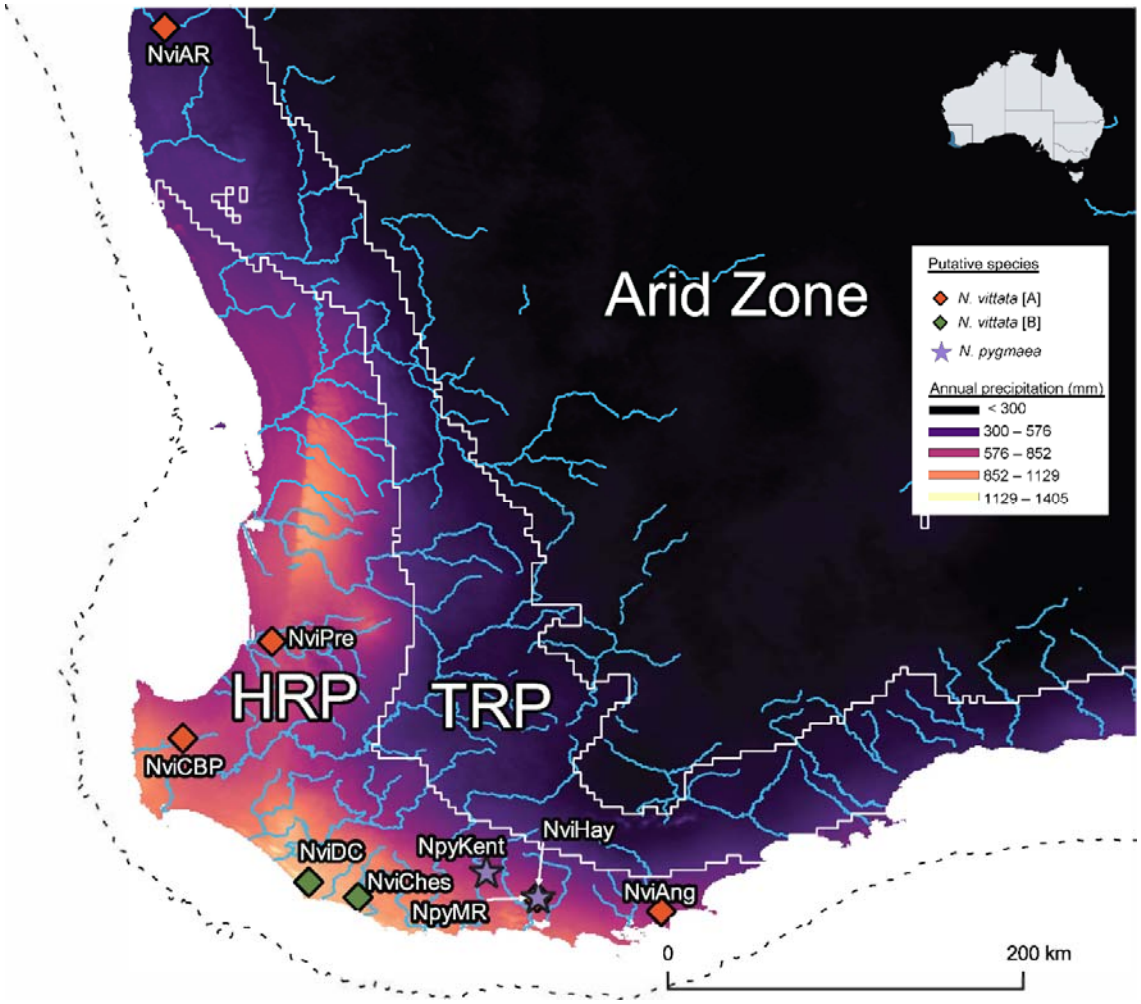
Figure 1: Map of sampling sites used within this study, and major rivers across the SWWA. Localities for *Nannoperca vittata* are indicated by diamonds and localities for *Nannoperca pygmaea* are indicated with stars. Locality colours indicate putative species (see Results). Colouration of the map indicates annual precipitation (derived from WorldClim 2.1). Solid white lines indicate biogeographic province boundaries. Dashed line indicates the maximum landmass extent exposed during the Last Glacial Maximum (LGM). TRP = Transitional Rainfall Province. HRP = High Rainfall Province.

