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Plio-Pleistocene sea-level changes drive speciation of freshwater fishes in north-western Australia

Running Title: Sea-level change drives freshwater biodiversity

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regarding data analysis and interpretation. We thank Gerry Allen, Jon Armbruster, Michael Baltzly, Joshua Brown, Chris Burridge, Stephen Caldwell, Adam Fletcher, David Galeotti, Mark Kennard, Adam Kerezszy, Alfred Ko'ou, Andrew McDougall, Masaki Miya, David Morgan, Tim Page, Colton Perna, Ikising Petasi, Michael Pusey, and Ross Smith and the Hydrobiology team for their efforts in helping to collect and/or provide specimens.

Abstract

Aim: Despite the influence of sea-level changes on biogeographic/phylogeographic patterns in freshwater ecosystems being well documented, studies that explicitly link the influence of sea-level change with speciation are rare. We aim to test the hypothesis that that sea-level changes during the Pliocene and Pleistocene have driven speciation in north-western Australia's (NWA's) largest freshwater fish family, Terapontidae, building upon a body of evolutionary literature focussed on the family.

Location: North-western Australian rivers including those draining the Kimberley Plateau

Taxon: Grunters (Family: Terapontidae, Genera: *Hannia*, *Hephaestus*, *Leiopotherapon*, *Syncomistes*)

Methods: A GIS was used to reconstruct palaeodrainages during lowered sea-levels and to delineate regions of high connectivity during low and high (current) sea-level conditions. For seven species, the degree of phylogenetic divergence among river basins in different regions was evaluated using a maximum likelihood phylogeny and analyses of the proportion of genetic divergence expressed with 601 base pairs of the mtDNA cytochrome *b* (cytb) gene.

Results: A low proportion of cytb haplotypes were shared among catchments not connected by the same receiving waters (e.g. estuaries) under current (high) sea-levels, indicating that contemporary dispersal is limited over fine spatial scales. Deeper phylogeographic patterns were largely congruent with reconstructed low sea-level drainage arrangements indicating that historic among-catchment connectivity was far more widespread under low sea-level conditions.

Main conclusions: The NWA landscape represents a geographic template that has shaped patterns of broad dispersal under low sea-levels, and fine-scale isolation under high sea-levels. The weight of evidence from recent literature on species boundaries and evolutionary patterns within the terapontids suggests that most NWA species were derived rapidly and

recently from a series of spatio-temporal vicariant events caused by such sea-level fluctuations during the late Pliocene and Pleistocene. Together, the findings provide a rare, comprehensively tested example of sea-level change driving speciation in the tropics.

Keywords: cryptic species, introgression, phylogeography, glacial cycles, Terapontidae, vicariance, tropics

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1 Introduction

Understanding the underlying mechanisms that generate biodiversity is a fundamental goal of evolutionary and conservation biology. Vicariance, caused by geological, climatic, or eustatic (sea-level change) processes, is considered a common mechanism for generating new species via allopatric speciation (e.g. Avise, 2000, 2004; Riginos, 2005). Comparative phylogeographic analysis provides a strong framework for investigating whether discontinuities in species assemblages and genetic structuring within species correspond to past or present geographic barriers (Unmack, Hammer, Adams, Johnson, & Dowling, 2013). Obligate freshwater taxa are particularly suited to such analyses because they are limited to well-defined habitat units (e.g. rivers and lakes) and any genetic signature of connectivity among habitat units implies a freshwater connection, either past or present (Unmack, 2013).

The glacial cycles during the Pliocene and Pleistocene have long been viewed as strong drivers of vicariant speciation around the globe, promoting the formation of new species through repeated geographic isolation and connection (Avise, Walker, & Johns, 1998; Haffer, 1969; Johnson & Cicero, 2004; Wallis, Waters, Upton, & Craw, 2016). However, this theory is the subject of debate since several studies have concluded that as many or more species arose prior to this period (Colinvaux & De Oliveira, 2001; Knapp & Mallet, 2003; Rull, 2008). During glacial cycles, regions in temperate latitudes of the world were most heavily influenced by the expansion and contraction of ice sheets that facilitated the isolation and connection of aquatic communities based on movement of the boundaries of unglaciated refuges (e.g. April, Hanner, Dion-Côté, & Bernatchez, 2013; Griffiths, 2006; Waters & Wallis, 2001). In contrast, in tropical latitudes, wet/dry climatic cycles and new land connections associated with the exposure of the continental shelf during lowered sea levels are considered the key factors influencing connectivity, distribution, and speciation (Hewitt,

2000; Voris, 2000).

