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2 **The roles of aridification and sea level changes in the**
3 **diversification and persistence of freshwater fish lineages**

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20 ABSTRACT

21 While the influence of Pleistocene climatic changes on divergence and speciation
22 has been well-documented across the globe, complex spatial interactions between
23 hydrology and eustatics over longer timeframes may also determine species
24 evolutionary trajectories. Within the Australian continent, glacial cycles were not
25 associated with changes in ice cover and instead largely resulted in fluctuations from
26 moist to arid conditions across the landscape. Here, we investigate the role of
27 hydrological and coastal topographic changes brought about by Plio-Pleistocene
28 climatic changes on the biogeographic history of a small Australian freshwater fish,
29 the southern pygmy perch *Nannoperca australis*. Using 7,958 ddRAD-seq (double
30 digest restriction-site associated DNA) loci and 45,104 filtered SNPs, we combined
31 phylogenetic, coalescent and species distribution analyses to investigate the relative
32 roles of aridification, sea level and tectonics and their associated biogeographic
33 changes across southeast Australia. Sea-level changes since the Pliocene and
34 reduction or disappearance of large waterbodies throughout the Pleistocene were
35 determining factors in strong divergence across the clade, including the initial
36 formation and maintenance of a cryptic species, *N. 'flindersi'*. Isolated climatic
37 refugia and fragmentation due to lack of connected waterways maintained the
38 identity and divergence of inter- and intraspecific lineages. Our historical findings
39 suggest that predicted increases in aridification and sea level due to anthropogenic
40 climate change might result in markedly different demographic impacts, both
41 spatially and across different landscape types.

42

43 Keywords: phylogeography, ddRAD, aridification, sea level rise, southeast Australia,
44 freshwater fish, Perciichthyidae

45 INTRODUCTION

46 Dramatic changes in climate, hydrology and topography have long been
47 recognised to have lasting impacts on the diversity, distribution and divergence of
48 species and populations (Pelletier et al. 2015). Understanding the relationship
49 between the historical environment and the genealogy of species remains critical for
50 interpreting how contemporary climate change may impact on species currently and
51 in the near future. Most notably, increasing aridification and rising sea-levels
52 predicted by climate change projections call into question the adaptive capacity and
53 resilience of organisms, especially those with poor dispersal potential and narrow
54 ranges (Davis et al. 2013, Falkenmark 2013, Grummer et al. 2019). These effects
55 are particularly exacerbated within regions of highly heterogeneous topography and
56 climatic variation which can lead to diverse and multifaceted impacts on species
57 (Guarnizo and Cannatella 2013, Graae et al. 2018). Applying broad-scale inferences
58 about environmental changes to understand historical biogeography and biodiversity
59 resilience in the future is further complicated by spatial variation in environmental
60 factors that might impact on how within-species responses vary across their ranges
61 (Razgour et al. 2019).

62

63 The relative role of Earth history events on the evolution and persistence of
64 species is expected to vary across regions (Barton et al. 2013). Even a single major
65 event may present multifaceted impacts on species evolution depending on how
66 local or regional environments are shaped (e.g. sea level changes; Lambeck et al.
67 2012). For example, studies have highlighted the role of glacial refugia throughout
68 Pleistocene glacial – interglacial cycles driving distributional shifts across the
69 northern hemisphere, particularly within the Americas and Europe (Carnaval et al.

70 2009, Duckett et al. 2013, Pelletier et al. 2015). Although glacial ice
71 expansion/retraction is unlikely to have affected much of the southern hemisphere
72 throughout these cycles *per se* (Duckett et al. 2013, Lamb et al. 2019), secondary
73 impacts such as intensifying aridity, sea-level changes and temperature shifts during
74 glacial maxima have impacted on the evolution, distribution and persistence of
75 southern hemisphere biotas (Williams et al. 2018, Ansari et al. 2019). Such
76 secondary impacts likely shape the environment of different regions based on their
77 local relevance, with eustatic changes having a larger influence on coastal or marine
78 ecosystems while aridification played a stronger role further inland (Beheregaray et
79 al. 2002, Pinceel et al. 2013). Additionally, environmental changes associated with
80 tectonic shifts or the formation and submergence of land-bridges are mostly locally
81 relevant and vary across landscapes. Thus, understanding the relative role of
82 different environmental changes between regions is important in more accurately
83 predicting species' responses.

84

85 Inferences of phylogeographic responses to past environmental change relies
86 upon a combination of genetic, spatial and modelling approaches. However,
87 determining the relative role and chronology of past climatic events is difficult when
88 resolution is low due to few genetic markers or limited model capability (Carstens et
89 al. 2012, Cutter 2013, Nakhleh 2013). To this end, the development of next-
90 generation sequencing allows for the collection of thousands of genetic markers
91 which better capture the diverse array of demographic processes influenced by Earth
92 history (Carstens et al. 2012, Edwards et al. 2016). In tandem, recent advancements
93 in coalescent modelling, informed by more detailed information of geological and
94 ecological history, have improved the ability to provide more nuanced inferences

95 (Cutter 2013, Excoffier et al. 2013). This combination of greater data and more
96 sophisticated modelling techniques provides the analytical framework to address
97 questions about the spatial variance of species responses to climate change.

98

99 An ideal biogeographic setting to test hypotheses of spatial and temporal variation
100 of Earth history on evolution is one including both inland and coastal regions. In this
101 regard, the temperate southeast of Australia is well-suited given it has been
102 influenced by aridification across the continent as well as by shifts in landmass
103 attributed to eustatic changes (Faulks et al. 2010, McLaren and Wallace 2010,
104 Chapple et al. 2011). This region is characterised by complex geography and
105 geology, affected by a history of uplift, subsidence and volcanism (Unmack 2001).
106 There is a strong gradient in temperature and precipitation across the region, with
107 cooler and wetter climates towards the south. The region is subdivided by the Great
108 Dividing Range, which runs parallel to the coastline from the top of Australia down to
109 the southern coast. This range acts as a barrier that separates the inland Murray-
110 Darling Basin (MDB) from coastal areas and is a key biogeographic feature of the
111 region (Fig. 1b; Unmack 2001, Chapple et al. 2011).

112

113 The MDB is one of the continent's largest freshwater basins and a key water
114 source for much of Australia. The MDB is hydroclimatically variable, with notable
115 differences in hydrology and climate between headwaters and the terminal lowland
116 lakes and wetlands near the Murray mouth (Pittock and Finlayson 2011). A major
117 environmental change that reshaped the MDB over time was the formation and
118 decline of the paleo megalake Bungunnia, which spanned 90,000 km² across the
119 lower section of the MDB at its largest size (McLaren et al. 2011, McLaren et al.

120 2012). Lake Bungunnia initially formed in the late Pliocene ~3 Ma when tectonic uplift
121 dammed the outlet of the ancestral Murray River. Its level fluctuated in accordance
122 with glacial and interglacial climate cycles; interglacial periods caused the lake to
123 reach overflowing where it resembled an open system of freshwater lakes (McLaren et
124 al. 2012). Predictions of the volume of inflow required to maintain Lake Bungunnia at
125 that size suggests 2 – 3 times higher rainfall than currently experienced was needed
126 (Stephenson 1986, McLaren and Wallace 2010). Repeated overflowing of Lake
127 Bungunnia during wetter climatic periods led to the eventual erosion of a new outlet
128 to the west, resulting in the removal of the barrier and complete drainage of the
129 inland lake system ~700 Ka (McLaren and Wallace 2010). Lake Bungunnia has been
130 suggested to be a relevant factor in the phylogeography of some terrestrial species
131 (Cooper et al. 2000, Joseph et al. 2008, Kawakami et al. 2009, Ansari et al. 2019,
132 Neal et al. 2019), and its fluctuation is likely an important biogeographic aspect of the
133 region for local aquatic species (Waters et al. 2019).

134

135 These attributes contrast with the coastal habitats south of the MDB, where major
136 changes in the environment are more associated with eustatic changes. One
137 example is the formation of the Bass Strait which separated Tasmania from the
138 mainland. Historically, Tasmania was connected to the mainland through a land
139 bridge known as the Bassian Isthmus: central to this landmass was a large
140 freshwater lake known as the Bass Lake (Blom and Alsop 1988, Porter-Smith et al.
141 2012). As sea levels rose during each interglacial of the Pleistocene, the Bassian
142 Isthmus was inundated. As a result, the land bridge narrowed into an eastern
143 corridor prior to its submergence, the remnants of which can be seen in the Flinders
144 and Cape Barren Islands. The isolation of Tasmania and the formation of the

145 Bassian Isthmus as the sole terrestrial connection are well documented drivers of
146 biogeographic patterns for a variety of both terrestrial, marine and freshwater taxa
147 (Schultz et al. 2008, Waters 2008). This combination of relevant Earth history factors
148 across southeast Australia, and the potentially interactive nature of these events,
149 provides an ideal scenario to investigate the relative role of different past
150 environmental changes on phylogeographic patterns.

151

152 Complex impacts of climatic change are particularly exacerbated in freshwater
153 ecosystems, as increasing temperature and aridity alters the stability and structure of
154 hydrological systems (Middelkoop et al. 2001, Nijssen et al. 2001, Pinceel et al.
155 2013, Blöschl et al. 2019). With limited dispersal capability and reliance on available
156 freshwater for survival, aquatic-dependent species demonstrate evolutionary
157 associations with hydrological changes. Even minor alterations to hydrologic
158 structure can have profound impacts on the evolution of a diverse array of freshwater
159 taxa (Inoue et al. 2014, Thomaz et al. 2017, Wallis et al. 2017). For example,
160 tectonic activity can reshape waterways, leading to river capture across new areas
161 and shifting distributional patterns of water-dependent species (Murphy and Austin
162 2004). Thus, freshwater biodiversity functions as an important indicator of the impact
163 of historical environmental changes.

