



Phylogenomics and biogeography of arid-adapted *Chlamydogobius* goby fishes

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ARTICLE INFO

Keywords:

Climate shift
Phylogenetics
Dispersal
Refuge
Fragmentation
Allopatric speciation
Connectivity

ABSTRACT

The progressive aridification of the Australian continent from ~ 20 million years ago posed severe challenges for the persistence of its resident biota. A key question involves the role of refugial habitats – specifically, their ability to mediate the effects of habitat loss and fragmentation, and their potential to shape opportunities for allopatric speciation. With freshwater species, for example, the patchiness, or absence, of water will constrain distributions. However, aridity may not necessarily isolate populations if disjunct refugia experience frequent hydrological connections. To investigate this potential dichotomy, we explored the evolutionary history of the *Chlamydogobius* gobies (Gobiiformes: Gobiidae), an arid-adapted genus of six small, benthic fish species that exploit all types of waterbodies (i.e. desert springs, waterholes and bore-fed wetlands, coastal estuarine creeks and mangroves) across parts of central and northern Australia. We used Anchored Phylogenomics to generate a highly resolved phylogeny of the group from sequence data for 260 nuclear loci. Buttressed by companion allozyme and mtDNA datasets, our molecular findings infer the diversification of *Chlamydogobius* in arid Australia, and provide a phylogenetic structure that cannot be simply explained by invoking allopatric speciation events reflecting current geographic proximity. Our findings are generally consistent with the existing morphological delimitation of species, with one exception: at the shallowest nodes of phylogenetic reconstruction, the molecular data do not fully support the current dichotomous delineation of *C. japalpa* from *C. eremius* in Kati Thanda-Lake Eyre-associated waterbodies. Together these findings illustrate the ability of structural (hydrological) connections to generate patterns of connectivity and isolation for an ecologically moderate disperser in response to ongoing habitat aridification. Finally, we explore the implications of these results for the immediate management of threatened (*C. gloveri*) and critically endangered (*C. micropterus*, *C. squamigenus*) congeners.

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1. Introduction

By changing the distribution and connectivity of habitat, climatic shifts represent some of the strongest drivers of biological diversification. Aridification in particular poses a substantial challenge for resident biota (Byrne et al., 2008; Pepper and Keogh, 2014, 2021). In the Australian context, the development of widespread aridity from the mid-Miocene imposed a unique biogeographic setting that forced existing flora and fauna to either adapt to environmental extremes, contract its distribution, or go extinct (Byrne et al., 2008). Reconstructions of the paleoenvironments of now-arid Australia chart dramatic environmental shifts (Fujioka and Chappell, 2010; Pepper and Keogh, 2021). A peak Cenozoic warm period saw high humidity that supported widespread rainforest and swamps and an associated tropical fauna including lungfishes, flamingos, and crocodiles (Fujioka and Chappell, 2010). However, a series of aridification events starting from around ~ 20 million years ago (Ma) (Martin, 2006), and reaching peak dryness around the Last Glacial Maximum (~18–16 thousand years ago [ka]), ultimately left water sources increasingly rare and isolated, and surface water almost entirely ephemeral (Alley, 1998; Silcock, 2009). Glacial-interglacial cycles over the last 400,000 years drove massive fluctuations in moisture, vegetation, and water levels. The nature and distribution of fresh water were further shaped by an intensification of the northern, seasonal monsoon (Hesse et al., 2004), the spread of the stony deserts in the south around 4–2 Ma (Fujioka et al., 2005), and the long term evolution of groundwater-fed spring groups over the Cenozoic, along with short term changes within spring groups over time frames of tens to hundreds of thousands of years as older springs “die” and new springs emerge at local scales (Ponder, 1986; Alley, 1998; Prescott and Habermehl, 2008).

This drying climate triggered a range of responses in different organismal groups. Genetic signatures in extant groups reveal consequences ranging from geographically large-scale divides between arid and mesic zones (e.g. in the lizard genus *Liopholis*: Chapple and Keogh, 2004) to heightened diversity and endemism, such as in dispersal-limited snails, amphipods and isopods restricted to groundwater habitats (Perez et al., 2005; Cooper et al., 2007, 2008; Murphy et al., 2012, 2013; Ponder et al., 2019). Nevertheless, patterns emerging from molecular studies signal several recurring themes (Pepper and Keogh, 2021). For instance, the Australian arid-zone biota is comparatively young, aligning with the relatively recent onset of fully arid and widespread conditions (Pepper and Keogh, 2021). Additionally, these lineages are usually derived from peripheral mesic or coastal environments such as the moisture-rich northern tropics or more highly vegetated zones of eastern-Australia (Byrne et al., 2008).

A complex picture of prolonged, widespread, and extreme aridity also offers excellent opportunities for testing the changing concept of evolutionary refugia (Byrne et al., 2008; Davis et al., 2013). Indeed, results for Australian desert-dwelling groups hint that evolutionary trajectories are more complex and idiosyncratic than have been seen for large environmental shifts in other parts of the world (e.g. expanding and contracting ice sheets in Pleistocene Europe: Hewitt, 2001; Kearns et al., 2014), highlighting the need for further study of processes underpinning persistence, refugia, and speciation after biome shifts (Pepper and Keogh, 2021).

Given their persistence and dispersal are intimately tied to the presence and configuration of water sources (Davis et al., 2013; Hughes et al., 2013), freshwater organisms represent powerful models for exploring the implications of aridification and loss of aquatic habitat (Murphy et al., 2015a; Attard et al., 2022). For example, investigations of the dispersal-limited invertebrates of the iconic, oasis-like artesian springs of central Australia have discovered high levels of diversification and endemism (Ponder et al., 1995, 2019; Fensham et al., 2011; Murphy et al., 2009; Murphy et al., 2013). In contrast, and despite suggestions of underestimated levels of cryptic diversity among Australia’s freshwater fish fauna (Hammer et al., 2013; Adams et al., 2014), its arid-zone fishes

have been less well explored (Unmack, 2001). Yet in this environment, fishes warrant increased attention because they represent an ecological ‘middle ground’ regarding dispersal capacity. Relative to winged organisms, their reliance on the presence of water generally constrains their mobility. However, their dispersal capacity should be greater than for more sedentary aquatic taxa, including exclusively aquatic invertebrates, such as snails and amphipods (Murphy et al., 2015b).

In arid lands across the world, springs are a critical aquatic habitat as they provide highly permanent stable habitats in an otherwise extremely hostile environment for aquatic organisms. They often harbour high numbers of endemic species (Rossini et al., 2018), often thought to be remnants of wetter periods of time. Springs are essentially islands in a sea of desert, in some ways similar to terrestrial islands in the ocean. Many aspects of the equilibrium model of island biogeography likely apply to springs, especially relative to the size, number, and proximity of springs to each other (Kodric-Brown and Brown, 1993; Rossini et al., 2018; Negus and Blessing, 2022). In addition, springs tend to be clustered in the landscape at multiple scales. They occur across larger areas around geologic elements such as faults or where aquifer sediments are close to the surface through to the finest scales where they group as “family units”, with older spring outlets or fossil springs sitting slightly higher in the landscape, a larger younger spring coming out at lower elevations, and other springs of various ages found in close proximity reflecting the slow gradual changes that occur in individual spring evolution (Ponder, 1986). Probably the best example of this is the Great Artesian Basin in Australia, with its massive geographic expanse covering a fifth of Australia and its old age (sediments were deposited from 250 to 100 million years ago) in the middle of a fairly stable continent. There are 13 defined larger clusters of springs called supergroups. Many of these supergroups of springs are likely to have existed for large portions of the Cenozoic, with more local clusters of springs changing in shorter time frames due to local geologic and geomorphic processes.

There are two relevant hypotheses for the radiation of obligate freshwater taxa. In one, species are relicts of wetter times, trapped in small, isolated refuge habitats. Such a model is exemplified by the highly genetically structured, desiccation-vulnerable snails of Great Artesian Basin (GAB) springs (Ponder et al., 1995; Perez et al., 2005; Murphy et al., 2015b), or arid-zone populations of *Liopholis* lizards (Chapple and Keogh, 2004). Alternatively, taxa with modest vagility have often revealed a “localised microgeographical refugia” response (sensu Byrne et al., 2008), in which diversification manifests as intraspecific lineages that are geographically distinct. This presents a “refugia within refugia” pattern inferred to reflect rapid colonisation and isolation in multiple, localised refuges within a species’ range (e.g. the trapdoor spider, *Moggridgea tingle*: Cooper et al., 2011).