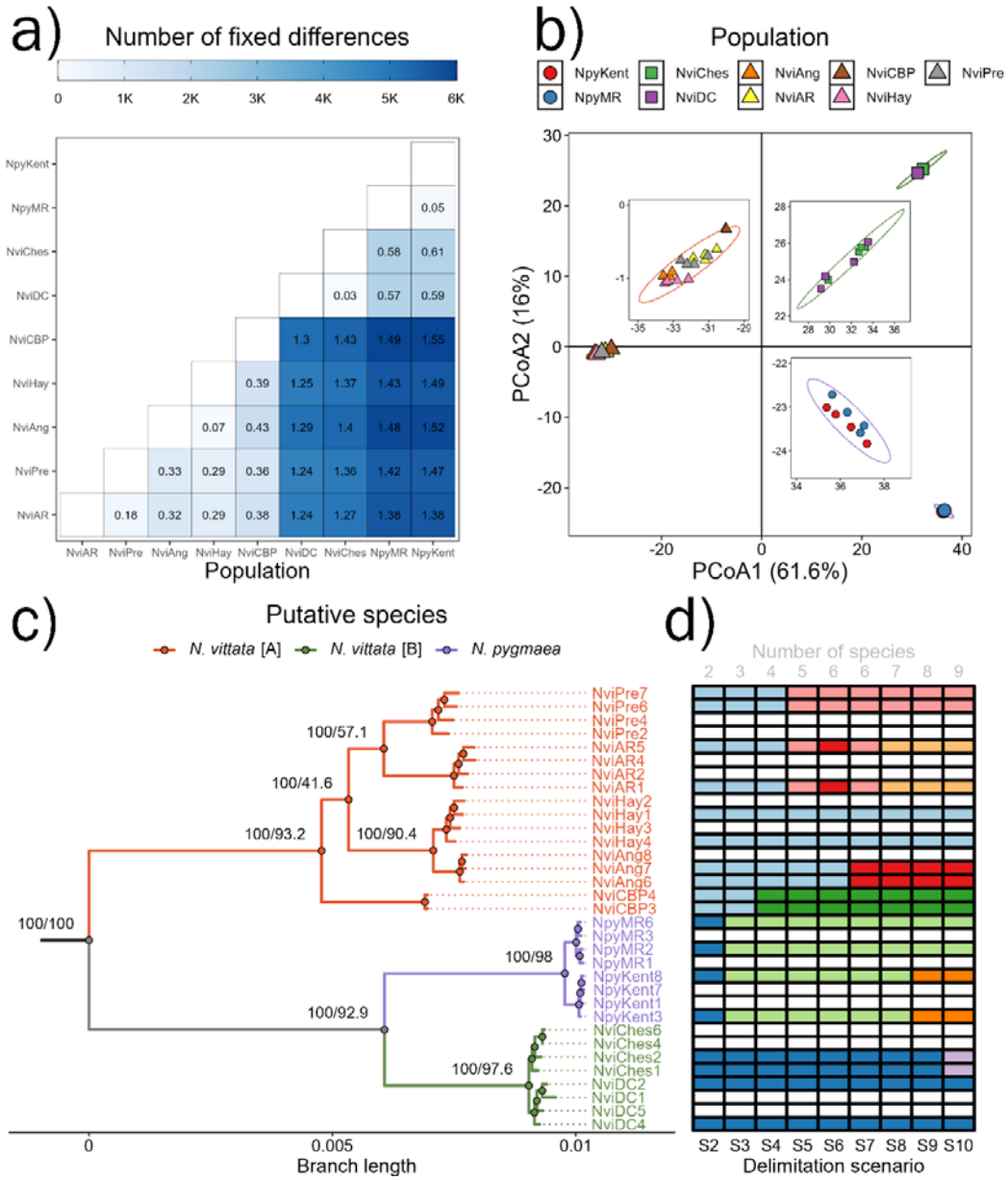
Figure 2: Population divergence and clustering. **a)** Heatmap of pairwise population summaries of divergence. Numbers within cells indicate pairwise uncorrelated genetic distances (p-distance; %) based on 19,426 ddRAD loci, whilst colours indicate the number of fixed differences within the 18,177 unlinked SNPs. **b)** PCoA of 18,177 unlinked SNPs. Each point represents the centroid per population with shapes indicating assignment to a cluster (i.e. a putative species: circles = *Nannoperca pygmaea*, triangles = *Nannoperca vittata* [A] and squares = *N. vittata* [B]). Insets depict close-ups of individuals within each cluster. **c)** ML tree estimated using 19,426 concatenated ddRAD loci and IQ-TREE2. Nodes are labelled with bootstrap support estimated using 1,000 RELL bootstraps and site concordance factors (respectively). Branches are coloured by putative species. **d)** Allocation of samples to species delimitation scenarios in SNAPP. Each column represents a single model, with each row corresponding to the aligned sample in the phylogenetic tree. Cell colours indicate allocation to species, with cells of the same colour indicating one species. Blank rows indicate samples that were not used in species delimitation.

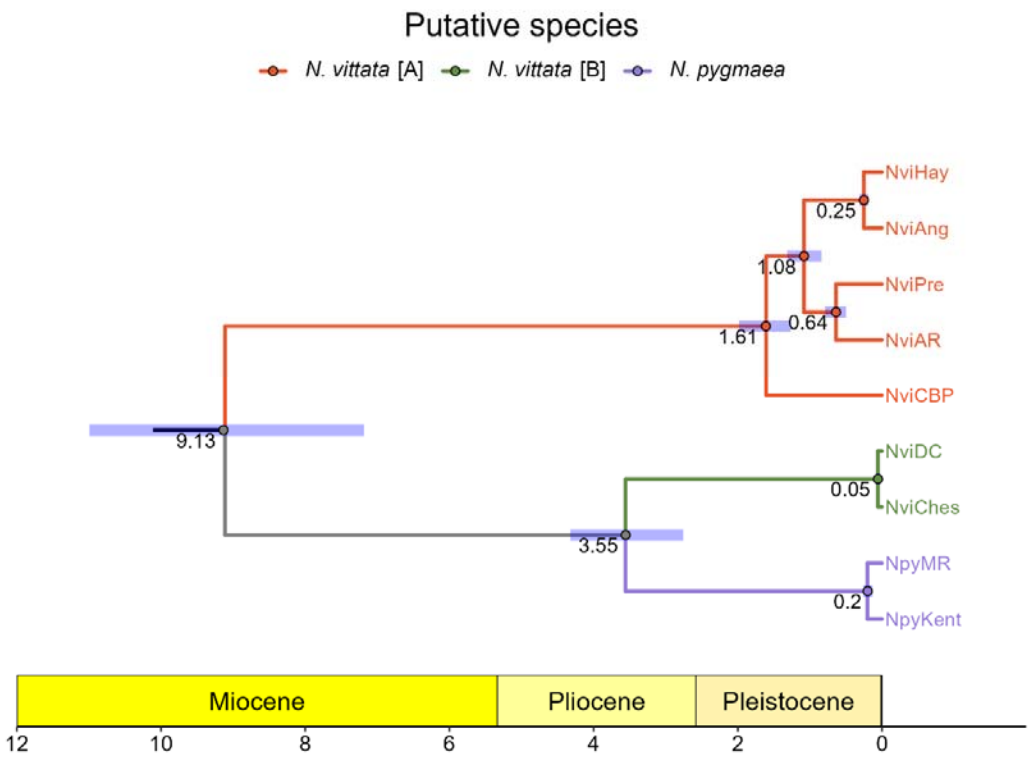
Figure 3: Chronogram of divergence time estimates across the species complex using SNAPP. Node labels indicate median divergence times, and error bars show 95% posterior probabilities from 1M simulations. Lineages are coloured according to putative species.