Despite many papers referring to the relative importance of sea-level changes to biogeographic/phylogeographic patterns in freshwater ecosystems (e.g. Dias et al., 2014; Perdices, Bermingham, Montilla, & Doadrio, 2002; Shelley, Dempster, et al., 2019), studies that explicitly test hypotheses relating to the influence of sea-level change on speciation in freshwater are rare. In the tropics, a substantial body of evidence from the east coast of southern Brazil and Uruguay provides support for sea-level change driving broad-scale, repeated events of vicariant speciation (Tschá, Bachmann, Abilhoa, & Boeger, 2017), but we are not aware of further examples. Consequently, the importance of vicariant speciation, driven by Plio- Pleistocene glacial cycles, in the diversification of tropical freshwater biodiversity globally remains unclear. Here, we assess the influence of Plio- Pleistocene sea-level changes on connectivity and speciation in tropical north-western Australia (NWA) using freshwater fishes as a model group.

NWA is a largely flat and ancient landscape (White, 2000). The dominant geological feature in the region is the elevated Kimberley Plateau, possibly the oldest continually exposed landform in the world (~700 Ma; Ollier, Gaunt, & Jurkowski, 1988). The region contains a disproportionately large number of endemic species within many plant and animal groups (Pepper & Keogh, 2014). Freshwater fishes conform with this broader pattern of high endemism (~35 species), and the region contains more than a quarter of Australia's freshwater fish diversity (~68 species) (Shelley, Morgan, et al., 2018; Shelley, Swearer, et al., 2018; Unmack, 2013). Within the endemic fish community, 24 (67%) of species are found exclusively in streams running through the plateau (Shelley, Morgan, et al., 2018). Regardless, due to NWA's remoteness, the biodiversity and evolutionary history of the region is still poorly studied (Bowman et al., 2010).

The rivers that run off the plateau are short, steep and flow through deeply-dissected gorges, often from their headwaters to the ocean. Consequently, connectivity among catchments at current sea-levels is expected to be low (Phillips, Storey, & Johnson, 2009; Shelley, Dempster, et al., 2019). Furthermore, due to the long-term geological stability of the plateau, historical river rearrangements or capture events are unlikely (Unmack, 2013). Alternatively, lowered sea levels exposed one of the widest continental shelves in the world (Yokoyama, Purcell, Lambeck, & Johnston, 2001), providing greater chances for rivers to coalesce together before they reached the ocean and, consequently, for aquatic organisms to disperse. Therefore, predictable phylogenetic signatures of Plio- Pleistocene connectivity among rivers that drain the plateau, repeated across multiple taxa, would provide strong evidence that range expansion was facilitated by the exposure of the continental shelf under low sea levels rather

than dispersal among catchments during chance events (e.g. river capture, flooding of low divides, or coastal dispersal) (Burridge, Craw, & Waters, 2006; Waters et al., 2007).

Geographically-widespread molecular studies have been lacking in NWA. A recent region-wide molecular analysis of the region's most speciose freshwater fish family, Terapontidae, provides a unique opportunity to investigate the potential influence of sea-level change on phylogenetic affinities and ultimately speciation in this freshwater group (Shelley, Swearer, et al., 2018). The previous study focussed on cryptic species discovery, employing bi-parentally inherited, multi-locus nuclear markers (allozyme markers and Recombination Activation Gene one (RAG1) sequences) to explore species boundaries, supported by sequence data from the mtDNA cytochrome *b* gene (*cytb*). The results indicated that 22 described and candidate terapontid species are present in the AMT.

Species relationships, the relative timing of speciation events, and diversification rates have also previously been estimated using the combined sequence data, providing insight into the evolutionary history of the family (Shelley, Swearer, et al., 2018; Shelley, Unmack, Dempster, Le Feuvre, & Swearer, 2019). Several findings from these studies lend support to the hypothesis that sea-level change has driven speciation in NWA terapontids. These include (1) the congeneric species in sympatry (12 NWA terapontid species exhibit sympatry of congeners) are not sister species (with one exception) suggesting that they arose in allopatry and have subsequently come into secondary contact, (2) 75% of speciation events in NWA endemic species occurred during the late-Pliocene and Pleistocene when sea-level fluctuations were at peak intensity, and diversification rates increased significantly at this time, and (3) reproductive boundaries of sympatric congeneric species appear to be incomplete in most cases, perhaps reflecting the consequences of independently derived species reconnecting early in their evolutionary history. However, without an understanding of whether patterns of dispersal in freshwater taxa have been influenced by changes in sea-level heights, the link between sea-level fluctuations and speciation remains tenuous.