Here, we use a fish genus with an unusually broad arid distribution (Fig. 1) to investigate the biogeographic consequences of aridification. *Chlamydogobius* (Gobiiformes: Gobiidae) is a genus of six, small-bodied, benthic fishes that exhibits an impressive physiological resistance to environmental extremes. While five *Chlamydogobius* congeners occur in arid rivers and/or individual spring complexes, the tropical primarily estuarine distribution of its single non-inland representative, the tadpole goby, *C. ranunculus* (Fig. 1), suggests a likely northern origin for the group. However, the nature of its colonisation of inland Australia (Lake Eyre Basin) remains uncertain. Additionally, these fishes are unusual among the local fish fauna in regularly exploiting springs (frequently small, isolated, and with depths prohibitively shallow for larger fishes) and not just riverine environments, of arid Australia (Larson, 1995), raising questions about the configuration and connectivity of aquatic habitat used by *Chlamydogobius* species.

Previously, we assessed the most widespread member of the genus, the desert goby *C. eremius*, in a spatially comprehensive mtDNA and allozyme-based phylogeography that included representatives of its nearest relative, the Finke goby, *C. japalpa* (Mossop et al., 2015). That study highlighted limited molecular distinctiveness and indications of

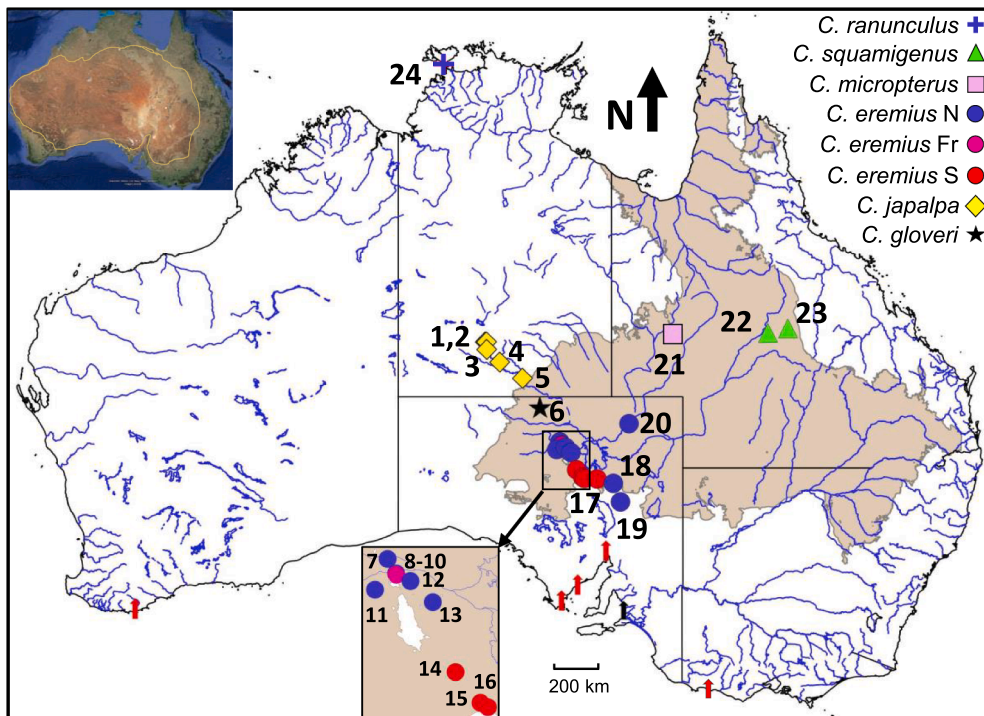


Fig. 1. Map of the Australian mainland continent displaying the location of all sites and comparative distribution of all *Chlamydogobius* species. Sites are designated by a taxon symbol, as per the legend provided, and numbered using the site codes used in Table 1. Inset map: Satellite image of the mainland, with the orange boundary depicting the extent of the arid/semi-arid zone (image: Google Earth). Light blue shading shows likely distribution of *C. ranunculus*. Locations for the outgroup specimens used are shown using an arrow symbol (red = *Pseudogobius*; black = *Mugilogobius*). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

recent geneflow between these two taxa, previously defined based on fine-scale morphological differences (Larson, 1995), and signalled the need for more comprehensive examination of the evolutionary and taxonomic affinities of all inland *Chlamydogobius*. Here, we use the ‘Anchored Hybrid Enrichment (AHE) method of Lemmon et al. (2012) and an extensive dataset (260 nuclear genes) to build a phylogeny and address three key objectives: 1) elucidate the biogeographic history of all desert-dwelling *Chlamydogobius* lineages; 2) assess the molecular validity of a species-level designation between *C. eremius* and *C. japalpa*; and 3) provide conservation and management insights for arid-zone biodiversity, including three species of immediate conservation concern (Dalhousie goby, *C. gloveri*; Elizabeth Springs goby, *C. micropterus*; and Edgbaston goby, *C. squamigenus*: IUCN, 2019). In so doing, we also provide a test of the utility of AHE loci at relatively shallow evolutionary time scales. Since allopatric speciation is frequently implicated in the diversification of Australia’s arid biota, we use the information gained to explore two relevant hypotheses for the radiation of water-sensitive taxa (mesic relicts or localised microgeographical refugia, *sensu* Byrne et al., 2008), and offer insights for understanding the extent to which arid refugia foster shared versus unique evolutionary responses in different aquatic species.

2. Materials and Methods

We used two approaches to explore the role of connectivity in shaping responses of an arid-adapted group. First, we examined the relationships among the *Chlamydogobius* gobies by using phylogenomics, underpinned by two more traditional molecular datasets (allozymes and mtDNA), to construct a comprehensive molecular phylogeny, and thus obtain insights into historical connectivity. Second, we explored the landscape characteristics of possible colonization pathways to assess the modern connectivity of extant populations, focussing on the three range-restricted, threatened, and spring-dependent species (*C. gloveri*, *C. micropterus* and *C. squamigenus*).

2.1. Historical connectivity: Genomic signatures

2.1.1. Sampling

We used the AHE sequencing method (Lemmon et al., 2012) to collect sequence data from 313 nuclear loci for 26 *Chlamydogobius* and 6 exemplars of two closely-related outgroups (blackspot mangrovegoby, *Mugilogobius stigmaticus* and bluespot goby, *Pseudogobius olorum*; Larson, 2001). Our tissue samples were muscle or caudal fin sourced from either recently-collected specimens or collections held at the South Australian Museum. We sequenced between three and five individuals for each *Chlamydogobius* species and for the two primary phylogroups evident within *C. eremius* (“northern” and “southern”: Mossop et al., 2015). Sampling thus covered both interspecific and intraspecific levels of divergence within the genus (details in Table 1, Fig. 1, and Supplementary Table S1).

2.1.2. Laboratory protocols

Genomic DNA was extracted and isolated from samples using the Qiagen Dneasy kit, following the manufacturer’s instructions. We used a Nanodrop spectrophotometer to measure DNA quantities, and confirmed the outcome using gel electrophoresis. Sequencing was attempted only for samples containing > 1.8 µg of DNA. The AHE method works by capturing and enriching the more variable regions that flank highly conserved areas common across vertebrate genomes, allowing the sequencing of DNA regions that are comparable among diverse taxa (Lemmon et al., 2012; Prum et al., 2015).