164

165 An ideal system for studying biogeographic changes in southeast Australia is the
166 southern pygmy perch, *Nannoperca australis* (Percichthyidae). This small-bodied
167 (<80mm), habitat-specialist fish prefers slow flowing and vegetated ephemeral
168 streams (Wedderburn et al. 2012, Hammer et al. 2013). It is distributed throughout
169 the temperate southeast Australia region, occupying the MDB, coastal Victoria and

170 northern Tasmanian rivers. Previous phylogenetic work indicated that southern
171 pygmy perch from eastern Victoria, Flinders Island and north-eastern Tasmania
172 belong to a genetically distinct cryptic species referred to as *Nannoperca* 'flindersi'
173 (Unmack et al. 2013, Buckley et al. 2018). Estimates of divergence time using a
174 biogeographic calibration point suggest this split occurred ~6.1 Ma (Buckley et al.
175 2018), but the biogeographic forces driving this speciation remain unknown. Being
176 an ancient lineage, *N. australis* has likely responded to a variety of environmental
177 changes across inland and coastal habitats since the Miocene. Furthermore, *N.*
178 *australis* is threatened, particularly within the MDB, due to extreme pressure from
179 anthropogenic changes to water flow, introduced predators and contemporary
180 climate change (Balcombe et al. 2011, Brauer et al. 2016). The low dispersal
181 capability, effective population size and high genetic structure of the species makes
182 their survivability of great concern (Brauer et al. 2016, Brauer et al. 2017). Here we
183 used genome-wide data assess the relative roles of hydrological and coastal
184 topographic changes as drivers of evolutionary diversification and lineage
185 persistence. We hypothesised that demographic changes and lineage diversification
186 linked to aridification would be older (Miocene – Pliocene) and stronger for
187 populations from inland basins, whereas changes linked to eustatic variation would
188 be comparatively younger (Pleistocene) and common for populations from coastal or
189 island habitats. We tested the impact of these factors using a hierarchical framework
190 that incorporated complex, hypothesis-driven coalescent modelling, model-free
191 demographic analyses and spatial (species distribution) modelling.
192

193 MATERIALS & METHODS

194 *Sample Collection and Genomic Library Preparation*

195 A total of 109 samples across 21 known populations of *N. australis* and three
196 populations *N. 'flindersi'* ($n = 4 - 5$ individuals per population) were collected. This
197 sample spans the full geographic range of the species and includes at least one
198 population from each management unit (MU) identified in previous genetic and
199 genomic studies (Table 1; Unmack et al. 2013, Cole et al. 2016). The sister species
200 *N. obscura* (Buckley et al. 2018) was included as the outgroup for phylogenetic
201 analyses ($n = 5$). Specimens were collected using a combination of electrofishing,
202 dip-, fyke- or seine-netting. Specimens (either caudal fin or entire specimen) were
203 stored dry at -80°C at the South Australian Museum, or in 99% ethanol at Flinders
204 University.

205

206 DNA was extracted from muscle tissue or fin clips using a modified salting-out
207 method (Sunnucks and Hales 1996) or a Qiagen DNeasy kit (Qiagen Inc., Valencia,
208 CA, USA). Genomic DNA quality was assessed using a spectrophotometer
209 (NanoDrop, Thermo Scientific), 2% agarose gels, and a fluorometer (Qubit, Life
210 Technologies). All ddRAD genomic libraries were prepared in-house following
211 Peterson et al. (2012), with modifications as described in Brauer et al. (2016).

212

213 Of the 109 samples, 73 were previously paired-end sequenced as part of a
214 landscape genomics study (Brauer et al. 2016) using an Illumina HiSeq 2000 at
215 Genome Quebec (Montreal, Canada). The additional 36 samples were single-end
216 sequenced on an Illumina HiSeq 2500 at the South Australia Health and Medical
217 Research Institute (SAHMRI).

218

219 *Bioinformatics*

220 The resultant reads (forward reads only for paired-end samples) were filtered and
221 demultiplexed using the 'process_radtags' module of Stacks 1.29 (Catchen et al.
222 2013), allowing ≤ 2 mismatches in the barcodes. Barcodes were removed and reads
223 trimmed to 80 bp to remove low-quality bases from the ends. Cut reads were then
224 aligned using PyRAD 3.0.6 (Eaton 2014), and further filtered by removing reads that
225 had > 5 bp with a Phred score of < 20 . Loci were retained at a minimum sequencing
226 depth of 5 and occurring in at least $\sim 90\%$ of samples (103). The final concatenated
227 alignment contained 7,958 ddRAD loci and 45,104 SNPs.

228

229 *Phylogenetic Analysis*

230 To determine evolutionary relationships as a basis for phylogeographic modelling,
231 a maximum likelihood (ML) phylogeny was estimated using RAxML 8.2.11
232 (Stamatakis 2014) and the 7,958 concatenated ddRAD loci dataset. This was done
233 using rapid hill-climbing and 1,000 resampling estimated log-likelihood (Pante et al.
234 2015) bootstraps under a GTR+ Γ substitution model. The resultant phylogenetic tree
235 was visualised using MEGA 7 (Kumar et al. 2016) and rooted with *N. obscura* as the
236 outgroup.

237

238 To determine if dendritic river hierarchy alone could explain phylogenetic patterns
239 across the Murray-Darling Basin lineage, and to clarify coalescent models (see
240 Results), linear correlations between genetic and riverine distance were estimated
241 using StreamTree (Kalinowski et al. 2008). StreamTree models genetic divergence
242 across a dendritic river system and assigns a cost to each riverine segment,

243 comparing this modelled distance with the empirical data. While StreamTree is often
244 used with pairwise F_{ST} values (e.g. Brauer et al. 2018) to assess contemporary
245 spatial patterns, we opted to use uncorrected genetic distances (p -distance) as this
246 more likely contains signal of historic patterns of divergence (Nei 2001). Pairwise p -
247 distances between individuals were estimated using PAUP* 4 (Swofford 2002) and
248 averaged per population using *R* for all 13 MDB populations.

249

250 *Divergence Time Estimation*

251 We estimated divergence times using r8s 1.81 (Sanderson 2003). Given the lack
252 of suitable fossils for pygmy perch (Buckley et al. 2018), we calibrated the node
253 between *N. australis* and *N. flindersi* at 5.9 – 6.1 Ma based on a previous estimate
254 that includes all pygmy perch species (Buckley et al. 2018). Divergence times for
255 each node were estimated using a penalized-likelihood model under a truncated
256 Newton algorithm (Nash 2000), which uses a parametric branch substitution rate
257 model with a nonparametric roughness penalty (Sanderson 2003). Cross-validation
258 was used to determine the best value of the smoothing parameter for the roughness
259 penalty between $\log_{10} 0$ and $\log_{10} 100$. The optimum smoothing parameter of \log_{10}
260 41.00, with a chi-square error of -12836.285, was used to estimate divergence times
261 between populations and higher order clades across the lineage.

262

263 *Ancestral Range Estimation*

264 We used a phylogenetic tree-based method to estimate ancestral ranges across
265 the maximum likelihood tree with the *R* package BioGeoBEARS (Matzke 2013). The
266 maximum likelihood tree was collapsed down to individual populations using the *R*
267 package ape (Paradis et al. 2004). Given the paraphyletic nature of the Albury

268 population (NauALB) within the phylogenetic tree (Fig. 2), this population was pruned
269 from the tree. The tree was then converted to ultrametric format using the divergence
270 time estimates from r8s.

271

272 Tips were assigned to one of six main geographic regions based on hydrogeology
273 (McLaren et al. 2011): the MDB, western Victoria coast (COAST), Wilson's
274 Promontory (WP), eastern Victoria (SRLO), Flinders Island (FLI) or Tasmania (ART).
275 Individual *N. flindersi* populations were assigned to unique geographic states given
276 their current isolation and to allow for the explicit testing of vicariance vs. dispersal
277 scenarios across the Bassian Isthmus. Ranges spanning multiple states were filtered
278 to only those composed of neighbouring ranges (total number of possible ranges =
279 21). Furthermore, given the historical marine inundation of the MDB which would
280 have precluded the presence of southern pygmy perch, time-stratification was used
281 to exclude 'MDB' as a geographic state prior to 5 Ma or 3 Ma. These times reflect a
282 conservative estimate of marine inundation (the most recent time at which marine
283 sediments have been accurately identified within paleolake Bungunnia; McLaren et
284 al. 2011) and a more relaxed estimate that is possibly the most recent time marine
285 water could have been present within the basin. Ancestral ranges were estimated
286 under all six available models (DEC, DIVA-LIKE and BAYAREA-LIKE, with and
287 without founder-event speciation, +J). All models were run under both time-
288 stratification scenarios and compared using the Akaike Information Criterion (AIC)
289 within each set.

290

291 *Biogeographic Hypothesis Testing Using Coalescent Modelling*

292 Specific hypotheses based on biogeographic events were tested using a
293 coalescent framework within FastSimCoal 2.6 (Excoffier et al. 2013). These
294 hypotheses expand on the broad interpretations based on phylogenetic analyses.
295 Predicted changes in population history were based on the expected impact of
296 biogeographic patterns previously identified within the literature (Table S2) and
297 information from both prior and current phylogenetic and coalescent analyses
298 (Buckley et al. 2018). These models were focused around particular divergence
299 events across the lineage, with hypotheses built around the separation of major
300 clades within the phylogeny (Fig. 2). Hypothesised biogeographical drivers of
301 divergences included marine inundation, tectonic shifts and hydrological
302 rearrangements. Specific biogeographic hypotheses for each divergence event, and
303 their predictive impacts on the evolution and demography of southern pygmy perch,
304 are described in Table S2 and Fig. S5.