In this study we compare the genetic structure of seven terapontid fishes to investigate the role of sea-level changes in driving phylogenetic patterns, vicariant speciation and freshwater fish diversification in NWA. We hypothesise that phylogenetic relatedness among populations of widespread species will be high among rivers that coalesced or had closely situated river mouths during lowered sea-levels, while evidence of recent dispersal will be rare or absent among rivers that are disconnected under current sea-levels. Together, with evidence from existing evolutionary studies of terapontids in the region, we propose that the weight of evidence favours the hypothesis that sea-level fluctuations during the Plio-Pleistocene have driven speciation in NWA terapontids.

2 Methods

2.1 Regional setting

We define NWA using the boundaries of the Kimberley freshwater fish biogeographic province (Shelley, Dempster, et al., 2019). The area is bounded by the Great Sandy Desert to the south, the Indian Ocean to the west and north, and the eastern boundary of the Fitzmaurice River catchment in the east; the land area is roughly the size of Spain (see Fig. 1). Dramatic shifts in sea-level and precipitation occurred during Quaternary glacial cycles that alternated every 100,000–150,000 years (reviewed in Byrne et al., 2008). Compared with today, sea-levels ranged from -120 to -140 m during cool and hyper-arid periods of glacial maxima to between +5 and +8 m during the warm and wet interglacial periods (Hope et al., 2004). Bathymetric analysis by Yokoyama et al. (2001) suggested that all catchments surrounding the Joseph Bonaparte Gulf coalesced into one brackish coastal lake, which we herein refer to as Lake Bonaparte (Fig. 1a). The region therefore provides an ideal setting for testing hypotheses regarding the phylogeographic affinities of freshwater communities under low sea-level (LSL) conditions.

2.2 Study taxa and sympatry of species

The family Terapontidae (~67 species in total) includes ~40 small-to-medium-sized, stout, obligate freshwater species, 32 of which are distributed broadly across northern Australia (Shelley, Swearer, et al., 2018; Vari, 1978). Shelley, Swearer, et al. (2018) presented evidence for 13 new candidate species in NWA, seven of which were formally described by Shelley, Delaval, & Le Feuvre (2017). We adopt both candidate and described species into the taxonomic framework used here. Of the 22 terapontids found in NWA, half are narrow-range endemics restricted to one or two river systems (Shelley, Morgan, et al., 2018).

Sympatry among congeners is relatively rare in Australia and, when found usually involves a widespread and a narrow range species (Unmack, 2013). However, it is a common phenomenon across NWA (11 genera, 33 species) and, on the Kimberley Plateau, sympatry is observed among narrow-range endemics (Shelley, Morgan, et al., 2018). Within the NWA terapontids, *Syncomistes* (eight species), *Hephaestus* (two species) and *Leiopotherapon* (two species) exhibit sympatry of congeners. The remaining seven species have discrete ranges that immediately neighbour a congeneric. The sympatric species either arose in sympatry (e.g. Pigeon, Chouinard, & Bernatchez, 1997) or in isolation followed by range expansion that led to secondary contact.

The clustering of many secondary contact zones is typical of regions influenced by glacial

expansion and contraction in temperate latitudes (Campton & Utter, 1987). Such regions have been described as ‘suture zones’ (Remington, 1968). Suture zones represent a unique opportunity for the study of speciation, as each region of secondary contact represents a test of reproductive isolation in which the permeability of gene exchange among the previously-allopatric genetic lineages can be assessed (Barton & Hewitt, 1985). As such, each case of sympatry in the terapontids provides insight into (a) likely instances of secondary contact, and (b) the strength of species boundaries, which provides supportive information regarding the relative timing of secondary contact (i.e. recent or not recent). The second point is based on the assumption that weaker species boundaries, evidenced by greater instances of hybridisation, will exist between recently derived species and stronger reproductive barriers will exist between species that diverged deeper in evolutionary time (Coyne & Orr, 2004).

2.3 Sampling

Fishes were sampled across NWA by J.J.S., M.C.L., T.D. AND S.E.S. between 2012 and 2015, covering 41 sites and an area of some 512,000 km² (Fig. 1). Samples from outside the study area were sourced by M.P.H. and P.J.U. and from several separate tissue collections (see acknowledgements). Most described or candidate species from NWA were sampled from all known major river systems, with minor gaps in coverage including *L. unicolor*, that was not sourced from the Isdell, Charnley, Drysdale, and Durack rivers. Phylogenetic analysis included 54 of the 67 described and candidate terapontid species, including all Australian species. Although, as we were ultimately only interested in the relationships recovered within each of our target species lineages we only present those results here. The details of the complete cytb dataset are discussed in the following section and the complete cytb terapontid phylogeny is presented in Shelley, Swearer, et al. (2018).