2.1.3. Locus selection and probe design

Although the AHE kit developed for vertebrates by Lemmon et al. (2012; v1 design) contains a fish reference (*Danio*) and has been utilized in teleosts with moderate success (Eytan et al., 2015), we desired an enrichment tool more efficient and appropriate for phylogenomics in teleosts. Because of the complex nature of teleost genome evolution, which involved multiple whole-genome duplications and lineage-specific gene losses (Glasauer and Neuhauss, 2014), it is impractical to identify a set of loci that are truly single-copy across all of Teleostei. Previous studies claiming to have identified single copy orthologs in teleosts (e.g. Li, 2007) likely only identified loci that were single-copy in

Table 1

Sampling details for all molecular datasets. Site codes match those used in Fig. 1; these are cross-referenced to the site codes [in square brackets] used for the two species represented in Mossop et al. (2015). The three genetic groups previously identified allozymically in *C. eremius* are designated here as N ('northern'), Fr ('Freeling') and S ('southern'). Sample sizes (n) for the three molecular appraisals are shown (AP = Anchored Phylogenomics), as are the AP library codes for each individual. (* asterisk indicates that one of these cyt b sequences was only 601 bp and was therefore not used in the final tree). Within Locality names, NT = Northern Territory; SA = South Australia; QLD = Queensland; WA = Western Australia; VIC = Victoria.

Site code	Species	Locality	Latitude	Longitude	n ^{AP}	AP library code	n ^{Alloz}	n ^{mtDNA}
1 [a1]	<i>C. japalpa</i>	Glen Helen Gorge, NT	-23.68	132.67	1	I2984	8	2
2 [a2]	<i>C. japalpa</i>	Pioneer Creek, NT	-23.68	132.72	1	I2983	6	
3 [a3]	<i>C. japalpa</i>	Finke River @ Hermannsburg, NT	-24.00	132.73			6	2
4 [a6]	<i>C. japalpa</i>	Finke River @ Henbury, NT	-24.53	133.28	2	I3006, I3007		
5 [a5]	<i>C. japalpa</i>	Finke River @ Horseshoe Bend, NT	-25.22	134.24	1	I2998		
7 [d1]	<i>C. eremius</i> N	Algebuckina Waterhole, SA	-27.90	135.81	1	I2994		
8 [f1]	<i>C. eremius</i> N	Freeling Spring #3, SA	-28.05	135.90			5	
9 [g1]	<i>C. eremius</i> Fr	Freeling Spring, largest, SA	-28.06	135.90			5	
10 [g2]	<i>C. eremius</i> Fr	Freeling Springs #2, SA	-28.07	135.90			3	
11 [m]	<i>C. eremius</i> N	Nilpinna Springs, SA	-28.22	135.68				2*
12 [e1]	<i>C. eremius</i> N	Peake Creek, SA	-28.13	136.05	2	I2997, I3009		2
13 [k1]	<i>C. eremius</i> N	Fountain Spring, SA	-28.35	136.28			10	
18 [y]	<i>C. eremius</i> N	Hergott Springs, SA	-29.65	138.07				2
19 [z]	<i>C. eremius</i> N	Leigh Creek, SA	-30.43	138.37	1	I3008		
20 [o]	<i>C. eremius</i> N	Ultoomurra Waterhole, SA	-27.13	138.73			5	
14 [p]	<i>C. eremius</i> S	Nunn's Bore, SA	-29.08	136.52			7	2
15 [r1]	<i>C. eremius</i> S	Coward Springs, SA	-29.40	136.78	2	I2995, I2996	8	2*
16 [aa]	<i>C. eremius</i> S	Bubbler Mound Spring group, SA	-29.45	136.86	2	I2982, I3010		
17 [v]	<i>C. eremius</i> S	Gosse Spring, SA	-29.46	137.38			5	
6	<i>C. gloveri</i>	Dalhousie Springs, SA	-26.47	135.48	3	I2991 – I2993	18	2*
21	<i>C. micropterus</i>	Elizabeth Springs, QLD	-23.35	140.58	3	I2988 – I2990	20	2
22	<i>C. squamigenus</i>	Bore 1652 Crossmore Station, QLD	-22.90	144.58			11	2
23	<i>C. squamigenus</i>	Edgbaston Mound Spring, QLD	-22.71	145.42	3	I2985 – I2987	6	2
24	<i>C. ranunculus</i>	Darwin, NT	-12.37	130.91	4	I3011 – I3014		2
Outgroup	<i>M. stigmaticus</i>	Port River, SA	-34.85	138.50	2	I3004, I3005		1
Outgroup	<i>P. olorum</i>	King River, WA	-34.94	117.90	1	I3002		
Outgroup	<i>P. olorum</i>	Tod River, SA	-34.59	135.88	1	I3001		
Outgroup	<i>P. olorum</i>	Arno Bay, SA	-33.92	136.57	1	I2999		
Outgroup	<i>P. olorum</i>	Port Augusta, SA	-32.49	137.76	1	I3000		
Outgroup	<i>P. eos</i>	Shaw River, VIC	-38.31	142.06				2

the species they considered (an over-fitting problem); evaluation of those loci in additional teleost lineages suggests that these loci are not universally single-copy (see below). Consequently, we aimed to target loci containing up to four gene copies in each of three diverse lineages of teleosts: zebrafish, platyfish, and cichlids.

Candidate target regions for Teleostei were derived by combining the 394 Vertebrate Anchor (v2) loci of Prum et al. (2015) and the 135 loci identified as *Takifugu-Danio* single-copy orthologs by Li (2007). For the vertebrate anchor loci, teleost orthologs were obtained for *Danio rerio* (danRer7) using the human (hg19) coordinates and the UCSC (University of California Santa Cruz) genome browser batch-coordinate (lift-over) tool (<https://genome.ucsc.edu/>; Kent et al., 2002). For the *Takifugu-Danio* orthologs, orthologous human (hg19) and chicken (galGal3) coordinates were obtained using the USCS lift-over tool and the *Danio* coordinates identified by Li (2007). Once the coordinates for *Danio*, *Homo*, and *Gallus* were obtained for all 529 candidate target regions, sequences corresponding to those regions (plus sufficient flanking region to obtain up to 3,000 bp total) were extracted from the genomes (downloaded on Apr 5, 2012 from <http://hgdownload.cse.ucsc.edu/downloads.html>) and aligned by locus using MAFFT (Katoh and Standley, 2013, v7.023b with “-genafpair” and “-maxiterate 1000” flags). The alignments were then used to generate a *Danio*-specific reference database containing spaced 20-mers. The *Danio* reference was then used to identify homologous regions in the genomes of zebrafish (Cypriniformes: Cyprinidae: *Danio rerio*: danRer7), platyfish (Cyprinodontiformes: Poeciliidae: *Xiphophorus maculatus*: Schartl et al., 2013), and cichlid (Perciformes, Cichlidae: *Maylandia zebra*: Loh et al., 2008). Details concerning the creation and use of the reference database are given in Supplementary Methods.

As expected, we recovered multiple homologs for many of the candidate loci (only 64 loci were single copy in all three species). Consequently, only 277 loci had fewer than five homologs per species

and were considered further. We aligned with MAFFT (Katoh and Standley, 2013, v7.023b with “-genafpair” and “-maxiterate 1000” flags) all homologs sequences (up to 12 per locus) for each of the 277 candidates together with the homologous human probe region sequence from the Vertebrate Anchor (v2) design. Alignments were then manually inspected for misplaced and grossly misaligned sequences, which were removed. Finally, alignments were trimmed to include regions best suited for AHE (conserved, low-gap, high taxon representation), taking care that the chosen region contained the human probe region. A total of 260 loci were retained.

Finally, to ensure efficient enrichment, we checked for high-copy regions (e.g., microsatellites and transposable elements) in each of the three teleost references as follows. First, a database was constructed for each species using all 15-mers found in the trimmed alignments for that species. We also added to the database all 15-mers that were 1 bp removed from the observed 15-mers. The genome for the species was then exhaustively scanned for the presence of these 15-mers and matches were tallied at the alignment positions at which the 15-mer was found. Alignment regions containing > 100,000 counts in any of the three species were masked to prevent probe tiling across these regions. Probes of 120 bp were tiled uniformly at 5.5x tiling density. Scripts used for locus selection and design, final probe region sequences, corresponding genomic coordinates, and probe design are available on request as supplemental materials.