305

306 *Estimating Effective Population Size Changes*

307 As a model-free and exploratory approach to clarify demographic history, changes
308 in effective population size (N_e) over time were estimated using the site-frequency
309 spectrum (SFS) and coalescent modelling in a stairway plot (Liu and Fu 2015).
310 Given the strong population genomic structure reported for southern pygmy perch
311 (e.g. $F_{ST} = 0 - 0.798$; Brauer et al. 2016) including for populations sampled in this
312 study, and to account for the biasing effect of population structure on the SFS
313 (Stadler et al. 2009, Xue and Hickerson 2015), loci were re-aligned for each
314 population independent of the rest of the dataset. As missing data can bias the
315 distribution of the SFS (Shafer et al. 2017), only loci present in all samples for each

316 population were retained. Unlinked biallelic SNPs from each independent alignment
317 were then used to generate the single-population SFS (Fig. S4) using the same in-
318 house script as above. Stairway plots were estimated assuming a mutation rate of
319 $10^{-8} - 10^{-9}$ per site per generation (Stobie et al. 2018) and a generation time of one
320 year (Humphries 1995).

321

322 *Species and Lineage Distribution Modelling*

323 The distribution of the species was modelled using MaxEnt 3.4 (Phillips et al.
324 2017) and 19 BioClim variables from WorldClim v1.4 (Hijmans et al. 2005),
325 summarising precipitation and temperature – two groups of climatic variables known
326 to impact on local adaptation and distribution of southern pygmy perch (Brauer et
327 al. 2016). To account for non-climatic environmental aspects that may limit the
328 distribution of the species (Paz et al. 2015), elevation (extracted from the Etopo1
329 combined bathymetry and topography dataset; Amante and Eakins 2009) and
330 topographic wetness index (twi, extracted from the ENVIREM database; Title and
331 Bemmels 2018) were also included. Species occurrence data was collected from the
332 Atlas of Living Australia (<http://www.ala.org.au>), with filtering for geographic accuracy
333 and removing outliers based on known distributional limits (final data included 6,106
334 occurrences). However, this dataset did not include some localities within the
335 Murrumbidgee River and mid-Murray River from which *N. australis* has been
336 extirpated due to post-European settlement habitat modification across the MDB
337 (Cole et al. 2016). Duplicates from the same coordinate point were removed to
338 minimise the biasing effect of uneven sampling effort (Elith et al. 2011), reducing the
339 dataset to 2,528 unique occurrences. Similar tests of spatial autocorrelation were
340 performed for the 19 BioClim variables using a Pearson's pairwise correlation test in

341 SDMToolbox (Table S4; Brown et al. 2017). Highly correlated ($|r| > 0.8$) variables
342 were removed to avoid overfitting of the model (Dormann *et al.* 2013), reducing the
343 environmental data down to 9 bioclimatic variables and the two topographic variables
344 (Table S5). A subset of 25% of occurrence sites were used to train the model.

345

346 Climatic data from the Last Glacial Maxima (LGM; 22 Ka) were extrapolated from
347 the WorldClim 1.4 database (Hijmans et al. 2005) to project the historic distribution of
348 the species. To evaluate environmental conditions more reflective of the divergence
349 between the two species, the SDM was also projected back to the mid-Pliocene
350 (~3.2 Ma) using a subset of 6 of the previous 9 BioClim variables (excluding
351 variables bio2, bio3 and bio6) from the PaleoClim database (Brown et al. 2018). The
352 fit of each SDM was determined using the area under the receiver operating curve
353 (AUC).

354

355 A lineage-specific distribution model (LDM) method described in Rosauer et al.
356 (2015) was used to determine the relative distributions of each lineage over time; this
357 was done with two 'intraspecific' lineages of *N. australis* and *N. flindersi*. A total of
358 72 site localities ($n = 61$ for *N. australis*; $n = 11$ for *N. flindersi*) were used based on
359 genetic assignment to a 'species' within this study, as well as based on mitochondrial
360 DNA results (Unmack et al. 2013). We estimated the LDMs for both species across
361 all three time periods (current, LGM, and Pliocene). Although the location of
362 intraspecific lineages is unlikely to remain constant in time, this method allows the
363 inference of probable relative distributions of each 'species' under past climatic
364 conditions.

365

366 RESULTS

367 *Bioinformatics*

368 A total of 340,950,849 raw sequenced reads were obtained from the 2 lanes of
369 Illumina sequencing. Quality control and alignment resulted in a concatenated
370 sequence dataset of 7,958 ddRAD loci with 45,104 variable sites (SNPs) and 30,485
371 parsimony-informative sites. This alignment had an average of 2.34% ($\pm 3.31\%$ SD)
372 missing data per individual. For coalescent analyses within FastSimCoal2, the
373 outgroup was removed and loci realigned and SNPs re-called, generating a dataset
374 of 8,022 ddRAD loci containing 38,287 SNPs and 22,820 parsimony-informative
375 sites. SNPs from this dataset were reduced to a single SNP per ddRAD locus for
376 estimating joint site frequency spectra, resulting in 7,780 biallelic SNPs.

377

378 *Phylogenetic Analysis*

379 The maximum likelihood phylogeny (Fig. 2) separated *N. 'flindersi'* from the rest of
380 *N. australis*, corroborating previous phylogenetic results (Unmack et al. 2013,
381 Buckley et al. 2018). Within *N. australis*, three major lineages were delineated; one
382 of the Wilson's Promontory population (NauWP), one of coastal Victoria populations
383 and another of populations within the MDB. The coastal Victorian lineage showed
384 relatively stronger phylogenetic structure than the MDB lineage, with its easternmost
385 populations diverging more recently compared to westernmost coastal Victorian
386 populations (Fig. 4). The MDB clade, however, generally featured shorter branches
387 and lower bootstrap support. Despite being geographically apart, lower MDB
388 populations (Lake Alexandrina and Angas) shared a MRCA with upper Murray
389 populations (Lachlan River and Coppabella Creek, respectively). The most basal
390 clade of the MDB lineage contained populations from the upper Murray River (Spring

391 Creek, Gap Creek, and Albury). Within this group, the Albury population was
392 paraphyletic with the other two; this is expected based on previously described levels
393 of admixture across the populations (Brauer et al. 2016). The breadth of the
394 phylogenetic tree was well supported, with the majority of population-level and above
395 divergences with bootstrap support of >80%.

396

397 The StreamTree results did not suggest that contemporary riverine hierarchy
398 alone could explain patterns of historical phylogeographic divergence across the
399 MDB (Fig. 3; $R^2 = 0.464$). Assessing the fit of the StreamTree model by comparing
400 the observed and expected genetic distance for each population individually
401 demonstrated that this was likely driven by several outlier populations (NauANG,
402 NauALE, NauALB and NauCOP) with significantly higher modelled genetic distance
403 (Fig. S2). Removal of these four populations from the StreamTree model led to much
404 higher correlation ($R^2 = 0.982$) with similar genetic distance penalties for river
405 segments common to both sets of populations (Fig. S3).

406

407 *Ancestral Range Estimation*

408 Comparison of ancestral range estimations from BioGeoBEARS identified the
409 DIVA-LIKE model as the best supported under both time-stratification scenarios (AIC
410 = 24.16 and 59.1 for models excluding presence in the MDB until 5 Ma and 2 Ma,
411 respectively). This model represents a likelihood approximation of the model
412 implemented in DIVA (Ronquist 1997) which broadly considers the relative role of
413 dispersal and vicariance (but not sympatric mechanisms) in driving biogeographic
414 patterns (Matzke 2013). Although patterns were similar across both time-stratification
415 criteria (Table S1), we choose to focus on the more conservative (5 Ma constraint)

416 results given the lack of precision in determining the end of marine inundation into
417 the MDB (McLaren *et al.* 2011). This DIVA-LIKE model demonstrated strong patterns
418 of vicariance, with weak contributions of dispersal ($d = 1.46 \times 10^{-2}$) and extinction (e
419 $= 1 \times 10^{-12}$) and all major geographic changes associated with vicariance events (Fig.
420 4). Including a parameter for founder event (+J) estimated very weak contributions of
421 founding events and contributed to negligible change in log likelihood across either
422 time-stratification scenario (Table S1). Ancestral states for major nodes were well
423 resolved across the phylogeny.

424

425 *Biogeographic Hypothesis Testing*

426 Comparison of biogeographic hypotheses under coalescent models clearly
427 supported one model over others for each focal divergence event (Fig. 5): these
428 results are detailed in Table S4. In general, most models including post-isolation
429 gene flow were better supported than those without, and models based on vicariance
430 due to hydrological changes were better supported than those invoking tectonic or
431 dispersal mechanisms.

432

433 *Divergence of species.* — Coalescent models including gene flow between the
434 two species after divergence was better supported than models without, suggesting
435 that secondary contact occurred at some point after their initial divergence (Fig. 5;
436 Model 1b). Models including bottlenecks did not have significantly higher support
437 than those without, and inferred bottlenecks were small in magnitude. The initial
438 divergence event between the two species was estimated at ~6.2 Ma. These results
439 suggest that range expansion and vicariant separation during the late Miocene drove
440 speciation of *N. 'flindersi'*.

441

442 *Divergence of Wilson's Promontory population.* — Coalescent models of the
443 Wilson's Promontory population and the two species separately also suggested likely
444 gene flow across adjacent lineages, with divergence of the Wilson's Promontory
445 population occurring ~3 Ma (Fig. 5; Model 2b). However, this gene flow was not
446 indicative of a hybridisation event and models simulating the WP population as
447 resulting from gene flow from either population coalesced nearly all alleles into the *N.*
448 *australis* ancestral population. Thus, NauWP represents an anciently diverged
449 population of *N. australis* that was isolated through vicariance, possibly due to either
450 marine inundation of the peninsula or tectonic changes across the region.

451

452 *Divergence of major N. australis lineages.* — Testing migration pathways from the
453 ancestral coastal population into the MDB suggested that colonisation most likely
454 occurred through the lower sections of the MDB and upstream into the upper
455 reaches (Fig 5.; Model 3a). The timing of this migration event would pre-date ~600
456 Ka, the estimated time of divergence between the coastal and MDB populations
457 within the best supported model. However, this model had only marginally better
458 likelihood than one estimating the divergence time of the coast and MDB populations
459 at ~1.2 Ma, suggesting that this estimate might not be overly precise. Regardless,
460 the biogeographic models suggest that migration facilitated by the presence of paleo
461 megalake Bungunnia allowed the ancestral southern pygmy perch to enter the MDB
462 following the withdrawal of marine water from the basin.