2.4 Sequence analysis

We used the cytb sequence dataset (601 bp) presented in (Shelley, Swearer, et al., 2018). Full details of the development of the dataset and analyses are found there. Briefly, tree building was performed with a likelihood approach in GARLI 2.0 (Zwickl, 2006). We identified the best-fitting model of molecular evolution for the analysis using the Akaike Information Criterion (AIC) in jModelTest 2.1.4 (Darriba, Taboada, Doallo, & Posada, 2012). jModelTest identified TIM1+I+G as the best model. GARLI was run using ten search replicates with the following default settings changed: attachmentspertaxon=294, gentreshfortopoterm=10,000. For bootstrapping, we ran 1000 replicates with the same settings except treerejectionthreshold was reduced to 20 as recommended in the GARLI manual.

2.5 Interpreting introgression

The mtDNA (cytb) and nuclear (RAG1) gene trees derived from the datasets in Shelley, Swearer, et al. (2018) contained several cases of discordance in the recovered species relationships, particularly within sympatric species from the genus *Syncomistes*. In these cases, sympatric species shared a single cytb haplotype, while the RAG1 dataset clearly distinguished among them and recovered their relationships. The RAG1 data were further supported by the allozyme and morphological datasets employed. Discordance between mitochondrial and nuclear gene trees may arise for several reasons, including sporadic introgression, a lack of lineage sorting and/or ancestral polymorphism, and paralogy, that is the inclusion of nuclear copies (Wallis et al., 2017). However, given that nearly all cases of heterospecific mtDNA haplotypes involved sympatric, nonsister species, and furthermore, allozyme analysis detected hybrid crosses among a variety of sympatric species pairs, sporadic mtDNA introgression was identified as the most likely reason for this pattern (Shelley, Swearer, et al., 2018). Therefore, we interpret these results as likely cases of introgression.

2.6 GIS bathymetry manipulation and defining low and high sea-level regions

To test the hypothesis that phylogenetic relatedness among populations of widespread species will be high among rivers that coalesced or were closely situated during lowered sea-levels, but notably lower among rivers that are disconnected under current sea-levels, we first developed a geographic framework of low and high sea-level regions. These regions were based on historic and current patterns of catchment connectivity and clear geological divides interpreted from bathymetry data from the continental shelf surrounding the Kimberley region.

Drainage patterns during low sea levels were modelled using Spatial Analyst 1.1 and ArcView 3.1, based upon a bathymetric 30 arc-second grid produced by the Australian Geological Survey Organization. Paleo-drainages are displayed from the current coastline to the sea floor depth of -135 m (the maximum estimated fall in sea level during the last glacial maximum; Clark & Mix, 2002). Based on these data, we identified four broad regions that we hypothesised would have experienced high intra-regional connectivity and inter-regional isolation during lowered levels (Fig. 1a). Furthermore, we defined 12 high sea-level sub-regions that we hypothesized would experience freshwater connection under current sea levels based on whether they flowed into the same receiving water body (e.g. estuary or bay) or were seasonally connected across a floodplain (Fig. 1b). Given that historical sea-level heights were only +5 to +8 m higher than current levels (Hope et al., 2004), and heightened sea-levels would only exacerbate isolation in catchments, we considered the conditions to be comparable and so we refer to current sea levels as ‘high’ sea-level conditions. Within this

framework, we refer to ‘widespread’ species as those that: a) occur across low sea-level boundaries, or b) are distributed across ≥ 3 high sea-level sub-regions or beyond the study region. ‘Narrow-range’ species do not meet these criteria.

2.7 Phylogeographic analysis

To assess genetic similarity within and among our hypothesized low sea-level regions, we calculated the proportion of genetic differences (p -distance) from the cytb data using MEGA 6.06. Given the last glacial maximum (sea-level minimum) occurred only 18 ka, our hypothesised high sea-level (HSL) sub-regions would have been formed more recently (current sea levels were reached around 6 ka) and the lineages would not have diverged enough to be analysed in the same fashion as the LSL regions (Yokoyama et al., 2001). Instead, we calculated the proportion of haplotypes in a sub-region that are shared across sub-regions, as an indicator of connectivity.

3 Results

3.1 Phylogeographic analysis of low sea-level regions

Based on the geographic framework derived from our GIS analysis, we identified four ‘widespread’ species or sibling pairs with either region-wide (*L. unicolor*, *He. jenkinsi*), or inter-regional (*Ha. greenwayi* I / *Ha. greenwayi* II, Western and Northern regions; *S. trigonicus* / *S. wunambal*, Northern, Mitchell, and Eastern regions) distributions that were suitable for our phylogeographic analysis of low sea-level boundaries. We also included *S. bonapartensis* in the analysis as it is distributed across the large Eastern region and could provide further insight into changed levels of connectivity around the catchments that drain into Lake Bonaparte. The final phylogeographic dataset included cytb sequences from 1–5 individuals from each catchment (154 samples in total) across the range of each target widespread species (Fig. 1; Table S1). A summary cytb sample sizes for each species used in our phylogeographic analysis, listed by catchment and low and high sea-level region is presented in Table 1. The mean cytb p -distances in and among LSL region lineages for these species are presented in Table 2 and summarised in Table 3.