2.1.4. Data collection

Phylogenomic data were collected following Lemmon et al. (2012) at the Center for Anchored Phylogenomics at Florida State University (www.anchoredphylogeny.com), with some adjustments. Each genomic DNA sample was sonicated to a fragment size of ~ 175–300 bp using a Covaris E220 Focused-ultrasonicator with Covaris microTUBES. Library preparation and indexing following the protocol of Meyer and Kircher

(2010) but adapted for use on a Beckman-Coulter Biomek FXP liquid-handling. One important modification is a size-selection step after blunt-end repair using SPRIselect beads (Beckman-Coulter Inc.; 0.9x ratio of bead to sample volume). Indexed libraries were then pooled at equal quantities (2 pools of 16 samples each), and the library pools were enriched using a custom Agilent Custom SureSelect kit (Agilent Technologies), designed as described above. The two enriched library pools were pooled equal quantities for sequencing on 1 PE150 Illumina HiSeq2000 lane with 8 bp indexing. Sequencing was performed in the Translational Science Laboratory in the College of Medicine at Florida State University.

2.1.5. Data analysis

Reads were quality filtered using Illumina's Casava software (<https://gaow.github.io/genetic-analysis-software/c/casava/>) with default parameters (i.e. with the chastity filter level set to high). Overlapping reads were then merged following Rokyta et al. (2012). Non-overlapping read pairs were kept separate but still used in the assembly. The merged and unmerged reads were then assembled into contigs following Prum et al. (2015) using mapping references derived the zebrafish, platyfish, and cichlid sequences used for probe design. Note that the assembler sorts gene copies with >5% sequence divergence into separate contigs. Contigs were then filtered by removing those derived from fewer than 100 reads. A summary of the sequence data collected and resulting assemblies is given as a supplemental file.

Filtered consensus sequences were grouped by target locus (across individuals) in order to produce sets of homologs. Orthology was then determined for each target locus as follows. First, a pairwise distance measure was computed for pairs of homologs, with distance being computed as the percentage of 20-mers observed in the two sequences that were found in both sequences. A neighbor-joining clustering algorithm was then used to cluster the consensus sequences in to orthologous sets, with at most one sequence per species in each orthologous set (see Prum et al., 2015 for details). In order to minimize the effects of missing data, clusters containing fewer than 16 (50%) of ingroup individuals) the species were removed from downstream processing. Following orthology assessment, alleles were phased using read assemblies following Pyron et al. (2016). Details are also given in Supplementary Methods.

Allele sequences in each orthologous set were aligned using MAFFT 7.023b (Kato and Standley, 2013) with `-genafpair` and `-maxiterate 1000` flags. The raw alignment for each locus was then trimmed and masked to remove poorly aligned regions following Prum et al. (2015), with the following adjustments: sites with > 40% similarity were identified as good, 20 bp regions containing < 13 good sites were masked, and sites with fewer than 16 unmasked bases were removed from the alignment. Loci containing fewer than 16 sequences were removed.

A maximum likelihood phylogeny was estimated with 100 bootstrap replicates for each of the separate loci using RAxML (Stamatakis, 2014) with GTR + rates model assumed. Alleles from the closely related goby genera *Pseudogobius* and *Mugilogobius* were used as outgroups. We then used the RAxML bootstrap trees to estimate a species tree using STAR (Liu et al., 2009) using default parameters.

2.2. Historic connectivity: Other molecular assessments

We also generated companion allozyme and mtDNA datasets, both to provide independent assessments of our genomic phylogeny for *Chlamydogobius* and to further integrate the present study with our previous study of the two central Australian species (Mossop et al., 2105). Our allozyme analyses encompassed 123 individuals (all ingroup species except *C. ranunculus*, for which no frozen tissues were available) from 15 sites, while the mtDNA dataset comprised cytochrome *b* (*cytb*) sequence data for 24 ingroup (all six species; 12 sites) and three outgroup specimens. For *C. eremius* and *C. japalpa*, selected populations and individuals genotyped by Mossop et al. (2015) were specifically chosen to

encompass the allozyme/mtDNA and geographic diversity found in these species, thus further linking the two studies. Sample sizes and locality details are presented in Table 1 and Supplementary Table S1. The methods for generating and analysing the allozyme dataset and for generating *cytb* sequences are all presented in Mossop et al. (2015).

Phylogenetic analyses of mitochondrial sequences were performed with maximum likelihood (ML) using GARLI 2.01 (Zwickl, 2006) on the CIPRES cluster (Miller et al., 2010). We evaluated different partition schemes and models of molecular evolution based on the Akaike Information Criterion with correction (AICc) in PartitionFinder 2.0 (Lanfear et al., 2012) using PhyML (Guindon et al., 2010). GARLI was implemented with 10 search replicates with the following default setting values changed: `streefname = random`; `attachmentspertaxon = 48`; `genthreshfortopoterm = 100,000`; `significanttopochange = 0.00001`. For bootstrapping we ran 500 replicates with the previous settings with the following changes: `genthreshfortopoterm = 10,000`; `significanttopochange = 0.001`; `treerejectionthreshold = 20`, as suggested in the GARLI manual to speed up bootstrapping. Trees were rooted using *M. stigmaticus* and *P. eos*. Mean between-species genetic divergences was calculated based on *cytb* using p-distances in MEGA 7.0.18 (Kumar et al., 2016).

Interpreting the timing of divergence is fraught with difficulty as we lack any clear fossils or geologic events that may provide a basis for calibrating a molecular clock. The only other option would be to use previous standard rates calculated for *cytb*; however, this is also challenging due to the small number of taxa in our study, especially given *Chlamydogobius* has short generation times, small body size and lives in warm environments, all being potentially associated with higher rates of molecular evolution (Gillooly et al., 2005; Thomas et al., 2010). Thus, any dates calculated would not be overly reliable. Despite these caveats, p-distances based on *cytb* provide some basis for indicating whether divergence is recent, or older based on previously published rates (while also ignoring the impacts of saturation). For instance, Burridge et al. (2008) summarised other studies on teleost fishes with calibrated molecular clocks for protein coding mtDNA genes (primarily *cytb*) and found estimated substitution rates of between 0.34 and 0.83% per lineage per million years.

2.3. Modern connectivity: Inferring isolation of contemporary mesic remnants

Dispersal in *Chlamydogobius* is thought to be flood-mediated (Unmack and Wager, 2007; Kerezszy, 2014; Mossop et al., 2015), and should therefore be influenced by the steepness of temporary waterways that could connect disparate patches of water (Kornis and Vander Zanden, 2010; Nicol et al., 2015). To investigate potential dispersal pathways of arid aquatic connectivity and guide inference of the most likely barriers to contemporary fish movement, we explored visually the river channel morphology and prevalence of ephemeral water in key areas of species' distributions (Worthington-Wilmer et al., 2008). Specifically, these were corridors of possible connection between the three spring-associated *Chlamydogobius* taxa (*C. gloveri*, *C. micropterus*, and *C. squamigenus*) and their nearest active river system; as well as pathways known to operate for spring populations of *C. eremius* (Mossop et al., 2015, 2017). Since the present-day geological setting (including landscape features such as sand dunes and gibber plains) and today's climate regime were established ≥ 1 Ma (Martin, 2006; Fujioka et al., 2009), surface topography and river morphologies have changed little since recent historical times; although the current expression of climate cycles means that conditions have never been drier (Fujioka and Chappell, 2010). However, due to their remoteness, scale, and high variability, Australia's inland rivers generally remain poorly characterised by hydrological data (Costelloe et al., 2005), limiting formalised modelling approaches (e.g. Isolation by Distance; McRae, 2006). We thus used a combination of surface water observations (derived from satellite imagery), and river channel morphology and topography to

identify extant water flow paths, landscape structure, and current river channel morphology.