463

464 *Divergence within coastal Victoria lineage.* — The coalescent model accounting
465 for the effects of both sea-level changes leading to isolation of rivers and the

466 shrinking of Lake Corangamite isolating eastern lineages was better supported than
467 models without these factors, or models only considering one (Fig. 5; Model 4e).
468 This suggests that while sea-level changes likely isolated many of the populations
469 from one another and prevented dispersal across river systems, the expanded Lake
470 Corangamite continued to facilitate gene flow across some of the more eastern
471 lineages.

472

473 *Divergence within MDB lineage.* — Coalescent modelling of the MDB lineage
474 suggested that some phylogeographic structure pre-dated European settlement, with
475 the divergence of the most basal lineage (containing the Spring Creek, Gap Creek
476 and Albury populations) estimated to have occurred ~2 Ka, albeit with low levels of
477 gene flow since divergence (Fig. 5; Model 5c). Models partitioning putatively upper
478 and lower populations into single demes did not converge, likely reflecting their
479 paraphyletic nature.

480

481 *Divergence within N. 'flindersi'.* — Coalescent models including migration between
482 adjacent populations gave much greater likelihood estimations than models without
483 gene flow, suggesting that migration between lineages had occurred in the past (Fig.
484 5; Model 6b). Including bottlenecks indicative of a dispersal event did not improve
485 likelihoods, supporting a range expansion and vicariance scenario. The central
486 population of Flinders Island had a much smaller population size than either of the
487 other two populations. Divergence time estimates for between populations suggest a
488 relatively ancient split, with the Snowy River population separating from the other two
489 lineages ~1.5 Ma and the secondary split between Flinders Island and Tasmania at
490 ~1.3 Ma.

491

492 *Historical Demography Reconstruction*

493 One-dimensional site-frequency spectra estimated from single-population SNPs
494 used a mean of 3,527 (1,045 – 7,389) SNPs (Fig. S4). Stairway plots indicated
495 significant declines in many populations of southern pygmy perch over the last 1 Myr
496 (Fig. 6). For many of these populations, gradual and concordant bottlenecks were
497 apparent within clades. Non-declining populations were typically relatively stable,
498 with none demonstrating an increase in N_e over this time. Almost all populations
499 across both putative species demonstrated population growth deep in the past (~ 1
500 Ma), although this may reflect fewer ancient coalescent events within the data.

501

502 Within *N. 'flindersi'*, the mainland population of NfISRLO demonstrated a strong
503 decline in N_e starting at ~10 Ka which contrasted with the more stable demographic
504 histories of the island populations. This decline resulted in much lower estimates of
505 recent N_e than the island counterparts, with the Tasmanian population NfIART
506 showing the highest consistent N_e of all *N. 'flindersi'* populations.

507

508 Demographic histories were variable across populations of *N. australis*. Within the
509 coastal ESU, most populations demonstrated relatively stable demographic histories,
510 with weak declines in N_e originating at ~100 Ka in NauMRG and NauGCH, and at
511 ~10 Ka in NauGRF. This contrasted with populations across the MDB, where
512 significant population declines at ~100 Ka were observed in the majority of
513 populations. Nevertheless, a few populations also demonstrated stable or weakly
514 declining N_e over time, with NauJHA and outlier showing significant growth at ~ 100
515 Ka followed by stable N_e . However, this is likely driven by the lower sample size ($n =$

516 3) within this population compared to others across the MDB, resulting in few
517 inferred coalescent events across the tree and a biased SFS. The highly divergent
518 Wilson's Promontory population showed a sharp population increase at ~100 Ka
519 followed by relatively stable and high N_e over time. Although all population stairway
520 plots inferred no changes in N_e <5 Ka, this likely reflects a lack of very recent
521 coalescent events within each population owing to small sample size.

522

523 *Species and Lineage Distribution Modelling*

524 Species distribution modelling for southern pygmy perch based on the nine
525 uncorrelated BioClim and 2 topographic variables effectively predicted the
526 contemporary distribution for the species, showing highest habitat suitability along
527 the Victorian coast, southern MDB and in northern Tasmania (including King and
528 Flinders Island; Fig. 7a). However, this SDM likely underestimates presence of *N.*
529 *australis* within the connective center of the MDB, where downstream migration
530 would have facilitated a mosaic of intermediate populations prior to extensive flow
531 abstraction and regulation over the last 200 years (Cole et al. 2016).

532

533 Historic projections of the SDM for both species together highlighted two glacial
534 refugia, one along the western coast of the distribution and another small and
535 isolated refugium closer to the southeast corner of the mainland. Together, these
536 results indicate a significant expansion in suitable habitat following the LGM (Fig.
537 7d). The AUC of the model was estimated at 0.908, indicating a good fit of the model
538 to the data.

539

540 Lineage distribution models under each time period demonstrated the disjunct
541 spatial nature of the two species. Under contemporary conditions, the LDMs showed
542 a geographic break near Wilson's Promontory, albeit with overlap at intermediate
543 localities (Fig. 7b-c). Additionally, the northern Tasmanian coastal habitat was
544 delimited into two equally sized sections for each species. During the LGM, the
545 disjunct LDMs of the two species in the east and west portion of the range supported
546 their long-term isolation and provided evidence for an environmental barrier
547 preventing the contact between the species (Fig. 7e-f). The Pliocene projection also
548 showed refugial habitat along the Victorian coast and within the upper MDB (Fig. 7g).
549 Similarly to contemporary conditions, the LDMs showed a narrow division between
550 species in northern Tasmania and central Victoria (Fig. 7h-i). The subset data used
551 for the Pliocene projection had marginally weaker support, with an AUC of 0.887.

552

553 DISCUSSION

554 Establishing how aquatic-dependent lineages responded to past hydroclimatic
555 changes contributes to our understanding about their contemporary ecological
556 requirements and to predicting demographic responses under ongoing climate
557 change. This study demonstrates the overarching impacts of varying hydrology due
558 to Plio-Pleistocene climatic change (e.g. reduction of lake systems and
559 rearrangement of river networks) on the evolution and diversification of a temperate
560 freshwater-dependent fish clade. However, coalescent analyses and species
561 distribution modeling show that the evolutionary consequences of major shifts in sea
562 level and hydroclimatic conditions varied substantially between coastal and inland
563 environments. Aridification altered the demography of populations from inland river
564 systems, whilst eustatic changes and marine inundation were major evolutionary

565 drivers of populations from coastal freshwater landscapes. Our findings suggest that
566 the long-term impact of key environmental changes associated with anthropogenic
567 climate change, such as increases in aridification and sea level, might vary
568 substantially for the same lineage, both spatially and across landscape types.

569

570 *Aridification Drives Phylogeographic Structure of Inland Basins*

571 Aridification of the Australian continent has dramatically altered the identity and
572 stability of ecosystems (Hawltischek et al. 2012), particularly in the formation of the
573 arid zone (Byrne et al. 2008) and reduction of temperate and wetter habitats (Crisp
574 et al. 2004, Byrne et al. 2011). Particularly for freshwater species, increasing
575 aridification since the Pliocene may be responsible for a number of divergent clades,
576 with water availability and river networks being critical for the long-term survival and
577 evolution of freshwater lineages (Faulks et al. 2010, Beheregaray et al. 2017).

578

579 Aridification and tectonics through the formation and demise of paleo-megalake
580 Bungunnia was a major event impacting the evolutionary history of inland lineages of
581 *N. australis*. Lake Bungunnia initially formed ~3 Ma when tectonics shifts along the
582 Padthaway High resulted in significant uplift across the region, damming the
583 ancestral Murray River which approximately aligned with the current Glenelg River
584 (McLaren et al. 2011, Waters et al. 2019). For many freshwater taxa across the
585 southeast of the continent, isolation of lineages between the MDB and the southwest
586 Victoria (SWV) drainages has been associated with this tectonic shift in the Pliocene
587 (Murphy and Austin 2004, Waters et al. 2019). Similar interpretations of tectonic
588 changes influencing river capture have been proposed for movement across other
589 sections of the Great Dividing Range into the MDB (McGlashan 2001, Murphy and

590 Austin 2004, Cook et al. 2006, Faulks et al. 2010). The isolating effect of the tectonic
591 changes between the two drainages is likely reflected in the strong differentiation
592 between clades and the ancient nature of the phylogenetic-based divergence time of
593 3.03 Ma.

594

595 Coalescent modelling instead points to more recent divergence (603 Ka),
596 suggesting the possibility that the edges of Lake Bungunnia at its largest extent
597 could have acted as suitable habitat for southern pygmy perch and potentially
598 facilitated further dispersal into the inland basin. With the eventual demise of the lake
599 ~700 Ka (McLaren et al. 2011), this secondary bout of isolation disconnected the two
600 basins fully, probably accounting for the results of coalescent models. Similar
601 patterns of initial isolation by vicariance during the Pliocene, followed by Pleistocene
602 secondary contact across the Great Dividing Range and into the MDB were
603 observed within mountain galaxias (*Galaxiis oliros* and *Galaxiis olidus*), which share
604 comparable ecological constraints to southern pygmy perch (Waters et al. 2019).
605 Similar to Lake Bungunnia, the reduction of Lake Corangamite to one seventh of its
606 original size over the course of the Holocene (White 2000) isolated several eastern
607 coastal Victoria *N. australis* populations. The reduced size (~160 km²) and
608 hypersalinity (>50 g/L) of Lake Corangamite likely prevents connectivity between
609 these populations under contemporary conditions (Williams 1995, White 2000).

610

611 Within the MDB clade there was weak evidence for historic phylogeographic
612 structure, with coalescent models suggesting divergences dating as ~2 Ka.
613 Correlating contemporary river structure and genetic distance *per se* did not predict
614 genetic divergence between populations across the MDB. A combination of

615 extensive flow and habitat modification since European settlement and naturally
616 complex metapopulation dynamics (Brauer et al. 2016) are probably better proxies
617 for contemporary patterns observed within the MDB. This was reflected in the
618 stairway plots, which showed a number of populations declining over the last 1 Myr
619 but with variance in demographic histories across the basin. The most likely
620 demographic scenarios include multiple waves of dispersal and colonisation,
621 possibly in response to local extinction or during rare environmental events such as
622 flooding, which altered patterns of genetic divergence.