All of these species except *L. unicolor*, Australia’s most widespread species and an extreme disperser (Bostock, Adams, Laurenson, & Austin, 2006), displayed phylogeographic structure broadly consistent with biogeographic regionalization shaped by low sea-levels. The phylogenetic relationships of samples within all species, except *L. unicolor*, are presented in Fig. 2. *Hephaestus jenkinsi* displayed three well-supported lineages (88 and 99% of bootstrap replicates) that mirrored a Western, Northern, and Mitchell/Eastern distribution (Fig. 2a).

Although nested in the Eastern region, the Mitchell was the most divergent population within that lineage (mean p -distance from Eastern lineage 1.0%). Within-region genetic variance was negligible in the Western (mean p -distance 0.18%), Northern (mean p -distance 0.09%), and Mitchell lineages (mean p -distance 0.00%), although was considerably higher in the Eastern lineage, mainly driven by divergences in the King Edward and Drysdale river populations on the northern Kimberley Plateau (mean p -distance 0.47%). *Syncomistes trigonicus* formed two deeper lineages with moderate support at the base node (72% of bootstrap replicates) that cluster according to the Northern and Eastern regions (mean p -distance 1.1%), while the sister taxa *S. wunambal* from the Mitchell formed a well-supported lineage that was sister to the neighbouring Northern lineage (97% of bootstrap replicates, mean p -distance 1.1%) (Fig. 2b). In the Eastern lineage, the Drysdale and King Edward rivers formed two shallower lineages, the latter being poorly supported, although the populations share haplotypes that implies some degree of mixing. *Hannia greenwayi* I showed little within-region divergence among the Western populations (mean p -distance 0.36%), while *Ha. greenwayi* II formed a moderately-supported (84% of bootstrap replicates) sister lineage in the neighbouring Northern region (mean p -distance 1.0%) (Fig. 2c). *Syncomistes bonapartensis* displayed little within-region genetic divergence over most of the region, between the Daly River (immediately north-east of the study region; Fig. 1) and Drysdale populations (mean p -distance 0.13%) (Fig. 2d). However, the King Edward population at the western boarder of the region, formed a moderately well supported (78% of bootstrap replicates) and deeply divergent lineage (mean p -distance 2.4%) that increased the overall within-region variance to 1.04%.

Considering all species analysed, the average genetic variation among low sea-level regions was at least 2.9x (and up to 7x) the within-region variance (Table 3). In addition to the intra-specific phylogenetic patterns amongst the widespread groups, 19 of the 22 NWA terapontid species were endemic to just one of the defined regions (exceptions are *He. jenkinsi*, *S. trigonicus*, and *L. unicolor*) (Table 3).

3.2 Phylogeographic analysis of high sea-level regions

We defined 12 HSL sub-regions that we hypothesized would be largely isolated under current sea-levels (Fig. 1b) and assessed the proportion of endemic haplotypes found in each sub-region versus the proportion shared among sub-regions. As predicted, there was little sharing of haplotypes across sub-regions (Fig. 2a-d, Table 3). In the Western region, only 13% of haplotypes were shared among neighbouring HSL sub-regions, specifically *He. jenkinsi* from Walcott Inlet and Doubtful Bay, and *Ha. greenwayi* from King Sound and Walcott Inlet. In the Northern region only one haplotype (7%) was shared between the St George Basin and

Prince Fredric Harbour HSL sub-regions (*Ha. greenwayi* II). The Mitchell region included only one major catchment and all haplotypes were unique to the region. Finally, only three haplotypes (10%) in the Eastern region were shared among HSL sub-regions; *S. trigonicus* from Deep Bay and Napier Broome Bay, *S. bonapartensis* from Napier Broome Bay and Cambridge Gulf, and *He. jenkinsi* from Cambridge Gulf and Queens Channel. Of these six shared haplotypes, all but one (*S. bonapartensis*) were only found in adjacent catchments.

4 Discussion

Our study suggest that the NWA landscape represents a predictable geographic template for a range of species that has been and is likely still influencing patterns of speciation. These results support a growing body of evidence from genetic and morphological analysis of the broader Terapontidae family that suggests a large proportion of terapontids in NWA were derived recently from a series of spatio-temporal vicariant events caused by late Pliocene and Pleistocene sea-level fluctuations. The combined evidence provides a rare, well supported example of sea-level change driving speciation in the tropics. It lends important empirical support to the hypotheses that eustatic processes in the tropics worked concurrently with glacial expansion and contraction in temperate regions to generate large numbers of freshwater species over small temporal and geographic scales during the Plio- Pleistocene ice ages (Hewitt, 2000).