Water Observations from Space (WofS; Mueller et al., 2016) is a web database that visualises historical surface water observations by collating satellite imagery from the years 1987 to present (a period spanning multiple years of large floods; Mossop et al., 2015) into a single, algorithm-based dataset that accounts for sources of error to provide qualitative confidence values. In conjunction, we used the “Elevation profile” function in Google Earth 7.1.5.1557 (Google Inc. 2015; Dolliver, 2012) as a dynamic tool with which to explore pathways between isolated spring localities (Witjira-Dalhousie Springs, Elizabeth Springs, and Edgbaston Springs; Fig. 1), and their nearest active river channels. Accessing both tools in September 2015, we extended the approach to explore flow paths known to operate for spring populations of *C. eremius* (Mossop et al., 2015, 2017). For simplicity, we traced the most direct routes of connection via meandering channels (while recognised that actual dispersal routes will be likely more circuitous). Although the representation of surface water from satellite images does not capture the maximum extent of flood events (Mueller et al., 2016), the method should indicate where flow occurs most readily, as the most active river channels will also reveal the most frequent water observations.

3. Results

3.1. AHE genomic phylogeny

The final AHE concatenated data matrix contained 32 individuals, 260 loci, 380,949 sites, and only 6.5% missing data. These 260 nuclear loci yielded a strongly-supported species phylogeny for *Chlamydogobius* fishes: well-resolved interspecific relationships included high bootstrap support at all but a few of the shallowest nodes involving conspecific individuals (Fig. 2). The five arid-adapted *Chlamydogobius* formed a monophyletic group, with the earliest branching lineage for the genus consisting of the coastally-distributed *C. ranunculus*. Four strongly-supported lineages, albeit with short branch lengths, are evident in the arid-adapted clade, corresponding to the three spring-associated species, *C. gloveri*, *C. micropterus*, and *C. squamigenus*, plus a clade comprising the two more widespread sister taxa, *C. eremius* and *C. japalpa*. Intriguingly, the four ‘northern’ *C. eremius* sites show a shallow but well-supported sister relationship with *C. japalpa*, rather than with their ‘southern’ counterparts.

Our well-resolved genomic phylogeny indicates that the sister species to the *C. eremius/japalpa* complex is *C. squamigenus* rather than its geographically more-proximate congeners *C. gloveri* and *C. micropterus* (Fig. 2). Furthermore, the earliest branching taxon in the arid-adapted clade is *C. gloveri*, the spring-restricted species found nestled within the broader geographic distribution of the Lake Eyre/Finke River *Chlamydogobius*.

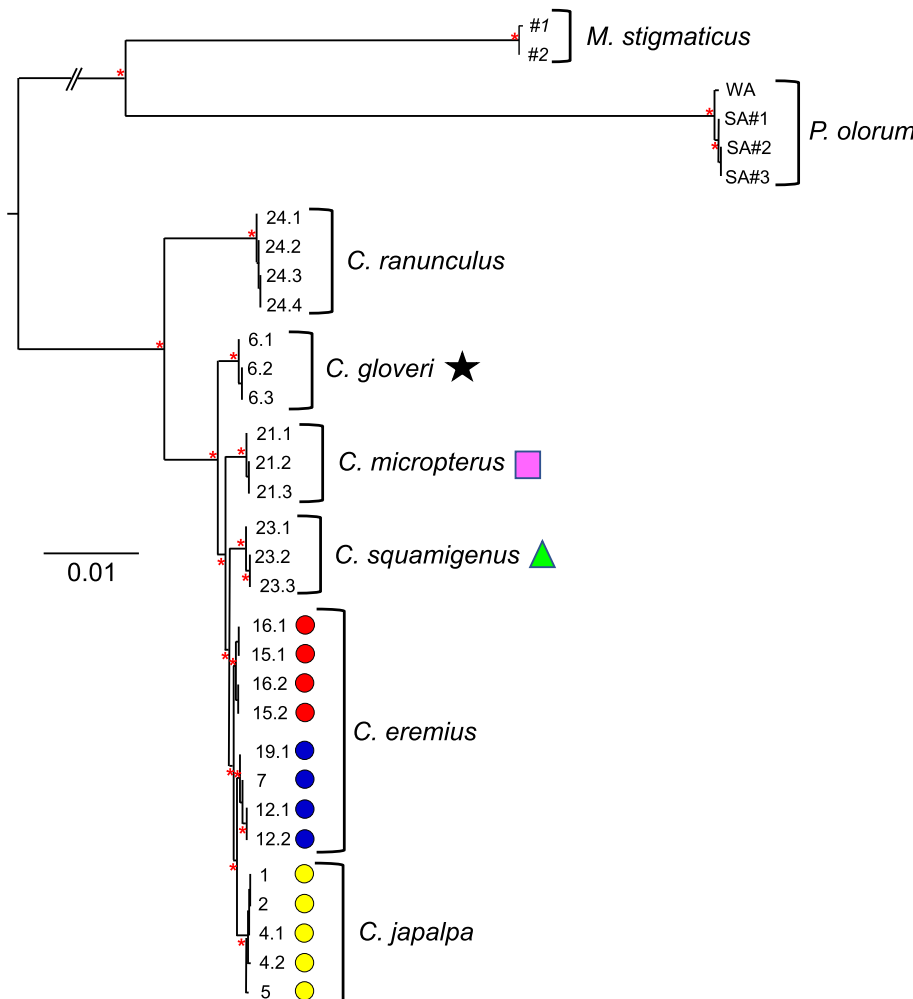


Fig. 2. Result of the RAxML maximum likelihood species tree for *Chlamydogobius* with meaningful branch lengths, estimated using STAR (Liu et al., 2009) and 260 anchored hybrid enriched (AHE) loci for all taxa (n = 26 ingroup and n = 6 outgroup taxa). Nodes receiving strong bootstrap support (>97%) are asterisked. The scale bar indicates expected changes per site. *Chlamydogobius* populations or species are identified with the legend and site codes used in Fig. 1. Individuals from the same site are labelled 0.1, 0.2 etc.

3.2. Allozyme perspectives

The final allozyme dataset comprised the genotypic profiles for 123 individuals at 51 putative loci. These data are summarized as allozyme frequencies by population across the five species available for analysis (Supplementary Table S2). An unrooted Neighbor-Joining network constructed from the pairwise unbiased Nei Distances among these 15 populations (Supplementary Table S3) revealed the presence of the same four primary genetic lineages evident in our genomics dataset, namely *C. gloveri*, *C. micropterus*, *C. squamigenus*, and *C. eremius/C. japalpa* (Fig. 3). All four groups were diagnosable by fixed allelic differences at multiple loci (range 7–14 loci; Supplementary Table S3). In contrast, *C. japalpa* was not reliably distinguishable from *C. eremius* at any locus (Supplementary Tables S2, S3) and its affinities lay more with ‘northern’ *C. eremius* (and in particular with two “Freeling” sites, which were not accessible for genomic analysis) than with sites representing the ‘southern’ genetic group (albeit still only 1–2 fixed allelic differences: Table S2).

3.3. Matrilineal gene tree

The final mtDNA dataset comprised of near complete *cytb* sequences (1,140 bp) for 21 ingroup and three outgroup individuals plus an additional 3 ingroup individuals sequenced for the first 601 bp of *cytb*. All sequences have been deposited in Genbank (accession numbers MW775536–MW775559). The ML gene tree (Fig. 4) was broadly concordant with the genomic species tree (Fig. 2), differing only in that *C. eremius* and *C. japalpa* were reciprocally monophyletic rather than split into three geographically-based lineages, and that branching order for the early-branching arid-adapted taxa *C. gloveri* and *C. micropterus* was not resolved with strong bootstrap support. The northern *C. ranunculus* was separated by large p-distances varying from 14.2 to 16.1%, while the five inland species varied from 1.6 to 5.8% (Table 2). Table 2 provides simplistic divergence time values based on rate of 0.5% per million years, which suggests the invasion of inland drainages are quite old, possibly occurring during mid-Miocene. Within the Lake Eyre Basin, divergence between most species was likely during Pliocene through to early-Pleistocene. Even when considering the potential for

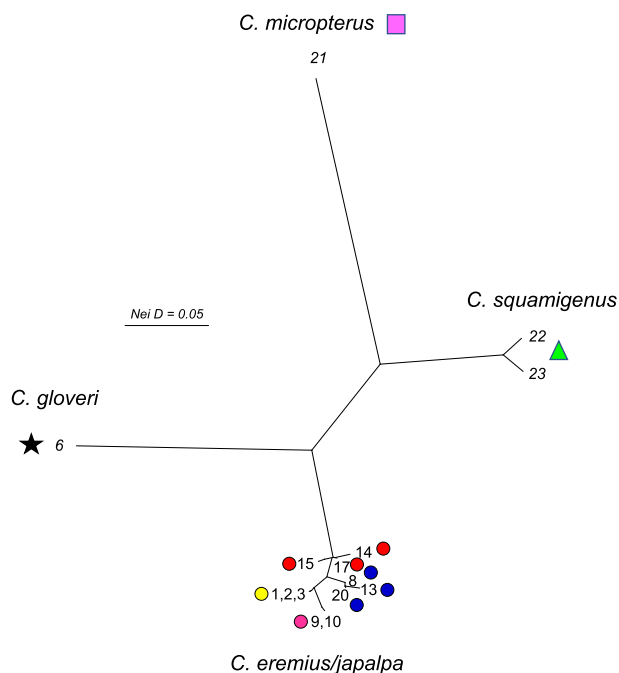


Fig. 3. Unrooted network based on unbiased Nei Distances among all *Chlamydogobius* sites surveyed in the allozyme study.

error, it appears likely the majority of divergence predates more the extreme aridity that occurred during mid to late-Pleistocene.