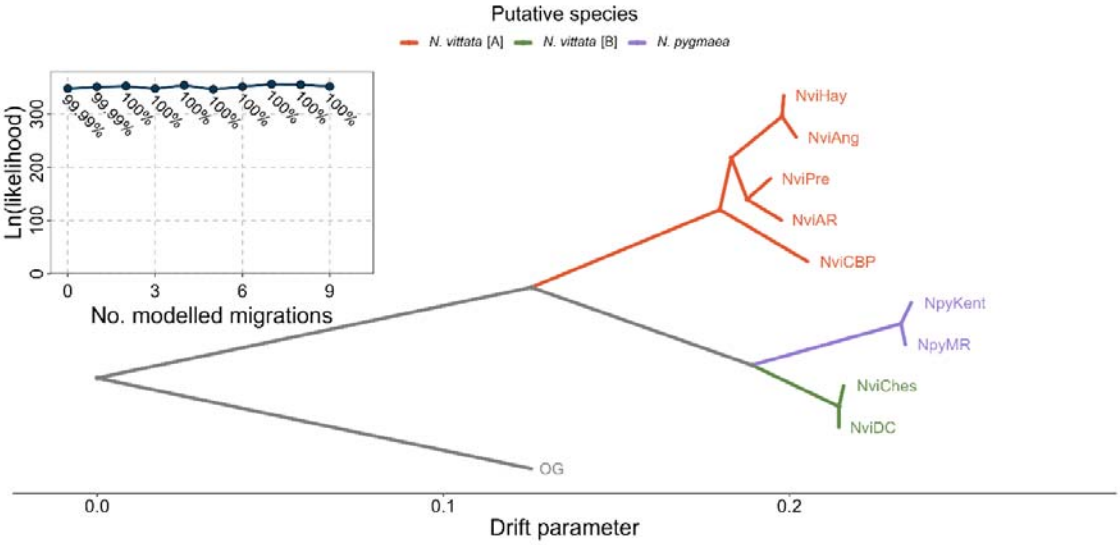
Figure 4: Best supported historical migration model using TreeMix, with no modelled migrations and lineages coloured by putative species. Inset depicts likelihood and percentage of variation explained (labels) per model under increasing number of migrations.

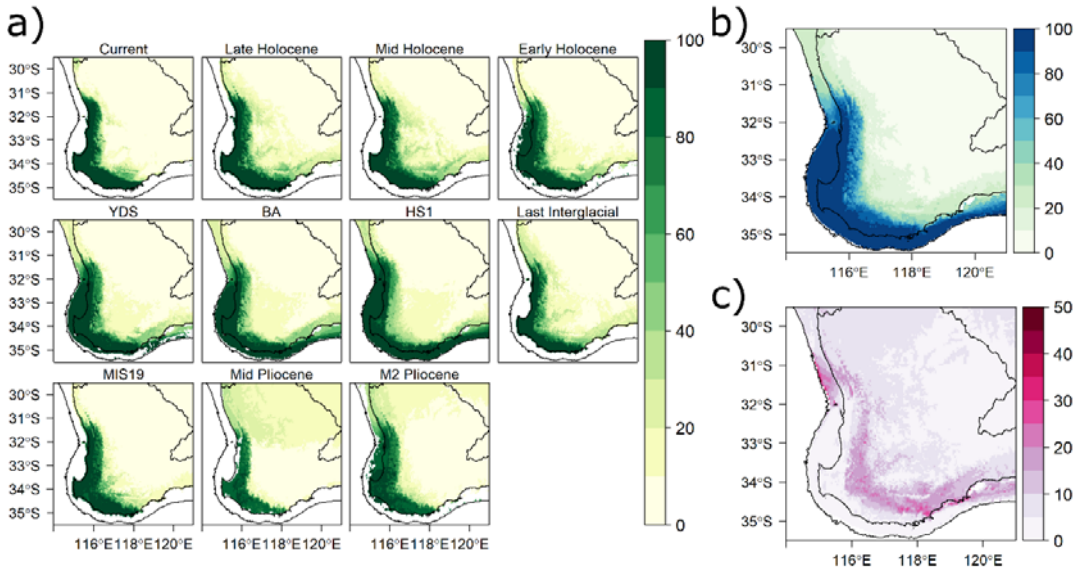
Figure 5: SDMs across eleven time periods, ranging from current conditions back to the Pliocene. **a)** Ensemble SDMs and projections summarising 45 individual models. **b)** Mean cell suitability across all eleven time periods. **c)** Standard deviation of cell suitability across all eleven time periods.


NviAR
Arid Zone
Putative species
N. vittata [A]
N. vittata [B]
N. pygmaea
Annual precipitation (mm)
< 300
300 – 576
576 – 852
852 – 1129
1129 – 1405
NviPre
HRP
TRP
NviCBP
NviHay
NpyKent
NviDC
NviChes
NpyMR
NviAng
0
200 km


a)
Number of fixed differences
0
1K
2K
3K
4K
5K
6K
NpyKent
NpyMR
NviChes
NviDC
NviCBP
NviHay
NviAng
NviPre
NviAR
Population
b)
Population
NpyKent
NpyMR
NviChes
NviDC
NviAng
NviAR
NviCBP
NviHay
NviPre
PCoA2 (16%)
PCoA1 (61.6%)
c)
Putative species
N. vittata [A]
N. vittata [B]
N. pygmaea
100/57.1
100/41.6
100/93.2
100/90.4
100/100
100/98
100/92.9
100/97.6
Branch length
d)
Number of species
Delimitation scenario
S2
S3
S4
S5
S6
S7
S8
S9
S10


Putative species
N. vittata [A]
N. vittata [B]
N. pygmaea
NviHay
NviAng
NviPre
NviAR
NviCBP
NviDC
NviChes
NpyMR
NpyKent
0.25
1.08
0.64
1.61
9.13
0.05
3.55
0.2
Miocene
Pliocene
Pleistocene
12
10
8
6
4
2
0


Putative species
N. vittata [A]
N. vittata [B]
N. pygmaea
Ln(likelihood)
0
100
200
300
99.99%
99.99%
100%
100%
100%
100%
100%
100%
100%
100%
0
3
6
9
No. modelled migrations
NviHay
NviAng
NviPre
NviAR
NviCBP
NpyKent
NpyMR
NviChes
NviDC
OG
0.0
0.1
0.2
Drift parameter


a)
Current
Late Holocene
Mid Holocene
Early Holocene
YDS
BA
HS1
Last Interglacial
MIS19
Mid Pliocene
M2 Pliocene
30°S
31°S
32°S
33°S
34°S
35°S
116°E118°E120°E
100
80
60
40
20
0
b)
100
80
60
40
20
0
c)
50
40
30
20
10
0

References

Adams, M., Raadik, T. A., Burridge, C. P. & Georges, A. (2014). Global biodiversity assessment and hyper-cryptic species complexes: more than one species of elephant in the room? *Systematic Biology, 63*(4), 518-533. doi:10.1093/sysbio/syu017.