4.1 Phylogeography of northwestern Australian terapontids

Our multi-species, region-wide mitochondrial dataset provides robust evidence that sea-level change has had a strong influence on patterns of connectivity among NWA terapontids. The high degree of genetic similarity among our hypothesized low sea-level regions, relative to among-region variability, is most easily explained by predicted connections during lowered sea levels rather than by chance dispersal which would be expected to exhibit less frequent patterns of connectivity and only among closely situated rivers. Furthermore, the low proportion of shared haplotypes among catchments not connected by the same receiving waters (e.g. estuaries or bays) or floodplains indicates that dispersal is limited over far smaller spatial scales under high sea-level conditions. A high level of current genetic subdivision among NWA rivers, particularly those that drain the northern Kimberley Plateau, has been further corroborated for a number of freshwater fish taxa (Huey, Cook, Unmack, & Hughes, 2014; Phillips et al., 2009). Herein, we describe the generalized patterns observed within and between the four identified ‘widespread’ species/ cryptic species pairs with either region wide or inter-regional distributions.

The comparatively high degree of genetic similarity in the large Eastern region (10 major

catchments) observed in widespread *He. jenkinsi* and *S. bonapartensis* matches the predicted connections during lowered sea levels when all of the region's catchments drained into a shallow coastal lake (Lake Bonaparte) (Fig 1a; Yokoyama et al., 2001). Under high sea-level conditions, the King Edward, Drysdale, and King George rivers drain north off the Kimberley Plateau into the Timor Sea, rather than east into what is now the Cambridge Gulf, severely limiting the opportunity for connectivity with other Eastern region catchments. This would explain the higher degree of genetic divergence observed amongst populations in these rivers and the presence of narrow-range endemics that have sister species in catchments to the east (*S. rastellus*, Shelley, Swearer, et al., 2018; *Melanotaenia gracillis*, Unmack, Allen, & Johnson, 2013). In particular, the King Edward River would be the last river to connect to the Lake Bonaparte paleo-drainage basin as sea-levels lowered and would be the first to disconnect as they rose again. The population of *S. bonapartensis* there exhibited unusually high cytb divergence relative to the other Eastern region catchments but fell just short of the cryptic species criterion set out in Shelley, Swearer, et al. (2018) based on nDNA markers. Regardless, the King Edward population appears to be progressing towards speciation. Additional to the genetic evidence, the north coast catchments contain fragmented populations of *M. nigrans* and *M. exquisita* that are found in the Victoria and Daly rivers to the far east of the Lake Bonaparte paleo-catchment (Allen, Midgley, & Allen, 2002). Given these findings, it seems likely that the Lake Bonaparte drainage basin provided a periodic freshwater connection for aquatic communities across the entire Eastern region and potentially as far as the Daly River outside our study region. Overall, the Eastern region contains nine endemic terapontids (out of 12), and fifteen endemic fish species (out of 50).

The Mitchell and Northern regions are isolated in the north of the Kimberley Plateau. Although they are closely situated and lie between the Eastern and Western regions, their genetic distinctiveness is high, matching our predictions. The low sea-level inter-regional barriers that define these regions were each attributed to the rugged topology and heterogeneous nature of the continental shelf edge along the northern Kimberley coastline. These regions also have a rich endemic fauna relative to the small number of streams they encompass. The Northern region (two major catchments) has five endemic terapontids (out of 8), and nine endemic fish species (out of 22), while the Mitchell region (one major catchment) has at least one endemic terapontid (out of 3) and two endemic obligate freshwater fish species (out of 10). An isolated population of *A. percoides* occurs in the lower Mitchell River, but its taxonomic status has not been assessed using genetics.

As predicted, a distinct genetic break was observed between the neighbouring Western and Northern regions. Although the Western region is comparatively large (8 major catchments),

genetic similarity in the region is high. The region also contains a rich endemic community, including four endemic grunTERS (out of 6) and 9 endemic obligate freshwater species (out of 29).

4.2 Allopatric origins and secondary contact

Our overarching hypothesis was that geographic isolation of terapontid lineages during the Plio-Pleistocene sea-level heights triggered the onset of reproductive isolation and consequent speciation within the family. Previous molecular clock analysis indicated that 75% of speciation events within Kimberley endemic terapontid species occurred since the late-Pliocene (< 3 Ma; Shelley, Swearer, et al., 2018), with this period marking a shift to a significantly higher diversification rate in the terapontids (Shelley, Unmack, et al., 2019). Our phylogeographic analysis provides additional insight into the process behind this divergence pattern. The results suggest that this period would have been characterized by cycles of fine-scale isolation, which would have promoted allopatric speciation, followed by increased connectivity among catchments that would have promoted broader dispersal and led to secondary contact among allopatrically derived congeners. In each case of sympatry, the contact zones included representatives of independently-derived lineages rather than sister taxa, thus providing strong evidence for allopatric speciation followed by secondary contact, rather than sympatric speciation (Shelley, Swearer, et al., 2018).