3.4. Connectivity or isolation of contemporary mesic remnants

Explorations of the elevation profiles and potential spring–river connections are summarised in Table 3, and for simplicity, generally represent the feasibility of movement away from the spring habitat patch (i.e. emigration; although some into-spring movements are represented for comparison). Patterns of elevation and surrounding surface water prevalence varied among the springs of interest. For example, pathways between Witjira-Dalhouse and adjacent river channels could theoretically take four routes. However, positive elevational differences demonstrate that the spring complex is depressed relative to the surrounding landscape on its western side, and intermittent flood extent and variable topography on the east suggested that all but a single (eastern) pathway are inactive. In contrast, the elevational variation potentially encountered by dispersing *C. squamigenus* (particularly in the south of its core range) was minimal, suggesting that dispersal attempts between the springs and the adjacent creek are less constrained by elevational differences. At Elizabeth Springs, the main extant pathway for potential movement was also topographically simple and short-distance, with little hydrological activity from either spring flow or river surface water. In comparison, pathways inferred from connections known to function between modern spring-based *C. eremius* populations revealed short distances, but also steeper slopes and greater flood activity.

4. Discussion

Here, we report a highly-resolved phylogenetic reconstruction of the evolutionary relationships of *Chlamydogobius* gobies. The current distribution of *Chlamydogobius* species corresponds to permanent water sources separated by increasing aridity, highlighting that while the group’s dispersal capacity has allowed it to exploit challenging dispersal opportunities in recent history, the levels of genetic divergence are consistent with the arid zone species being mesic relicts that now rely on access to permanent relictual habitats for persistence. Our molecular data largely agree with Larson’s (1995) morphological study, noting that she had access to unpublished allozyme data (from M. Adams), which delimited six species using characters including the number of caudal vertebrae, scale extent, body colouring, and the size and ray counts of pectoral and dorsal fins. However, while supporting the validity of four species, the genomic data in particular question the taxonomic distinctiveness of *C. japalpa* and *C. eremius*, currently separated on the basis of modal differences in three fine-detail meristic characters and previously flagged as not allozymically diagnosable (Mossop et al., 2015).

4.1. Inland invasion and arid-zone diversification

The geographically defined phylogenetic tree inferred that *Chlamydogobius* was monophyletic, with the northern distributed *C. ranunculus* (Fig. 1) being the first branching lineage to a single inland invasion. Patterns of speciation point to hydrological isolations that are not determined simply by distance or drainage boundaries. There is no evidence of the more structured signature that would be expected from repeated colonisation events, or isolation and then reconnection between inhabited regions with climatic fluctuations. Instead, the phylogeny is consistent with the gradual isolation of areas from a broadly distributed ancestor.

The northern species, *C. ranunculus*, provides an excellent proxy for how Lake Eyre Basin populations may have had broader distributions that diverged over time. This species is found in mangrove and estuarine habitats which has enabled it to have a broad distribution around the coast of northern Australia, but it has also dispersed far inland into



Fig. 4. Maximum likelihood tree for *Chlamydogobius*, rooted using the three outgroup sequences and based on 1,140 bp of Cytochrome *b*. Nodes receiving strong bootstrap support (>97%) are asterisked. Symbols and individual codes follow Fig. 2.

Table 2

Mean between-species Cytochrome *b* p-distances (lower left) for *Chlamydogobius* with a “crude” estimate of divergence times based on a standard molecular clock rate of 0.5% per lineage per million years (upper right).

Species	<i>C. eremius</i>	<i>C. japalpa</i>	<i>C. squamigenus</i>	<i>C. micropterus</i>	<i>C. gloveri</i>	<i>C. ranunculus</i>
<i>C. eremius</i>	–	1.6	2.6	5.7	5.5	16.1
<i>C. japalpa</i>	0.016	–	2.7	5.6	5.6	15.7
<i>C. squamigenus</i>	0.026	0.027	–	5.3	5.5	15.8
<i>C. micropterus</i>	0.057	0.056	0.053	–	5.8	14.2
<i>C. gloveri</i>	0.055	0.056	0.055	0.058	–	15.1
<i>C. ranunculus</i>	0.161	0.157	0.158	0.142	0.151	–

drainages in the Gulf of Carpentaria (Fig. 1) to at least within 50 km of the drainage divide with the Lake Eyre Basin. In this region, it occupies a diverse range of freshwater habitats, such as floodplain wetlands, intermittent creeks as well as springs and spring-like habitats provided by flowing bores. Once the ancestral *Chlamydogobius* invaded the Lake Eyre Basin from the north, it could have easily dispersed across the massive, interconnected Lake Eyre Basin, as climate was wetter during the Miocene. A variety of suitable habitats would have existed, along with the various spring supergroups present in the Lake Eyre Basin (Ponder, 1986). Over time, these habitats gradually became increasingly isolated, either as a result of landscape processes, such as the isolation of

Dalhousie Springs by high stream gradients, and/or as aridity increased, making it more difficult for extensive dispersal to occur through less hospitable intervening habitats.

We assume that during earlier wet climatic phases *Chlamydogobius* would have been ubiquitous across the Lake Eyre Basin, similar to how *C. ranunculus* is in the Gulf of Carpentaria today. The first diverged species is *C. gloveri* from Dalhousie Springs Supergroup, which consists of 80 active springs over an area of 70 km² (Zeidler and Ponder, 1989). Several springs in this complex are massively larger than any other springs found in the Lake Eyre Basin today, with large pools and discharges up to ~ 140 l/s. Based on geological interpretations, Krieg

Table 3

Summary of landscape characteristics of potential dispersal routes between perimeters of key spring habitats and adjacent river systems. All listed species have been recorded from riverine habitats (*C. squamigenus*, *C. micropterus*, *C. eremius*), or use overland flow to colonise new springs (*C. gloveri*; [Kodric-Brown and Brown, 2007](#)).

Spring complex or group	Species present	Pathway	Direction of movement modelled	Elevational difference (from spring to nearest river) (m)	Maximum slope (%)	Average slope (%)	Distance (km)
Witjira-Dalhousie	<i>C. gloveri</i>	Dal_1	Into river	75.5	1.9	0.5	20.7
		Dal_2	Into river	-33.5	-1.9	-0.3	17.3
		Dal_3	Into river	-30.0	-1.9	-0.3	14.2
		Dal_4	Into river	-48.9	-5.6	-0.4	21.6
Elizabeth Springs Edgbaston	<i>C. micropterus</i>	Eliz SpCk	Into river	-2.24	-1.6	-0.3	1.2
	<i>C. squamigenus</i>	SE60_overland	Into river (overland flow)	-1.16	-2.7	-0.4	0.6
		Edg_Aram*	Into river	-55.7	-1.2	-0.2	41.6
		Edg_Cros*	Into river	-98.9	-0.5	-0.1	158.0
Wabma-Kadarbu Mound Springs	<i>C. eremius</i>	Blanche_overland (east)	Into river (overland flow)	-7.96	-5.2	-2.2	0.5
			Into spring	4.58	66.0	2.5	0.5
		Blanche_overland (north)	Into river (overland flow)	-7.95	-5.2	-1.9	0.7
			Into spring	4.74	5.8	1.7	0.7
		The Bubbler_over	Into river (overland flow)	-36.3	-13.4	-1.8	3.75
Peake Station		Fountain Spring to Levi Creek	Into river	-20.5	-3.9	-0.7	5.85

*Indicate pathways implied by long-distance connectivity, rather than simply entry into a functional river or creek.