623

624 *Eustatic Changes Drive Phylogeography and Speciation Along Coastal Habitats*

625 Sea level changes associated with interglacial periods and more arid climates
626 played a significant role in the divergence of coastal lineages. Marine inundation
627 across the East Gippsland region during the Mio-Pliocene (~6 Ma), prior to the
628 climatic cycles of the Pleistocene, likely drove the initial divergence and speciation of
629 *N. flindersi*. Marine sediments and low elevation of the region indicates that this
630 marine inundation was significant (Gallagher et al. 2001, Holdgate et al. 2003), and
631 was correlated with the onset of major aridification in the continent (Garrick et al.
632 2004, Faulks et al. 2010, McLaren and Wallace 2010). Ancient marine inundation of
633 East Gippsland has been proposed to influence vicariant speciation in various
634 terrestrial species (Chapple et al. 2005, Norgate et al. 2009). This low-lying region
635 approximately forms the interface between the distribution of the two putative study
636 species (Fig. 1b) and the timing of this inundation corresponds well with the
637 estimated molecular clock-based divergence time. This period of sea level rise is
638 also associated with inundation of the lower parts of the MDB which ostensibly
639 precluded the presence of pygmy perch (McLaren et al. 2011).

640

641 Previous hypotheses of the mechanisms driving the initial divergence of *N.*
642 ‘flindersi’ have suggested that the separation of drainages by tectonic shifts across
643 the region (Dickinson et al. 2002, Gallagher et al. 2003) isolated populations
644 following a dispersal event facilitated by river capture or flooding (Unmack et al.
645 2013). Regardless of the mechanism, divergence between Tasmanian and mainland
646 lineages prior to the Pleistocene has been reported for birds (Lamb et al. 2019),
647 lizards (Dubey and Shine 2010, Chapple et al. 2011, Kreger et al. 2019), butterflies
648 (Norgate et al. 2009) and other freshwater fish (Coleman et al. 2010), suggesting
649 that climatic oscillations during the LGM alone did not drive the speciation of *N.*
650 ‘flindersi’.

651

652 More recent sea-level changes also likely impacted within-basin phylogeographic
653 patterns. Within *N.* ‘flindersi’, relatively ancient estimates of divergence times
654 between populations (1.5 – 2 Ma) suggested that early glacial cycles of the Plio-
655 Pleistocene resulted in strong differentiation. However, coalescent models
656 suggested that gene flow across these disparate populations was possible during
657 glacial maxima. At lowered sea levels, river systems occupied by *N.* ‘flindersi’ all
658 drained eastward towards the continental shelf (Unmack et al. 2013), with shorter
659 overland distances between river mouths than today (Fig. 1b). Given the presence of
660 a small glacial refugia in the far eastern extreme of the distribution, gene flow may
661 have resulted from contraction into a singular locale followed by expansion back
662 across the Bassian Isthmus during more favourable environmental conditions
663 (Lambeck and Chappell 2001).

664

665 Within the coastal *N. australis* lineage, isolation of river catchments during
666 aridification in the Pleistocene led to the strong structure observed within the
667 phylogenetic tree. During glacial maxima, lowered sea levels significantly increased
668 the extent of the mainland Australian coastline, particularly across the southeast
669 corner (Williams et al. 2018). Although it does not appear that the current rivers of
670 coastal Victoria ever fully connected together before meeting the shoreline (Unmack
671 et al. 2013), climatic modelling has suggested that the low topographic relief and
672 evaporation across this region would have allowed overland networks to form
673 through small lakes and floodplains (Williams et al. 2018). Sea-level rise and
674 aridification during the Pleistocene inundated much of this habitat and subsequently
675 isolated river systems from one another. Thus, along coastal Victoria aridification
676 and eustatic changes demonstrated interactive effects on phylogeographic patterns.
677 The more historic nature of divergences within the coastal lineage compared to the
678 MDB lineage were corroborated by the well-resolved phylogenetic structure and
679 relative stability of population sizes over time across the clade.

680

681 *Identity and Maintenance of Cryptic Species, N. 'flindersi'*

682 Although the initial divergence between *N. australis* and *N. 'flindersi'* was
683 associated with older biogeographic events during the Miocene, our results indicated
684 weak post-divergence gene flow between the two species. Distribution modelling
685 indicated a likely overlap in suitable habitat under contemporaneous conditions
686 across the Victorian and Tasmanian coastlines, with little divergence in
687 environmental ranges between the two species (Fig. S6). However, environmental
688 changes during glacial maxima likely caused the two species to retract to isolated
689 refugia. These factors together suggest a history of alternating periods of isolation

690 and connectivity during glacial cycles, with isolated glacial refugia and weak
691 interspecific interglacial gene flow limited to a narrow hybrid zone at the point of
692 contact. Other studies of terrestrial species diversification across recurrently
693 connected islands suggest patterns of gene flow in accordance with lower sea levels
694 (Paulay and Meyer 2002, Jordan and Snell 2008, Parent et al. 2008, Papadopoulou
695 and Knowles 2017). In this case, gene flow with *N. australis* does not appear to have
696 impeded divergence and our results support the previous denotation of *N. 'flindersi'*
697 as an independent species (Unmack et al. 2011, Unmack et al. 2013, Buckley et al.
698 2018).

699

700 *Implications for Conservation Management*

701 Southern pygmy perch are currently listed as Near Threatened on the
702 International Union for Conservation of Nature (IUCN, 2019) and Vulnerable or
703 Endangered within state government management lists (Hammer et al. 2013).
704 Ongoing conservation management has sought to recover their numbers, particularly
705 across the MDB (Attard et al. 2016, Brauer et al. 2016, Cole et al. 2016). Across the
706 species range, a number of clades have been identified and used as the basis for
707 management practices (Unmack et al. 2013, Cole et al. 2016). Traditionally,
708 conservation managers have adopted a 'local is best' paradigm, maintaining
709 independence of populations within captive breeding and translocation programs to
710 prevent outbreeding depression (Frankham et al. 2011, Love Stowell et al. 2017).
711 However, the complex nature of the southern pygmy perch populations across the
712 MDB indicates a history likely dictated by metapopulation dynamics with natural
713 patterns of local extinction, recolonization and sporadic gene flow (Cole et al. 2016).
714 A growing body of literature suggests that the propensity and magnitude of

715 outbreeding depression has been overestimated (Frankham et al. 2011). Given the
716 low levels of genetic diversity and high imperilment of MDB populations (Brauer et al.
717 2016), as well as the recent pattern of within-basin divergence detected here, we
718 argue that *in situ* and *ex situ* conservation efforts should use a basin-wide context
719 when selecting populations as source for demographic and genetic rescue (e.g.
720 captive breeding and translocations).

721

722 *Implications Under Climate Change*

723 Historic climatic fluctuations have often been used to predict future responses to
724 anthropogenic climate change (MacDonald et al. 2008, Dawson et al. 2011).
725 Primarily, these studies have focused on how species ranges and survival have
726 responded to changes in temperature, precipitation and sea level. Correspondingly,
727 however, many bioregions across the world are expected to increase in aridity with
728 ongoing climate change (Christensen et al. 2007), impacting on the stability and
729 structure of freshwater ecosystems globally (Middelkoop et al. 2001, Nijssen et al.
730 2001), including for the MDB (Cai and Cowan 2008, Pittock and Finlayson 2011).
731 Additionally, drought events are projected to occur at higher frequency and with
732 higher severity within Australia (Christensen et al. 2007). However, impacts of
733 climatic change on the availability and reliability of water resources are uncertain
734 (Middelkoop et al. 2001), as it is also the case on the influence of hydrological
735 change on the evolution and persistence of species. Projections of sea level rise
736 associated with melting glacial ice similarly predict major inundation of coastal
737 habitats globally (Rotzoll and Fletcher 2012). This poses a threat to freshwater
738 species not adapted to high salinity, and salinification of ecosystems to more
739 estuarine or marine environments across coastal regions threatens swathes of

740 biodiversity (Courchamp et al. 2014). Indeed, terrestrial species extinctions have
741 already been directly linked to inundation of island habitats (Waller et al. 2017).

742

743 Our study highlights how spatial variation in the role and extent of environmental
744 changes may result in variable impacts on the demography, distribution and
745 divergence of populations. Particularly, we show how aridification of inland
746 waterbodies and sea level rise leading to marine inundation of habitat impacted
747 different regions across the distribution of a freshwater fish, operating on different
748 timescales and to different extents. These environmental changes caused significant
749 divergence across the clade, resulting in a hierarchy of lineages spanning from a
750 cryptic species to intraspecific clades. While further increases in temperature will
751 directly impact on the long-term survival of many species broadly, additional impacts
752 on hydrological systems through aridification will have compounding effects on
753 freshwater species. Our findings suggest that ongoing impacts from anthropogenic
754 climate change may be complex in nature and vary across biogeographic regions
755 depending on the role and identity of environmental forces that operate locally. We
756 suggest that future management scenarios should consider this spatial variation in
757 prediction of responses to climate change, particularly in how specific aspects (e.g.
758 aridification, sea level changes) may act heterogeneously across species
759 distributions.

760

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766

767 DATA ACCESSIBILITY

768 The sequence alignment used for phylogenetic-based analyses is available in
769 NEXUS format in Dryad (**Dryad ref here**).

770

771 AUTHOR CONTRIBUTIONS

772 S.B. contributed to all sections of data analysis as well as drafting the manuscript.

773 L.B.B. designed the study, obtained resources, and helped with manuscript drafting.

774 C.B. generated the data. P.U. and M.H. contributed with samples and field expertise.

775 All authors contributed to the interpretation of results and critically revised the

776 manuscript.