The identification of fixed allozyme differences between species in each of the three sympatric species groups tested, and/or the presence of conserved and highly distinct morphological features among all sympatric groups (Shelley, Delaval, & Le Feuvre, 2017; Shelley, Swearer, et al., 2018; Vari, 1978), indicates that they have largely remained reproductively isolated in their zone of secondary contact (Adams, Raadik, Burridge, & Georges, 2014). Nevertheless, introgressive hybridization was detected among all sympatric species, except *S. bonapartensis* and *He. epirrhinos* (Shelley, Swearer, et al., 2018). Both *S. bonapartensis* and *He. epirrhinos* represent early-branching lineages of the NWA *Syncomistes* and *Hephaestus* groups and have had longer to develop stronger reproductive barriers, mediated by either prezygotic (genetic) or post-zygotic (behavioural) processes, than their more recently-derived congeners (Scribner, Page, & Bartron, 2000). Overall, naturally occurring introgression was found to be fairly common within NWA terapontids, and further observations on the wider *Hephaestus* genus suggests that it is prevalent in the family (Pusey et al., 2016). Regardless, it was observed to occur at far higher frequencies within sympatric members of the recently evolved *Syncomistes* complex (Shelley, Swearer, et al., 2018). This introgression appeared to be recent as each pairing mostly shared one individual haplotype. The high frequency of mtDNA introgression, contrasting with highly conserved morphologies

and fixed allozyme differences, indicates that significant, although incomplete, reproductive isolating barriers have developed in these species (Gay, Crochet, Bell, & Lenormand, 2008).

4.3 Vicariant speciation and biodiversity in NWA

The combined evidence suggests that NWA terapontids are at different stages of allopatric divergence and speciation, a mosaic likely caused by the same vicariant processes. As such, this study supports the hypothesis that changing sea levels during late Pliocene and Pleistocene glacial cycles are a key driver of speciation and distributional patterns in the region. Alternative explanations for the patterns of speciation observed here, such as chance dispersal events following river capture or flooding followed by founder event speciation, seem far less likely given the geological stability of the region (White, 2000). Also, the patterns of speciation were highly predictable based on our hypothesised low and high sea-level divisions, whereas random dispersal would not be expected to follow these patterns.

Comparative phylogeographic analysis matched to molecular clock estimates showed that a large proportion of NWA terapontids likely arose via vicariance during Plio- Pleistocene glacial cycles, when fluctuating sea levels caused periodic connection and isolation among catchments over relatively small spatial scales. These taxa display varying degrees of genetic and morphological differentiation, although in each instance of secondary contact, moderate to strong reproductive barriers are apparent, sufficient to maintain species integrity despite habitat sharing and sporadic (or historic) introgression (Shelley, Swearer, et al., 2018). Therefore, we can generalize that isolation during interglacial high sea-level periods occurred at time scales large enough to induce speciation in fishes, as has been suggested in other taxonomic groups (Avice et al., 1998; Haffer, 1969; Johnson & Cicero, 2004).

The impact of fluctuating sea levels on phylogenetic and community structure was predictable based on continental shelf geography and modelled low sea-level drainage patterns. This process is likely to influence speciation and biogeography across all obligate freshwater communities in NWA and deep mtDNA structuring across the region has been identified in a number of other fishes (Huey et al., 2014; Shelley, Swearer, et al., 2018) and crustaceans (De Bruyn, Wilson, & Mather, 2004). A productive line of future research would include a comprehensive biodiversity assessment of remaining groups to compare phylogeographic patterns and detect additional cryptic species. This would allow generalisations as to the influence of vicariance, driven by fluctuating sea levels, on speciation and distribution of aquatic fauna in the region and, as similar studies mount, more broadly across the tropics.

479 **5 Competing interests**

480 The authors declare that they have no competing interests.

481 **6 Consent for publication**

482 Not applicable

483 **7 Ethics approval and consent to participate**

484 All work had animal ethics approval from the Research Ethics and Integrity office at the
485 University of Melbourne, ID 1212470.1. All collections by the authors were made under
486 Government of Western Australia, Department of Fisheries permit, Ref. 220/12. Collections
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492 Charitable Trust, and the Nature Conservancy (Australian Conservation Taxonomy Award).