Addo-Bediako, A., Chown, S. L., & Gaston, K. J. (2000). Thermal tolerance, climatic variability and latitude. *Proceedings of the Royal Society of London. Series B: Biological Sciences, 267*(1445), 739-745. doi:10.1098/rspb.2000.1065

Allen, M.G., Beatty, S.J., & Morgan, D.L. (2017). Aquatic fauna refuges in Margaret River and the Cape to Cape region of Australia's Mediterranean-climatic Southwestern Province. *FiSHMED: Fishes in Mediterranean Environments*, 2017.002, 1-26. doi:10.29094/FiSHMED.2017.002

Allen, M. G., Morgan, D. L., Close, P. G., & Beatty, S. J. (2020). Too little but not too late? Biology of a recently discovered and imperilled freshwater fish in a drying temperate region and comparison with sympatric fishes. *Aquatic Conservation: Marine and Freshwater Ecosystems, 30*(7), 1412-1423. doi:10.1002/aqc.3346

Andrich, M. A., & Imberger, J. (2013). The effect of land clearing on rainfall and fresh water resources in Western Australia: a multi-functional sustainability analysis. *International Journal of Sustainable Development & World Ecology, 20*(6), 549-563. doi:10.1080/13504509.2013.850752

Baele, G., Lemey, P., Bedford, T., Rambaut, A., Suchard, M. A., & Alekseyenko, A. V. (2012). Improving the accuracy of demographic and molecular clock model comparison while accommodating phylogenetic uncertainty. *Molecular Biology and Evolution, 29*(9), 2157-2167. doi:10.1093/molbev/mss084

Beatty, S., & Morgan, D. (2013). Introduced freshwater fishes in a global endemic hotspot and implications of habitat and climatic change. *BioInvasions Records, 2*(1), 1-9. doi:10.3391/bir.2013.2.1.01

Beatty, S. J., Morgan, D. L. and Lymbery, A. J. (2014). Implications of climate change for potamodromous fishes. *Global Change Biology*, 20 (6), 1794-1807. doi.org/10.1111/gcb.12444

Beatty, S., Morgan, D., Rashnavadi, M., & Lymbery, A. J. (2011). Salinity tolerances of endemic freshwater fishes of south-western Australia: implications for conservation in a biodiversity hotspot. *Marine and Freshwater Research, 62*(1), 91-100. doi:https://doi.org/10.1071/MF10100

Beheregaray, L. B., & Caccone, A. (2007). Cryptic biodiversity in a changing world. *Journal of Biology, 6*(4), 9-9. doi:10.1186/jbiol60

Bell, D. A., Robinson, Z. L., Funk, W. C., Fitzpatrick, S. W., Allendorf, F. W., Tallmon, D. A., & Whiteley, A. R. (2019). The exciting potential and remaining uncertainties of genetic recsue. *Trends in Ecology and Evolution, 34*(12): 1070-1079. doi: 10.1016/j.tree.2019.06.006.

Benson, J., Stewart, B., Close, P., & Lymbery, A. (2022). Evidence for multiple refugia and hotspots of genetic diversity for *Westralunio carteri*, a threatened freshwater mussel in south-western Australia. *Aquatic Conservation: Marine and Freshwater Ecosystems, 32*(4), 559-575. doi:https://doi.org/10.1002/aqc.3780

Bouckaert, R., Vaughan, T. G., Barido-Sottani, J., Duchêne, S., Fourment, M., Gavryushkina, A., . . . Drummond, A. J. (2019). BEAST 2.5: An advanced software platform for Bayesian evolutionary analysis. *PLoS Computational Biology, 15*(4), e1006650. doi:10.1371/journal.pcbi.1006650

Brauer, C. J., & Beheregaray, L. B. (2020). Recent and rapid anthropogenic habitat fragmentation increases extinction risk for freshwater biodiversity. *Evolutionary Applications, 13*(10), 2857-2869. doi: 10.1111/eva.13128

Brauer, C. J., Hammer, M. P., & Beheregaray, L. B. (2016). Riverscape genomics of a threatened fish across a hydroclimatically heterogeneous river basin. *Molecular Ecology, 25*(20), 5093-5113. doi:10.1111/mec.13830

Brouwers, N. C., Mercer, J., Lyons, T., Poot, P., Veneklaas, E., & Hardy, G. (2012). Climate and landscape drivers of tree decline in a Mediterranean ecoregion. *Ecology and Evolution, 3*(1), 67-79. doi:10.1002/ece3.437

Brown, J. L., Bennett, J. R., & French, C. M. (2017). SDMtoolbox 2.0: the next generation Python-based GIS toolkit for landscape genetic, biogeographic and species distribution model analyses. *PeerJ, 5*, e4095. doi:10.7717/peerj.4095

Brown, J. L., Hill, D. J., Dolan, A. M., Carnaval, A. C., & Haywood, A. M. (2018). PaleoClim, high spatial resolution paleoclimate surfaces for global land areas. *Scientific Data, 5*(1), 180254. doi:10.1038/sdata.2018.254

Bryant, D., Bouckaert, R., Felsenstein, J., Rosenberg, N. A., & RoyChoudhury, A. (2012). Inferring species trees directly from biallelic genetic markers: Bypassing gene trees in a full coalescent analysis. *Molecular Biology and Evolution, 29*(8), 1917-1932. doi:10.1093/molbev/mss086

Buckley, S. J., Brauer, C. J., Unmack, P. J., Hammer, M. P., & Beheregaray, L. B. (2021). The roles of aridification and sea level changes in the diversification and persistence of freshwater fish lineages. *Molecular Ecology, 30*(19), 4866-4883. doi:https://doi.org/10.1111/mec.16082

Buckley, S. J., Domingos, F. M. C. B., Attard, C., Brauer, C. J., Sandoval-Castillo, J., Lodge, R., . . . Beheregaray, L. B. (2018). Phylogenomic history of enigmatic pygmy perches: implications for biogeography, taxonomy and conservation. *Royal Society Open Science, 5*(172125). doi:10.1098/rsos.172125

Byrne, M., Steane, D. A., Joseph, L., Yeates, D. K., Jordan, G. J., Crayn, D., . . . Weston, P. H. (2011). Decline of a biome: evolution, contraction, fragmentation, extinction and invasion of the Australian mesic zone biota. *Journal of Biogeography, 38*(9), 1635-1656. doi:10.1111/j.1365-2699.2011.02535.x