777

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1173 TABLES

1174 **Table 1:** Locality data for samples used in this study. Abbreviations described in the
 1175 table were those used for further analyses, while *n* refers to the number of
 1176 individuals sequenced per population. *N. obscura* samples were only included as an
 1177 outgroup in the phylogenetic analysis.

Species	Population	Abbreviation	Field code	<i>n</i>
<i>N. australis</i>	Angas R., Strathalbyn	NauANG	F-FISH84	5
	Lake Alexandrina	NauALE	SPPBrA*	4
	Middle Ck, Warrenmang, Avoca	NauAVO	F-FISH75: PU99-33SPP	5
	Jew Harp Ck, Sidonia	NauJHA	F-FISH78: PU00-01SPP	3
	Tributary to Seven Creeks	NauSEV	PU13-65SPP	4
	Merton Ck, Goulburn Rvr.	NauMER	F-FISHY6: PU09-01SPP	5
	Broken R., Lima South	NauBRO	F-FISHY6: PU09-02SPP	5
	King R., Cheshunt, Ovens Rvr.	NauKIN	F-FISHY6: PU09-06SPP	4
	Spring Ck, Mitta Mitta	NauSPR	F-FISHY6: PU09-13SPP	5
	Gap Ck, Kergunyah, Kiewa	NauGAP	F-FISHY6: PU09-12SPP F-FISH77: PU99-81SPP	5
	Murray R. lagoon, Albury	NauALB	F-FISH53: IW94-47	4
	Coppabella Ck, Coppabella	NauCOP	F-FISH75: PU99-82SPP	5
	Blakney Ck, Lachlan Rvr.	NauLRT	F-FISH98: LPP-*	5
	Glenelg R., Glenisla	NauGRG	F-FISH78: PU0014-SPP	5
	Merri R., Grassmere	NauMRG	F-FISH78: PU00-22SPP	5
	Curdies R., Curdie	NauCRC	F-FISH78: PU00-24SPP	4
	Gellibrand R. floodplain	NauGRF	F-FISH97: PU02-92SPP	5
	Barongarook Ck, Colac	NauBAR	SPP08-13	4
	Mundy Gully	NauMG	F-FISHY8: PU08-11SPP	4
	Gnarkeet Ck, Hamilton	NauGCH	F-FISHY2: PU00-27SPP	4
Wilsons Promontory	NauWP	F-FISH97: PU02-70SPP	5	
<i>N. flindersi</i>	Snowy R. lagoon, Orbost	NfiSRLO	F-FISH77: PU99-85SPP	5
	Flinders Island	NfiFI	F-FISH84: FI-*	4
	Anson R. tributary	NfiANS	F-FISH82: HT-2*	5
<i>N. obscura</i>	Lake Alexandrina	Outgroup	YPBR*	5
Total		24		109 (114)

1178

1179

1180 FIGURE CAPTIONS

1181 **Figure 1: a)** Distribution and sampling map for southern pygmy perch. Inset depicts
1182 extent of distribution within Australia. The shaded area denotes the putative
1183 distribution of the species, spanning multiple major basins (black lines). Colours
1184 denote major clades explored within coalescent models (refer to Results) whilst
1185 shapes denote 'species' (circles = *N. australis*; squares = *N. 'flindersi'*). The extent of
1186 the continental shelf (-121m), which was exposed during glacial periods, is indicated
1187 in dark blue. **b)** Topographic map (including bathymetry) of southeast Australia,
1188 highlighting topographic heterogeneity and major biogeographic regions across the
1189 area. Solid black lines indicate major basin boundaries whilst the bold dashed line
1190 indicates the drainage divide across the Bassian Isthmus. The maximum extent of
1191 Lake Bungunnia (at 1.2 Ma) is also indicated with a narrow dashed line.

1192

1193 **Figure 2:** Maximum likelihood phylogeny of *N. australis* and *N. 'flindersi'* using 7,958
1194 concatenated ddRAD loci containing 45,104 SNPs. As all samples within a
1195 population formed monophyletic clades (excluding NauALB, shown in dashed lines),
1196 the phylogeny was collapsed to individual populations. Nodes with 100% bootstrap
1197 support are indicated by asterisks. The tree was rooted using *N. obscura* as the
1198 outgroup. The full phylogenetic tree with all 119 samples is shown in Figure S1.

1199

1200 **Figure 3:** Dendritic riverine network of the Murray-Darling Basin (MDB), with streams
1201 colour-coded according to the StreamTree model that determines the contribution
1202 (as a penalty) of each segment in driving genetic divergence across the basin.
1203 Segments coloured in yellow confer little penalty (i.e. genetic divergence between

1204 populations at either end of the segment is low) whereas red segments confer higher
1205 genetic differentiation.

1206

1207 **Figure 4:** Most likely ancestral areas under the best supported model (DIVA-LIKE),
1208 with presence in the Murray-Darling Basin (MDB) excluded until 5 Ma (indicated by
1209 the dashed line). A biogeographic timeline of major alterations to the MDB is
1210 included for reference. Colours denote one of six contemporary areas, or ranges
1211 combining more than one area, as described by the legend. **a)** Most likely state at
1212 each node or branch where state changes occurred. **b)** Probability of the most likely
1213 state (black) for each node.

1214

1215 **Figure 5:** Representative diagrams of the best supported coalescent models under
1216 each model set. The full set of tested models and the biogeographic hypotheses
1217 underpinning them are described within the Supplementary Material. Red arrows
1218 denote divergence time parameters whilst blue arrows denote migration rate
1219 parameters. Population sizes are reported as the number of diploid individuals ($N/2$).
1220 Gene flow parameters are reported in terms of proportion of alleles moving in the
1221 direction of the arrow forward in time. Δ Likelihoods = difference between estimated
1222 (simulated) model likelihoods and observed (empirical) likelihoods.

1223

1224 **Figure 6:** Stairway plot reconstructions of demographic history for individual
1225 populations. Both axes are reported in \log_{10} scale. Dark blue lines indicate medians
1226 with 95% confidence intervals shaded. Top two rows = Murray-Darling Basin (MDB)
1227 and WP populations; third row = coastal Victoria populations; bottom row = *N.*
1228 'flindersi' populations.

1229

1230 **Figure 7:** Species distribution models for all lineages and lineage-specific

1231 distribution models for each putative species based on 9 bioclimatic and 2

1232 topographic variables. **a-c)** Distributions under contemporary climate conditions. **d-f)**

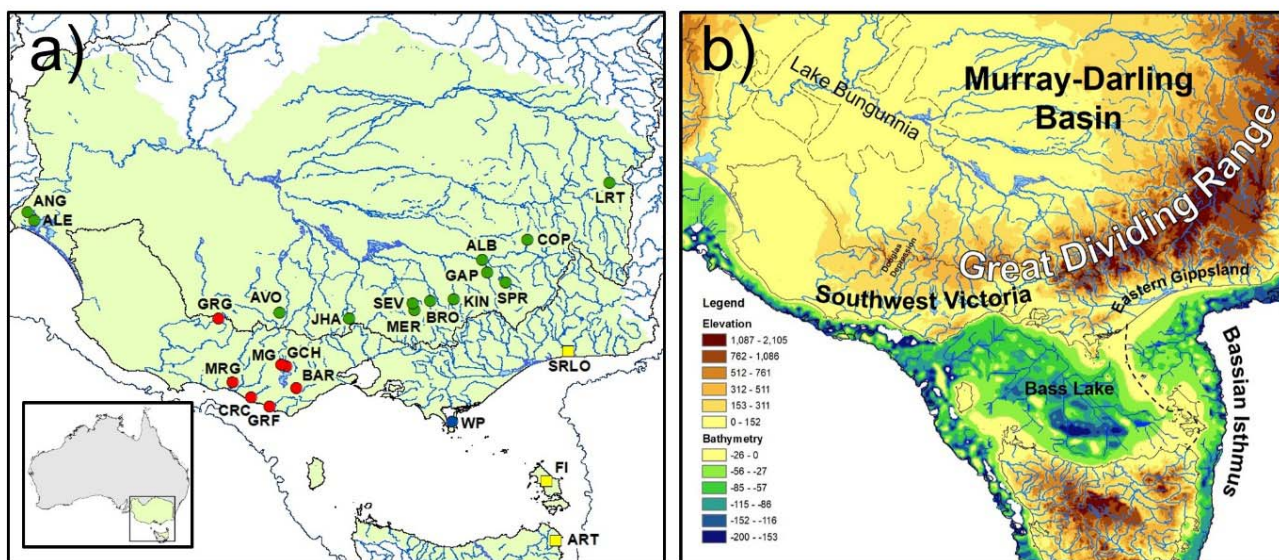
1233 Distributions under last glacial maximum (LGM; 22 Ka) climate. **g-i)** Distributions

1234 under mid Pliocene (3.2 Ma) climate conditions.