(1986) argued Dalhousie Springs are around 7 million years old, but possibly up to 20 million years, which is consistent with them having formed prior to the evolution of *C. gloveri* (Table 2). Dalhousie Springs are one of the most biodiverse spring complexes in Australia (Rossini et al., 2018) with a number of endemic aquatic animals, highlighting that they must have been accessible by the broader aquatic biota during wetter climatic periods and have likely provided a suitable refuge over several million years (Zeidler and Ponder, 1989). The isolation of Dalhousie Springs was also supported by exploration of rainfall and elevation data that found flow pathways draining from Dalhousie Springs are substantially more challenging than those successfully navigated by other *Chlamydogobius* species (Table 3, Mossop et al., 2015) via steep topography (e.g. an increase of > 70 m across a 20 m stretch) and a relative absence of nearby stream activity.

The next species to diverge is *C. micropterus*, which is endemic to Elizabeth Springs, one spring group within the Springvale Supergroup. Most springs in this supergroup today are quite small and scattered, with only Elizabeth Springs containing endemic biota. Today, Elizabeth Springs consists of ~ 40 very small springs, seeps, and inactive outlets in an area ~ 1600 m by 600 m (Unmack and Wager, 2007; Roberts, 2015). Prior to bore drilling and subsequent loss of pressure in the Great Artesian Basin this spring group was reported to flow for at least 5 km, or possibly 30 or more kilometres (Fairfax and Fensham, 2002). Thus, while isolated from other springs in this supergroup, its sheer size (it would have been one of the largest springs outside of Dalhousie Springs) would have provided considerable refuge over a long period of time. In addition, Spring Creek (which Elizabeth Springs is adjacent to) has five permanent waterholes, which is a higher number than any other part of the Diamantina catchment (Silcock, 2009). These waterholes may have helped provide additional refuges in recent time. It remains unclear though why this species diverged so early given there are no substantial barriers other than distance to Lake Eyre and the other *Chlamydogobius* species. The only key difference is the Diamantina has a much lower number of permanent and near permanent waterholes than Cooper Creek (Silcock, 2009) as well as lower fish species richness (Wager and Unmack, 2000).

The three remaining *Chlamydogobius* species have lower genetic divergences and are more similar in meristic counts, appearance and colouration (Larson, 1995), which is indicative of their more recent isolation during the Pleistocene. While geographically *C. squamigenus* is the most distant species from Lake Eyre, it has probably had longer-term

connectivity due to the higher number of waterholes present in Cooper Creek (Silcock, 2009). Today, *C. squamigenus* is primarily known from the upstream reaches of the Aramac Creek subcatchment, but it has also been found in two distant bore drains lower down in this catchment (Kerezy, 2020). This could either indicate a high level of gene flow across Aramac Creek (distances of over 100 km) or early colonisation from now extinct springs. There are many smaller springs in the Barcaldine Supergroup along parts of Aramac Creek, which may have historically provided refuge to *C. squamigenus* prior to bore drilling. The Edgbaston Springs (also now known as Byarri) group contains around 100 mostly very small springs and seeps across an area of ~ 30 km². Despite its small size and geographic extent, the Edgbaston Springs group contains a disproportionate number of endemic species (26 total vs 13 in the next most diverse spring group) when compared to all other Great Artesian Basin springs (Rossini et al., 2018). The unusually large number of springs in a small area, often separated by only a few hundred meters or less apart, has likely aided long term persistence of a diverse biota, as does probably the presence of other springs (many of which are extinct today) along parts of Aramac Creek that may have facilitated past persistence of the biota during the Pleistocene.

The remaining two species are in close geographic proximity but are hydrologically isolated today, as the Finke River no longer appears to reach Kati Thanda-Lake Eyre under current climatic conditions (Kotwicki, 1989). Even if rare paleofloods (Pickup et al., 1988) connected the Finke to Kati Thanda-Lake Eyre, successful dispersal would still likely be problematic, as floods from different rivers would need to be synchronous to allow this. *Chlamydogobius jalalpa* represents the only fully riverine/non-spring species in the Lake Eyre Basin, being restricted to the Finke River and its waterholes. While primarily known from waterholes in the vicinity of the MacDonnell Ranges, they also disperse into more ephemeral habitats in the lower Finke River.

The last species, *C. eremius* is the most widespread and is found across the Lake Eyre Supergroup, which is the largest in geographic area (~400x20 km) and number of springs (~100 named groups consisting of 1000 s of small individual springs), as well as being commonly found in riverine habitats as well. The Lake Eyre Supergroup is crisscrossed by many smaller creek catchments, which may facilitate dispersal between individual spring groups. It also sits at the crossroads for access between all rivers that flow into Lake Eyre. Populations in catchments draining into Kati Thanda-Lake Eyre South also appear to be more isolated (Mossop et al., 2015) due to limited connections with Kati Thanda-Lake

Eyre North. While the species clearly disperses well in catchments flowing into Kati Thanda-Lake Eyre North, it only ever penetrates a short distance up Warburton Creek (which is the lower part of Diamantina River) and is yet to be recorded from lower Cooper Creek. This may be due to multiple factors, such as the available time to move upstream as flows in upstream reaches start to decrease by the time flow reach Kati Thanda-Lake Eyre, the lack of permanent waterholes, and a larger number of fish species to co-exist with.

4.2. Taxonomic status of Kati Thanda-Lake Eyre-associated taxa

An apparently important landscape element is evident in the fact that, at the shallowest nodes of the nuclear phylogenetic tree, the separation of groups – *C. japaipa*, *C. eremius* “North”, and *C. eremius* “South” – corresponds to the structure that the contemporary Kati Thanda-Lake Eyre imposes on the hydrological landscape. Indeed, these nodes reveal very recent disjunctions that presumably arose with the cessation of hydrological connectivity, first between drainages associated with the south and north branches of the lake, and subsequently, between Lake Eyre North and the Finke River system. Interestingly, this finding agrees with patterns found previously using allozyme markers (Mossop et al., 2015). In contrast though, the mtDNA *cytb* data presents a different picture, with *C. japaipa* being sister to *C. eremius* (Fig. 4). This suggests differing evolutionary histories for the matrilineal and nuclear genomes. Overall, it seems likely the onset of playa (ephemeral) lake conditions represents an important shift for the potential connectivity of *Chlamydogobius* around Kati Thanda-Lake Eyre. Kati Thanda-Lake Eyre reached its current form at around 50–47 ka, when increasing aridity severed its last connection with the adjacent paleo megalake, Lake Mega-Frome (Cohen et al., 2011; for a detailed description of associated dispersal barriers, see Mossop et al., 2015).

Importantly, the current findings offer strong molecular support for a taxonomic revision of the status of *C. eremius* and *C. japaipa* populations. Detailed analyses found the absence of any fully-diagnostic nuclear genetic markers (51 allozyme loci) screened by Mossop et al. (2015), coupled with their finding that a few individuals from the Freeling Spring complex (within the range of *C. eremius* ‘North’) displayed *C. japaipa* nuclear genetic ancestry. These two taxa were differentiated morphologically on the basis of fine meristic details but did not incorporate many exemplars of *C. eremius* ‘North’ (Larson, 1995). However, the current results demonstrate the divergence within *C. eremius* likely pre-dates the separation of *C. japaipa*, indicating their current dichotomy is not an appropriate one. In this light, a morphological reappraisal focussing on a comparison of representatives of all three phylogroups is clearly desirable. Here, we envisage three basic outcomes: i) designating a single, variable species with discrete conservation units, ii) a division of two taxa (*eremius*-South, and *eremius*-North plus ‘japaipa’), or iii) a split into three taxa (Adams et al., 2014). A similar scenario is presented for two other co-located fishes, the Finke and Lake Eyre hardyheads (*Craterocephalus centralis* and *Craterocephalus eyresii*), where genetic data also suggest these sibling ‘species’ are likely conspecific (Adams et al., 2011), in contrast to the species-level divergence displayed by the Finke and Dalhousie populations of the more habitat specialised eleotrid genus *Mogurnda* (Adams et al., 2013).