Carnaval, A. C., Hickerson, M. J., Haddad, C. B., Rodrigues, M. T., & Moritz, C. (2009). Stability predicts genetic diversity in the Brazilian Atlantic forest hotspot. *Science, 323*(5915), 785-789. doi: 10.1126/science.1166955

Catchen, J., Hohenlohe, P. A., Bassham, S., Amores, A., & Cresko, W. A. (2013). Stacks: an analysis tool set for population genomics. *Molecular Ecology, 22*(11), 3124-3140. doi:10.1111/mec.12354

Chambers, E. A., & Hillis, D. M. (2020). The multispecies coalescent over-splits species in the case of geographically widespread taxa. *Systematic Biology, 69*(1), 184-193. doi:10.1093/sysbio/syz042

Coates, D. J., Byrne, M., & Moritz, C. (2018). Genetic diversity and conservation units: Dealing with the species-population continuum in the age of genomics. *Frontiers in Ecology and Evolution, 6*(165), 165. doi:10.3389/fevo.2018.00165

Collen, B., Whitton, F., Dyer, E. E., Baillie, J. E., Cumberlidge, N., Darwall, W. R., . . . Bohm, M. (2014). Global patterns of freshwater species diversity, threat and endemism. *Global Ecology and Biogeography, 23*(1), 40-51. doi:10.1111/geb.12096

Cooper, S. J., Harvey, M. S., Saint, K. M., & Main, B. Y. (2011). Deep phylogeographic structuring of populations of the trapdoor spider *Moggridgea tingle* (Migidae) from

southwestern Australia: evidence for long-term refugia within refugia. *Molecular Ecology, 20*(15), 3219-3236. doi:10.1111/j.1365-294X.2011.05160.x

Cowling, R. M., & Lombard, A. T. (2002). Heterogeneity, speciation/extinction history and climate: explaining regional plant diversity patterns in the Cape Floristic Region. *Diversity and Distributions, 8*(3), 163-179. doi:10.1046/j.1472-4642.2002.00143.x

Crisp, M. D., & Cook, L. G. (2007). A congruent molecular signature of vicariance across multiple plant lineages. *Molecular Phylogenetics and Evolution, 43*(3), 1106-1117. doi:10.1016/j.ympev.2007.02.030

Dalmaris, E., Ramalho, C. E., Poot, P., Veneklaas, E. J., & Byrne, M. (2015). A climate change context for the decline of a foundation tree species in south-western Australia: insights from phylogeography and species distribution modelling. *Annals of Botany, 116*(6), 941-952. doi:10.1093/aob/mcv044

Davis, J., Pavlova, A., Thompson, R., & Sunnucks, P. (2013). Evolutionary refugia and ecological refuges: key concepts for conserving Australian arid zone freshwater biodiversity under climate change. *Global Change Biology, 19*(7), 1970-1984. doi:10.1111/gcb.12203

Derkarabetian, S., Castillo, S., Koo, P. K., Ovchinnikov, S., & Hedin, M. (2019). A demonstration of unsupervised machine learning in species delimitation. *Molecular Phylogenetics and Evolution, 139*, 106562. doi:10.1016/j.ympev.2019.106562

Dinnage, R., Skeels, A., & Cardillo, M. (2020). Spatiophylogenetic modelling of extinction risk reveals evolutionary distinctiveness and brief flowering period as threats in a hotspot plant genus. *Proceedings of the Royal Society B: Biological Sciences, 287*(1926), 20192817. doi:10.1098/rspb.2019.2817

Drummond, A. J., & Rambaut, A. (2019). TreeAnnotator (Version 2.6). http://beast.bio.ed.ac.uk.

Dynesius, M., & Jansson, R. (2000). Evolutionary consequences of changes in species' geographical distributions driven by Milankovitch climate oscillations. *Proceedings of the National Academy of Sciences, 97*(16), 9115-9120. doi:10.1073/pnas.97.16.9115

Eaton, D. A. (2014). PyRAD: assembly of de novo RADseq loci for phylogenetic analyses. *Bioinformatics, 30*(13), 1844-1849. doi:10.1093/bioinformatics/btu121

Elith, J., Phillips, S. J., Hastie, T., Dudík, M., Chee, Y. E., & Yates, C. J. (2011). A statistical explanation of MaxEnt for ecologists. *Diversity and Distributions, 17*(1), 43-57. doi:10.1111/j.1472-4642.2010.00725.x

Funk, W. C., McKay, J. K., Hohenlohe, P. A. & Allendorf, F. W. (2012). Harnessing genomics for delineating conservation units. *Trends in Ecology and Evolution, 27*(9), 489-496. doi:10.1016/j.tree.2012.05.012.

Funnekotter, A. V., Millar, M., Krauss, S. L., & Nevill, P. G. (2019). Phylogeographic analyses of *Acacia karina* (Fabaceae) support long term persistence of populations both on and off banded iron formations. *Australian Journal of Botany, 67*(3), 194. doi:10.1071/bt18045

Gioia, P., & Hopper, S. D. (2017). A new phytogeographic map for the Southwest Australian Floristic Region after an exceptional decade of collection and discovery. *Botanical Journal of the Linnean Society, 184*(1), 1-15. doi:10.1093/botlinnean/box010

Goldberg, E. E., Roy, K., Lande, R., & Jablonski, D. (2005). Diversity, endemism, and age distributions in macroevolutionary sources and sinks. *The American Naturalist, 165*(6), 623-633. doi:10.1086/430012

Gruber, B., Unmack, P. J., Berry, O. F., & Georges, A. (2018). dartr: An r package to facilitate analysis of SNP data generated from reduced representation genome

sequencing. *Molecular Ecology Resources, 18*(3), 691-699. doi:10.1111/1755-0998.12745

Grummer, J. A., Bryson, R. W., Jr., & Reeder, T. W. (2013). Species delimitation using Bayes factors: Simulations and application to the *Sceloporus scalaris* species group (Squamata: Phrynosomatidae). *Systematic Biology, 63*(2), 119-133. doi:10.1093/sysbio/syt069

Gutiérrez-Pesquera, L. M., Tejedo, M., Olalla-Tárraga, M. Á., Duarte, H., Nicieza, A., & Solé, M. (2016). Testing the climate variability hypothesis in thermal tolerance limits of tropical and temperate tadpoles. *Journal of Biogeography, 43*(6), 1166-1178. doi:10.1111/jbi.12700

Habel, J. C., Rasche, L., Schneider, U. A., Engler, J. O., Schmid, E., Rödder, D., . . . Stork, N. E. (2019). Final countdown for biodiversity hotspots. *Conservation Letters, 12*(6). doi:10.1111/conl.12668

Hammer, M. P., Bice, C. M., Hall, A., Frears, A., Watt, A., Whiterod, N. S., … Zampatti, B. P. (2013). Freshwater fish conservation in the face of critical water shortages in the southern Murray–Darling Basin, Australia. *Marine and Freshwater Research, 64*(9), 807-821. doi:10.1071/MF12258.