1235

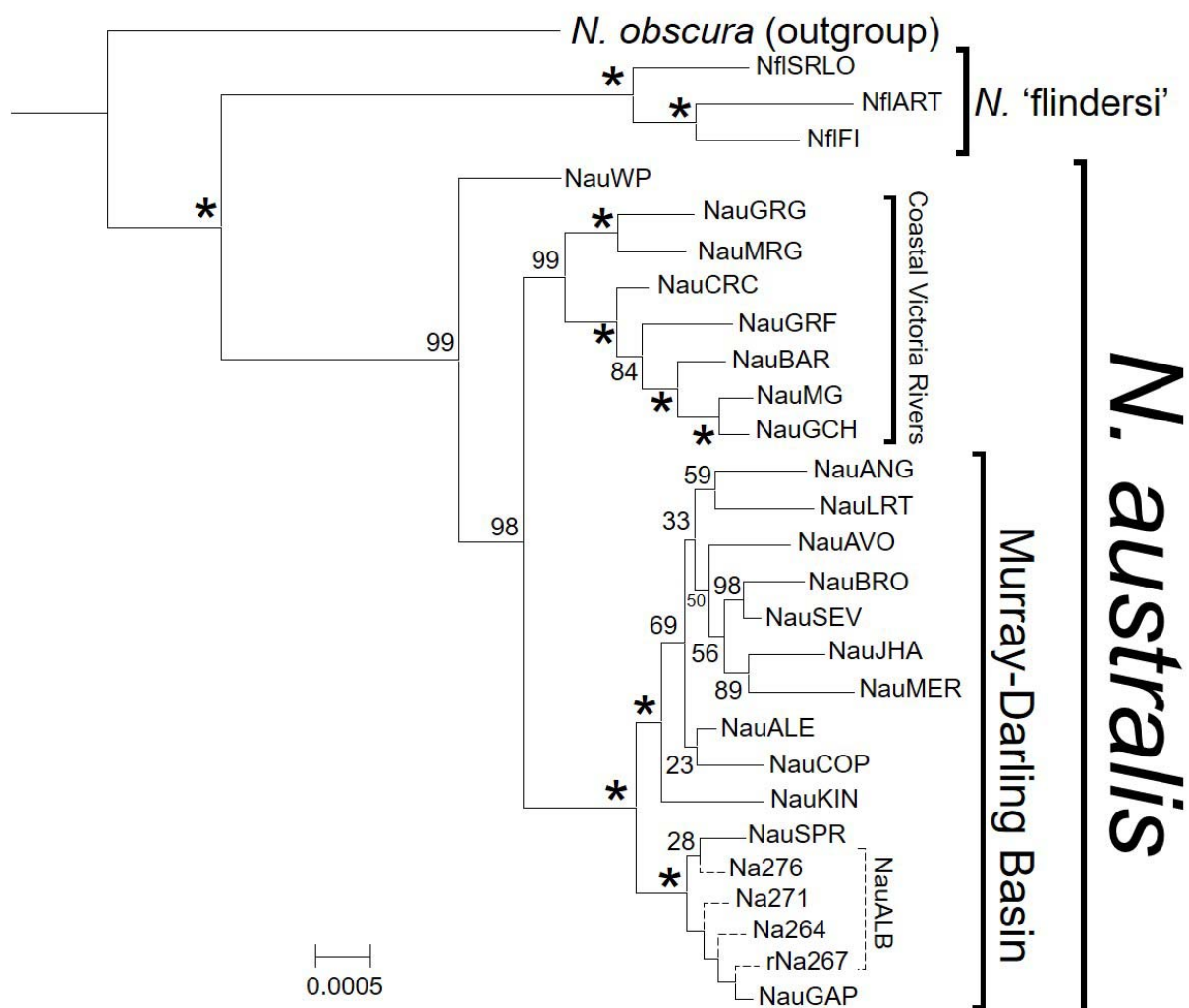
1236 FIGURES

1237 **Figure 1**



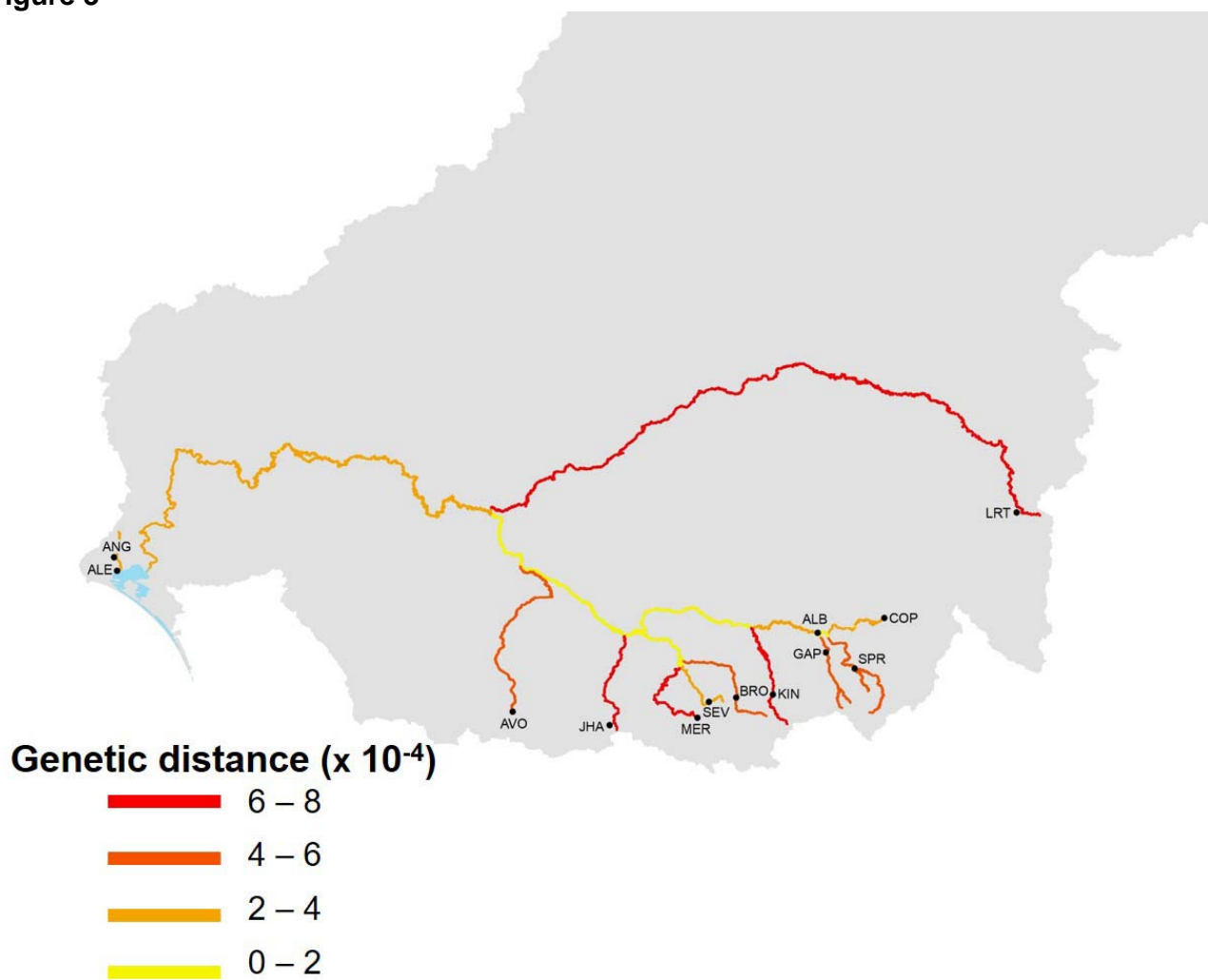
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1240 **Figure 2**



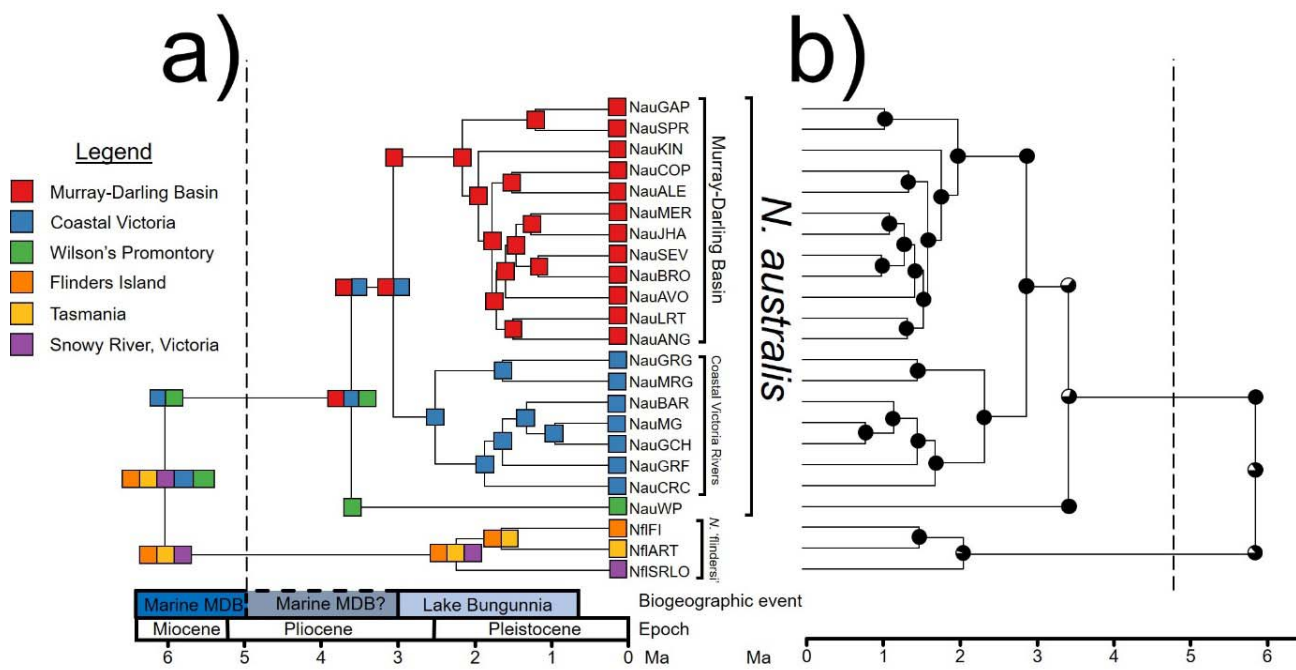
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1243 **Figure 3**