Interestingly, other studies of inland fish fauna have reported species-specific responses to limited or lost hydrological connectivity (Huey et al., 2011), but the use of such a diversity of habitat types (including extremely shallow, spring-fed wetlands) is restricted to only the *Chlamydogobius* fishes. Nonetheless, there are intriguing parallels with the *Craterocephalus* spp (hardyheads); that group’s distribution similarly includes the Finke River; the direct Kati Thanda-Lake Eyre tributaries via two sympatric endemics (as above, *C. centralis*, *C. eyresii*), although there is no evidence for a north–south disjunction for *Craterocephalus* in waters connecting to Kati Thanda-Lake Eyre (Unmack and Dowling, 2010).

4.3. Conservation implications

Insights into the consequences of historical connectivity regimes have immediate relevance for managing taxa with fragmented or spatially restricted ranges. Despite their isolation, Australia’s arid water bodies are of intensifying conservation concern following rapid the anthropogenic change accompanying European settlement (Fensham et al., 2010, 2011; McNeil et al., 2011; Davis et al., 2013; Gotch, 2013; McBurnie et al., 2015; Rossini et al., 2018). While Australia’s Lake Eyre Basin is one of the few large drainages worldwide to remain unregulated (Pisanu et al., 2015), arid waterbodies are increasingly subject to the threat of invasive fishes, the impacts of pastoralism and mining/extraction activities, and the effects of climate change (Davis et al., 2013). In particular, populations in artesian springs are impacted by the large-scale, extraction of water from the GAB aquifer, including the extinction or degradation of many (up to 88%) spring wetlands across the region (Fensham and Fairfax, 2003; Gotch, 2013).

In this context, our study also provides insights into the species-specific nature of arid aquatic refugia. The concept of ecological and evolutionary refugia has been subject to ongoing revision and discussion (Ponder & Slatyer, 2007; Byrne et al., 2008; Fensham et al., 2011; Davis et al., 2013; Costelloe and Russell, 2014). In this case, the effects of historical isolation amidst the challenges of arid survival are, on contemporary scales, severely exacerbated by anthropogenic threats. The widescale extraction of GAB artesian aquifers, and subsequent extinction and reductions of spring habitats is a critical threat affecting inland *Chlamydogobius* species and populations. In several cases, this imposes reductions on the number and connectivity of available habitat patches: a problem especially pertinent for species with restricted geographic ranges and small population sizes (Fagan et al., 2002).

In this respect, insights into the roles of elevation, topography, and flow regime are highly relevant. For example, at Elizabeth Springs, a relatively homogenous topography means that topographic barriers are not overt, but (in common with Dalhousie Springs) nor does an elevated position facilitate downstream aquatic connections with lower-lying creek channels (as in other cases; Table 3). Rather, the impact of arid aquatic refuges will then depend on surface water availability, which is highly limited in nature (Duckett and Stow, 2013). Historic records show that the unchecked extraction of GAB waters has reduced flow at Elizabeth Springs by 70% (Fensham and Fairfax, 2003): an outcome made more visible in recent years by the further extinction of individual springs, and the loss of an adjacent waterhole in the creek that was apparently sustained by flow from the springs themselves (Unmack and Wager, 2007; Roberts, 2015). Combined with the stochasticity of temporary, flood-driven dispersal opportunities, the number of extant populations and their location in the landscape will be important for future persistence.

Currently, *C. micropterus* is listed as Vulnerable (IUCN, 2019). Recent further habitat losses mean that it probably numbers far less than the most recent population size estimates of just 1–2,000 individuals (Wager, 1995), and is possibly currently constrained to only 3–5 individual springs (Unmack and Wager, 2007; Roberts, 2015). Yet capping of unused GAB bores has yielded some promising results for the partial restoration of artesian pressure, particularly when implemented strategically and on local scales (Fensham and Fairfax, 2003; Hassall and Associates Pty Ltd, 2003). Since Unmack and Wager’s (2007) assessment, the refuge of Elizabeth Springs has only further declined (primarily via cattle damage and choking by vegetation), highlighting the crucial role of water availability in both maintaining the extent and ecological function of spring wetlands, and increasing their resilience to co-occurring threats and natural environmental variation. Restoring artesian pressure at Elizabeth Springs should now be urgently prioritised via ongoing capping of bores in the vicinity of Spring Creek. A dual benefit here would be to reduce the risk of catastrophic invasion by the prolific and aggressive live bearing eastern mosquitofish, *Gambusia holbrooki*, as bore drains also provide key refugia for them. Prioritising

groundwater flow rates to boost the extent and number of habitat patches can also render refuge sites more resilient to co-occurring threats, such as physical and chemical damage by cattle and feral herbivores, the choking of open water by excessive vegetation growth, natural fluctuations in flow rates, and the threats imposed by small population sizes.

Introduced fishes, especially *G. holbrooki*, represent pervasive and ongoing threats to desert spring faunal communities globally. At Edgbaston Springs, *G. holbrooki* has had a devastating effect on the critically endangered red-fin blue-eye, *Scaturiginichthys vermeilipinnis*, a co-occurring endemic fish (Fairfax et al., 2007; Kerezszy, 2014; Nicol et al., 2015), with strong evidence that it has also induced population declines and extirpations of *C. squamigenus* populations (Fairfax et al., 2007; Kerezszy, 2014; Nicol et al., 2015; Faulks et al., 2017). Programs such as the restoration of local aquifer pressures (e.g. government initiatives to reduce the artesian water wastage that decreases water supply to springs; Gotch, 2013) that raise the possibility of increasing the size or number of habitat patches, and the management of invasive species (e.g. *G. holbrooki* control at Edgbaston Springs; Kerezszy and Fensham, 2013) are invaluable for securing long-term persistence.

5. Conclusions

Here, we provide a highly resolved phylogeny of the *Chlamydogobius* gobies, contributing a comprehensive insight into the evolution of Australia's arid-adapted fish fauna. In so doing, we provide insights into the consequences of aridity for an ecologically moderate disperser, the species-specific nature of aquatic arid refugia, and the conservation of threatened desert biota. We infer a mid-Miocene diversification of the *Chlamydogobius* fishes of arid Australia, and suggest that is similar to the earlier origins of many other arid-zone lineages. Species that occur in restricted evolutionary refugia in harsh arid environments require careful consideration in conservation and natural resource management.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We extend deep respect to the many Traditional Owners of the lands and waters surrounding Kati Thanda-Lake Eyre and beyond, and their ongoing connection to and custodianship of Country. In particular, we are grateful to Dean Ah Chee and Reg Dodd for their advice, conversations, and natural and cultural insights. Thanks to many people for field assistance: Nicholas Moran, Nicholas Deal, Rob Mossop, David Mossop, Marcus Michelangeli, Minna Saaristo, and P. Andreas Svensson. Thank you to Dave Wilson, and Adam Kerezszy for generous support with samples. We also thank Michelle Kortyna at the Center for Anchored Phylogenomics for assistance with AHE data collection. Thanks to Winston Ponder for providing comments on the manuscript. Procedures for field sampling were approved by the Monash University Animal Ethics Committee (BSCI/2012/14). The South Australian Department of Environment and Natural Resources granted collection permits (Q25886-2; Q25886-3) and the South Australian Department of Primary Industries and Regions granted S115 Ministerial Exemptions for fishing activity (Numbers: 9902391, 9902523, 9902598, and 9902599).

Funding sources

The research was supported by grants from the Great Artesian Basin Coordinating Committee, the Nature Foundation South Australia, and the Holsworth Wildlife Research Endowment (to KM) and the Australian Research Council (DP120103010; to BMW).

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ympev.2023.107757>.

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