Hansen, B. B., Pedersen, Å. Ø., Peeters, B., Le Moullec, M., Albon, S. D., Herfindal, I., . . . Aanes, R. (2019). Spatial heterogeneity in climate change effects decouples the long-term dynamics of wild reindeer populations in the high Arctic. *Global Change Biology, 25*(11), 3656-3668. doi:https://doi.org/10.1111/gcb.14761

Hao, T., Elith, J., Guillera-Arroita, G., & Lahoz-Monfort, J. J. (2019). A review of evidence about use and performance of species distribution modelling ensembles like BIOMOD. *Diversity and Distributions, 25*(5), 839-852. doi:10.1111/ddi.12892

Harms, D. (2018). The origins of diversity in ancient landscapes: Deep phylogeographic structuring in a pseudoscorpion (Pseudotyrannochthoniidae: *Pseudotyrannochthonius*) reflects Plio-Pleistocene climate fluctuations. *Zoologischer Anzeiger, 273*, 112-123. doi:10.1016/j.jcz.2018.01.001

Harrison, S., & Noss, R. (2017). Endemism hotspots are linked to stable climatic refugia. *Annals of Botany, 119*(2), 207-214. doi:10.1093/aob/mcw248

Hopper, S. D., & Gioia, P. (2004). The Southwest Australian Floristic Region: Evolution and conservation of a global hot spot of biodiversity. *Annual Review of Ecology, Evolution, and Systematics, 35*(1), 623-650. doi:10.1146/annurev.ecolsys.35.112202.130201

Jeremias, G., Barbosa, J., Marques, S. M., Asselman, J., Gonçalves, F. J. M., & Pereira, J. L. (2018). Synthesizing the role of epigenetics in the response and adaptation of species to climate change in freshwater ecosystems. *Molecular Ecology, 27*(13), 2790-2806. doi:10.1111/mec.14727

Johnson, P. L. F., & Slatkin, M. (2007). Accounting for bias from sequencing error in population genetic estimates. *Molecular Biology and Evolution, 25*(1), 199-206. doi:10.1093/molbev/msm239

Joppa, L. N., Roberts, D. L., Myers, N., & Pimm, S. L. (2011). Biodiversity hotspots house most undiscovered plant species. *Proceedings of the National Academy of Sciences, 108*(32), 13171-13176. doi:10.1073/pnas.1109389108

Klausmeyer, K. R., & Shaw, M. R. (2009). Climate change, habitat loss, protected areas and the climate adaptation potential of species in mediterranean ecosystems worldwide. *PLoS ONE, 4*(7), e6392. doi:10.1371/journal.pone.0006392

Klunzinger, M. W., Lopes-Lima, M., Gomes-dos-Santos, A., Froufe, E., Lymbery, A. J., & Kirkendale, L. (2020). Phylogeographic study of the West Australian freshwater mussel, *Westralunio carteri*, uncovers evolutionarily significant units that raise new conservation concerns. *Hydrobiologia, 848*, 2951-2964. doi:10.1007/s10750-020-04200-6

Le Roux, J. J., Hui, C., Castillo, M. L., Iriondo, J. M., Keet, J. H., Khapugin, A. A., . . . Hirsch, H. (2019). Recent anthropogenic plant extinctions differ in biodiversity hotspots and coldspots. *Current Biology, 29*(17), 2912-2918 e2912. doi:10.1016/j.cub.2019.07.063

Leaché, A. D., Fujita, M. K., Minin, V. N., & Bouckaert, R. R. (2014). Species delimitation using genome-wide SNP data. *Systematic Biology, 63*(4), 534-542. doi:10.1093/sysbio/syu018

Lintermans, M., Geyle, H. M., Beatty, S., Brown, C., Ebner, B. C., Freeman, R., . . . Garnett, S. T. (2020). Big trouble for little fish: identifying Australian freshwater fishes in imminent risk of extinction. *Pacific Conservation Biology*, *26*(4), 365-377. doi:https://doi.org/10.1071/PC19053

Liu, L., Xi, Z., & Davis, C. C. (2015). Coalescent methods are robust to the simultaneous effects of long branches and incomplete lineage sorting. *Molecular Biology and Evolution, 32*(3), 791-805. doi:10.1093/molbev/msu331

Malinsky, M., Matschiner, M., & Svardal, H. (2021). Dsuite - fast D-statistics and related admixture evidence from VCF files. *Molecular Ecology Resources*, *21*, 584-595. doi: 10.1111/1755-0998.13265

Marmion, M., Parviainen, M., Luoto, M., Heikkinen, R. K., & Thuiller, W. (2009). Evaluation of consensus methods in predictive species distribution modelling. *Diversity and Distributions, 15*(1), 59-69. doi:10.1111/j.1472-4642.2008.00491.x

Marshall, I. R., Brauer, C. J., Wedderburn, S. D., Whiterod, N. S., Hammer, M. P., Barnes, T. C., Attard, C. R. M., Möller, L. M., & Beheregaray, L. B. (2022). Longitudinal monitoring of neutral and adaptive genomic diversity in a reintroduction. *Conservation Biology, 36*, e13889. doi:10.1111/cobi.13889.