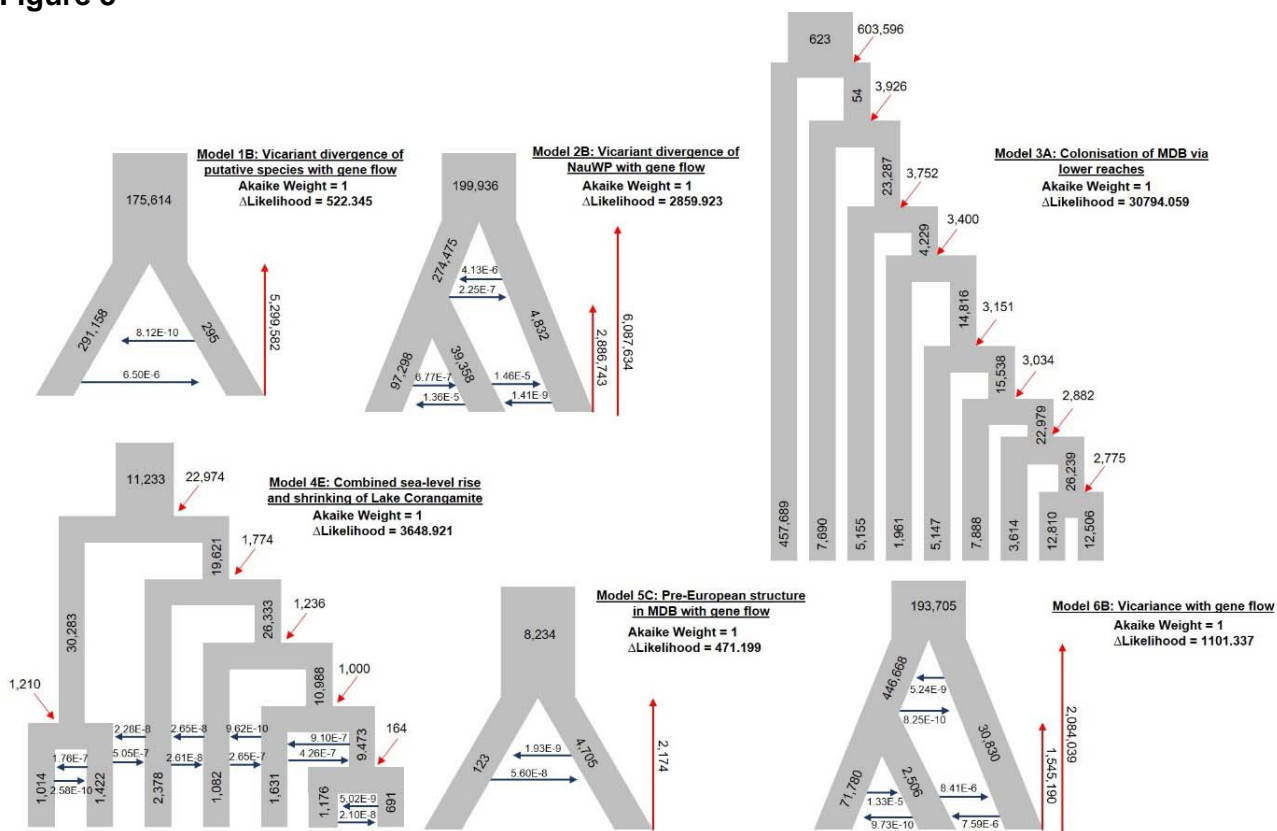
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1246 **Figure 4**



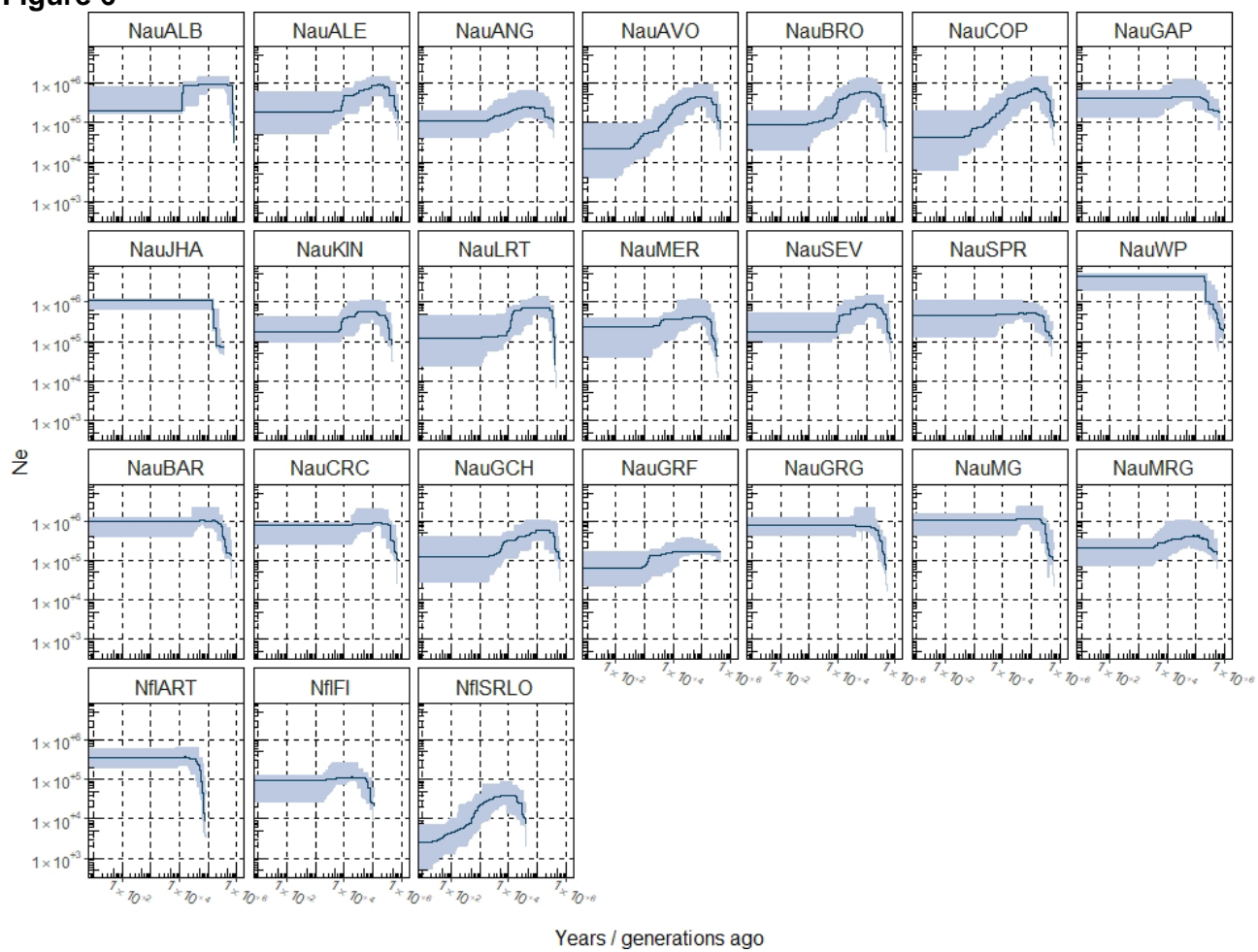
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1249 **Figure 5**



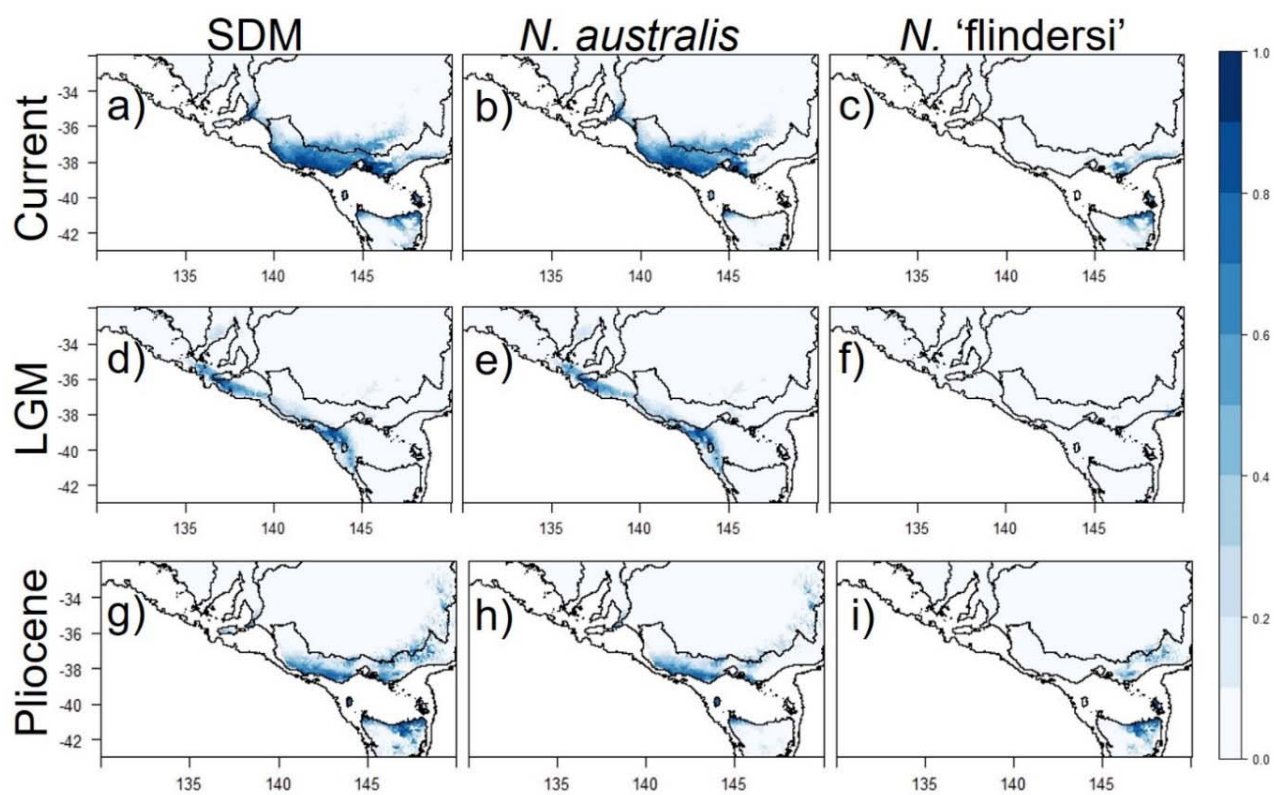
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1252 **Figure 6**



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1255 **Figure 7**



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