Martin, S. H., Davey, J. W., & Jiggins, C. D. (2014). Evaluating the use of ABBA–BABA statistics to locate introgressed loci. *Molecular Biology and Evolution, 32*(1), 244-257. doi:10.1093/molbev/msu269

Mercier, K. P., Vasconcellos, M. M., Martins, E. G. A., Pirani, J. R., Michelangeli, F. A., & Carnaval, A. C. (2023). Linking environmental stability with genetic diversity and population structure in two Atlantic Forest palm trees. *Journal of Biogeography, 50,* 197-208. doi: 10.1111/jbi.14523

Minh, B. Q., Hahn, M. W., & Lanfear, R. (2020a). New methods to calculate concordance factors for phylogenomic datasets. *Molecular Biology and Evolution. 37*(9), 2727-2733. doi:10.1093/molbev/msaa106

Minh, B. Q., Schmidt, H. A., Chernomor, O., Schrempf, D., Woodhams, M. D., von Haeseler, A., & Lanfear, R. (2020b). IQ-TREE 2: New models and efficient methods for phylogenetic inference in the genomic era. *Molecular Biology and Evolution, 37*(5), 1530-1534. doi:10.1093/molbev/msaa015

Mittelbach, G. G., Schemske, D. W., Cornell, H. V., Allen, A. P., Brown, J. M., Bush, M. B., . . . Turelli, M. (2007). Evolution and the latitudinal diversity gradient: speciation, extinction and biogeography. *Ecology Letters, 10*(4), 315-331. doi:10.1111/j.1461-0248.2007.01020.x

Monks, L., Barrett, S., Beecham, B., Byrne, M., Chant, A., Coates, D., . . . Yates, C. (2019). Recovery of threatened plant species and their habitats in the biodiversity hotspot of

the Southwest Australian Floristic Region. *Plant Diversity, 41*(2), 59-74. doi:10.1016/j.pld.2018.09.006

Morgan, D. L., Beatty, S. J., & Adams, M. (2013). *Nannoperca pygmaea*, a new species of pygmy perch (Teleostei: Percichthyidae) from Western Australia. *Zootaxa, 3637*(4), 401. doi:10.11646/zootaxa.3637.4.1

Morgan, D.L., Gill, H.S., Maddern, M.G. & Beatty, S.J. (2004). Distribution and impacts of introduced freshwater fishes in Western Australia. *New Zealand Journal of Marine and Freshwater Research,* 38, 511-523.

Morgan, D.L., Gill, H.S. & Potter, I.C. (1998). Distribution, identification and biology of freshwater fishes in south-western Australia. *Records of the Western Australian Museum*, Supplement 56, 1-97.

Morgan, D.L., Thorburn, D.C. & Gill, H.S. (2003). Salinization of south-western Western Australian rivers and the implications for the inland fish fauna – the Blackwood River, a case study. *Pacific Conservation Biology*, 9, 161-171.

Moritz, C., Patton, J. L., Schneider, C. J., & Smith, T. B. (2000). Diversification of rainforest faunas: An integrated molecular approach. *Annual Review of Ecology and Systematics, 31*(1), 533-563. doi:10.1146/annurev.ecolsys.31.1.533

Myers, N., Mittermeier, R. A., Mittermeier, C. G., da Fonseca, G. A. B., & Kent, J. (2000). Biodiversity hotspots for conservation priorities. *Nature, 403*(6772), 853-858. doi:10.1038/35002501

Nevill, P. G., Bradbury, D., Williams, A., Tomlinson, S., & Krauss, S. L. (2014). Genetic and palaeo-climatic evidence for widespread persistence of the coastal tree species *Eucalyptus gomphocephala* (Myrtaceae) during the Last Glacial Maximum. *Annals of Botany, 113*(1), 55-67. doi:10.1093/aob/mct253

Nistelberger, H., Gibson, N., Macdonald, B., Tapper, S. L., & Byrne, M. (2014). Phylogeographic evidence for two mesic refugia in a biodiversity hotspot. *Heredity, 113*(5), 454-463. doi:10.1038/hdy.2014.46

Ogsten, G., Beatty, S., Morgan, D., Pusey, B., & Lymbery, A. (2016). Living on burrowed time: Aestivating fishes in south-western Australia face extinction due to climate change. *Biological Conservation, 195*, 235-244. doi: 10.1016/j.biocon.2016.01.008

Patterson, N., Moorjani, P., Luo, Y., Mallick, S., Rohland, N., Zhan, Y., . . . Reich, D. (2012). Ancient admixture in human history. *Genetics, 192*(3), 1065-1093. doi:10.1534/genetics.112.145037

Pickrell, J. K., & Pritchard, J. K. (2012). Inference of population splits and mixtures from genome-wide allele frequency data. *PLOS Genetics, 8*(11), e1002967. doi:10.1371/journal.pgen.1002967

Pittock, J., Hansen, L. J., & Abell, R. (2008). Running dry: Freshwater biodiversity, protected areas and climate change. *Biodiversity, 9*(3-4), 30-38. doi:10.1080/14888386.2008.9712905

Rabiee, M., Sayyari, E., & Mirarab, S. (2019). Multi-allele species reconstruction using ASTRAL. *Molecular Phylogenetics and Evolution, 130*, 286-296. doi:10.1016/j.ympev.2018.10.033

Raftery, A. E., Newton, M. A., Satagopan, J. M., & Krivitsky, P. N. (2006). Estimating the integrated likelihood via posterior simulation using the harmonic mean identity. *Bayesian Statistics*.

Rambaut, A., & Drummond, A. J. (2009). Tracer v1.5. http://beast.bio.ed.ac.uk.

Rix, M. G., Edwards, D. L., Byrne, M., Harvey, M. S., Joseph, L., & Roberts, J. D. (2015). Biogeography and speciation of terrestrial fauna in the south-western Australian biodiversity hotspot. *Biological Reviews, 90*(3), 762-793. doi:10.1111/brv.12132

Rix, M. G., & Harvey, M. S. (2012). Phylogeny and historical biogeography of ancient assassin spiders (Araneae: Archaeidae) in the Australian mesic zone: evidence for Miocene speciation within Tertiary refugia. *Molecular Phylogenetics and Evolution, 62*(1), 375-396. doi:10.1016/j.ympev.2011.10.009

Rosauer, D. F., Catullo, R. A., VanDerWal, J., Moussalli, A., & Moritz, C. (2015). Lineage range estimation method reveals fine-scale endemism linked to Pleistocene stability in Australian rainforest herpetofauna. *PLoS ONE, 10*(5), e0126274. doi:10.1371/journal.pone.0126274

Rotzoll, K., & Fletcher, C. H. (2012). Assessment of groundwater inundation as a consequence of sea-level rise. *Nature Climate Change, 3*, 477. doi:10.1038/nclimate1725

Sandel, B., Arge, L., Dalsgaard, B., Davies, R. G., Gaston, K. J., Sutherland, W. J., & Svenning, J.-C. (2011). The influence of Late Quaternary climate-change velocity on species endemism. *Science, 334*(6056), 660-664. doi:10.1126/science.1210173

Sandoval-Castillo, J., Gates, K., Brauer, C. J., Smith, S., Bernatchez, L., & Beheregaray, L. B. (2020). Adaptation of plasticity to projected maximum temperatures and across climatically defined bioregions. *Proceedings of the National Academy of Sciences, 117*(29), 17112-17121. doi:10.1073/pnas.1921124117

Smith, I., & Power, S. (2014). Past and future changes to inflows into Perth (Western Australia) dams. *Journal of Hydrology: Regional Studies, 2*, 84-96. doi:https://doi.org/10.1016/j.ejrh.2014.08.005

Spooner, M. I., De Deckker, P., Barrows, T. T., & Fifield, L. K. (2011). The behaviour of the Leeuwin Current offshore NW Australia during the last five glacial–interglacial cycles. *Global and Planetary Change, 75*(3-4), 119-132. doi:10.1016/j.gloplacha.2010.10.015

Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics, 30*(9), 1312-1313. doi: 10.1093/bioinformatics/btu033.

Stange, M., Sanchez-Villagra, M. R., Salzburger, W., & Matschiner, M. (2018). Bayesian divergence-time estimation with genome-wide single-nucleotide polymorphism data of sea catfishes (Ariidae) supports Miocene closure of the Panamanian Isthmus. *Systematic Biology, 67*(4), 681-699. doi:10.1093/sysbio/syy006

Stanton, D. W. G., Frandsen, P., Waples, R. K., Heller, R., Russo, I.-R. M., Orozco-terWengel, P. A., . . . Bruford, M. W. (2019). More grist for the mill? Species delimitation in the genomic era and its implications for conservation. *Conservation Genetics, 20*(1), 101-113. doi:10.1007/s10592-019-01149-5

Stewart, B. A., Ford, B. J. & Benson, J. A. (2022). Using species distribution modelling to identify ‘coldspots’ for conservation of freshwater fishes under a changing climate. *Aquatic Conservation: Marine and Freshwater Ecosystems, 32*(4), 576-590. doi: 10.1002/aqc.3793.

Struck, T. H., Feder, J. L., Bendiksby, M., Birkeland, S., Cerca, J., Gusarov, V. I., . . . Dimitrov, D. (2018). Finding evolutionary processes hidden in cryptic species. *Trends in Ecology & Evolution, 33*(3), 153-163. doi:10.1016/j.tree.2017.11.007

Sundaram, M., Donoghue, M. J., Farjon, A., Filer, D., Mathews, S., Jetz, W., & Leslie, A. B. (2019). Accumulation over evolutionary time as a major cause of biodiversity

hotspots in conifers. *Proceedings of the Royal Society B: Biological Sciences, 286*(1912), 20191887. doi:10.1098/rspb.2019.1887

Sunnucks, P., & Hales, D. F. (1996). Numerous transposed sequences of mitochondrial cytochrome oxidase I-II in aphids of the genus *Sitobion* (Hemiptera: Aphididae). *Molecular Biology and Evolution, 13*(3), 510-524. doi:10.1093/oxfordjournals.molbev.a025612

Swofford, D. L. (2002). *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.0b10* (Vol. Version 4.0). Sunderland, Massachusetts: Sinauer Associates.

Thomaz, A. T., Malabarba, L. R., & Knowles, L. L. (2017). Genomic signatures of paleodrainages in a freshwater fish along the southeastern coast of Brazil: genetic structure reflects past riverine properties. *Heredity, 119*(4), 287-294. doi:10.1038/hdy.2017.46

Thuiller, W., Lafourcade, B., Engler, R., & Araújo, M. B. (2009). BIOMOD – a platform for ensemble forecasting of species distributions. *Ecography, 32*(3), 369-373. doi:10.1111/j.1600-0587.2008.05742.x

Unmack, P. J., Adams, M., Hammer, M. P., Johnson, J. B., Gruber, B., Gilles, A., … Georges, A. (2022). Plotting for change: an analytical framework to aid decisions on which lineages are candidate species in phylogenomic species discovery. *Biological Journal of the Linnean Society, 135*(1), 117-137. doi:10.1093/biolinnean/blab095

Unmack, P. J., Hammer, M. P., Adams, M., & Dowling, T. E. (2011). A phylogenetic analysis of pygmy perches (Teleostei: Percichthyidae) with an assessment of the major historical influences on aquatic biogeography in southern Australia. *Systematic Biology, 60*(6), 797-812. doi:10.1093/sysbio/syr042

Vercoe, P., Lawrence, C., & de Graaf, M. (2009). Rapid replacement of the critically endangered hairy marron by the introduced smooth marron (Decapoda, Parastacidae) in the Margaret River (Western Australia). *Crustaceana, 82*(11), 1469-1476. doi:10.1163/156854009x463937

Waldvogel, A. M., Feldmeyer, B., Rolshausen, G., Exposito-Alonso, M., Rellstab, C., Kofler, R., . . . Pfenninger, M. (2020). Evolutionary genomics can improve prediction of species' responses to climate change. *Evolution Letters, 4*(1), 4-18. doi:10.1002/evl3.154

Waters, J. M., Burridge, C. P., & Craw, D. (2020). River capture and freshwater biological evolution: A review of galaxiid fish vicariance. *Diversity, 12*(6), 216. doi:10.3390/d12060216

Zhang, C., Rabiee, M., Sayyari, E., & Mirarab, S. (2018). ASTRAL-III: polynomial time species tree reconstruction from partially resolved gene trees. *BMC Bioinformatics, 19*(6), 153. doi:10.1186/s12859-018-2129-y

Biosketch

This work represents a component of Sean Buckley's PhD work in the Molecular Ecology Lab at Flinders University (MELFU) studying the phylogeographic history and conservation of pygmy perches. The MELFU team focuses on applying genomic and environmental information to address pure and applied research questions in the

conservation management, ecology and evolution of (predominantly) aquatic taxa. For more information visit https://molecularecology.flinders.edu.au/.

Author contributions: Sean James Buckley contributed to all sections of data generation and analysis, as well as drafting the manuscript. Luciano Beheregaray designed and supervised the study, and helped with manuscript drafting. Peter Unmack, Michael Hammer, Mark Adams, Stephen Beatty and David Morgan all contributed with samples and field expertise. Chris Brauer assisted with population genomic analyses and interpretation. All authors contributed to the interpretation of results and critically revised the manuscript. All authors read and approved the final manuscript.