493 **9 Availability of data and materials**

494 All unique gene sequences used in this analysis are available from Genbank and the full
495 details of the accession numbers presented in Supplementary Material, Table S2.

496 **10 Authors' contributions**

497 J.J.S., S.E.S, T.D. and M.C.L. conceived the ideas; J.J.S., M.C.L, P.J.U., S.E.S., T.D. and
498 M.P.H collected the data; J.J.S. conducted the analysis under the supervision of P.J.U. and
499 M.A.; J.J.S. led the writing, and all authors contributed through editing.

500

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sequence datasets under the maximum likelihood criterion. PhD Thesis. Austin: University of Texas.

Biosketch

James Shelley conducted this research as a PhD student in the School of Biosciences at the University of Melbourne. His thesis investigated biodiversity, evolution and conservation of freshwater fishes in the Kimberley region of Western Australia. He is now based at the Arthur Rylah Institute for Environmental Research in Melbourne, Australia.

Table Captions

Table 1. Cytochrome *b* sample sizes each species used in our phylogeographic analysis, listed by catchment and low and high sea-level region. Letters in parentheses refer to river catchment codes in Fig.1. Dashes refer to catchments where the species occurred but was not sampled.

Table 2. Mean cytochrome *b* *p*-distances (as percentages) within and among low sea-level region lineages for widespread terapontid species. Mean within-lineage *p*-distance is shown in brackets on the diagonal. Comparisons among closely-related sister species, rather than within species, are marked with an (*). The results for *S. bonapartensis* marked with (**) refer to within-region variation calculations with the outlying King Edward River lineage removed. The capital letters in brackets following low sea-level region names refer to specific rivers depicted in Fig. 1.

Table 3. Summary of low sea-level and high sea-level region comparisons with measures of among and within-region genetic variability for widespread terapontid species, based on the cytochrome *b* dataset. Results for average within and among low sea-level (LSL) region variance are presented as the average variance across all widespread species analysed, followed by the number of widespread species analysed and the range of values in brackets. The outlying population of *S. bonapartensis* from King Edward River was removed from the genetic variance analysis of the Eastern region as it obscured the dominant pattern. No. haps. shared refers to number of haplotypes that are shared among the hypothesized high sea-level (HSL) sub-regions (Fig. 1b) that are nested within the corresponding LSL region.

Table 1

Low sea-level region	High sea-level region	Catchment	<i>Leiopotherapon unicolor</i>	<i>Hannia greenwayi</i> I	<i>Hannia greenwayi</i> II	<i>Hephaestus jenkinsi</i>	<i>Syncomistes bonapartensis</i>	<i>Syncomistes trigonicus</i>	<i>Syncomistes wunambal</i>
Western	King Sound	Fitzroy (A)	2	4		5			
	Walcott Inlet	Isdell (B)	–	2		2			
		Charnley (C)	–	2		3			
		Calder (D)	2	2		4			
	Doubtful Bay	Sale (E)	2			4			
		Glenelg (F)	1	5		4			
Northern	St George Basin	Prince Regent (G)			5	5			
	Prince Fredrick H.	Roe (H)	2		4	5		3	
Mitchell	Walmesly Bay	Mitchell (I)	2			3			5
Eastern	Deep Bay	King Edward (J)	2			7	5	12	
	Napier Broome Bay	Drysdale (K)	2			5	4	8	
	Koolama Bay	King George (L)	3						
	Cambridge Gulf	Durack (N)	–			1	5		
		Pentecost (O)	2			6	2		
		Ord (P)	2			5	4		
	Queens Channel	Victoria (Q)	–			3	–		

Table 2

Low sea-level region	Eastern (J–Q)	Mitchell (I)	Northern (G–H)	Western (A–F)
<i>Hephaestus jenkinsi</i>				
Eastern	(0.47)			
Mitchell	1.0	(0.00)		
Northern	2.0	2.4	(0.09)	
Western	1.7	2.0	2.7	(0.18)
<i>Leiopotherapon unicolor</i>				
Eastern	(0.40)			
Mitchell	0.3	(0.00)		
Northern	0.4	0.3	(0.00)	
Western	0.4	0.5	0.5	(0.30)
<i>Syncomistes trigonicus</i> + <i>Syncomistes wunambal</i> *				

Eastern		(0.45)			
Low sea-level region	No. rivers	Avg. % within LSL region variance	Avg. % variance among LSL regions	Endemic taxa in LSL regions	No. haps. shared among HSL sub-regions (%)
Eastern	6	0.36 (3; 0.13-0.47)	1.05 (2; 1-1.1)	9/12	3/30 (10)
Mitchell	1	0.02 (2; 0.00-0.07)	1.40 (2; 1-2.4)	1/3	N/A
Northern	2	0.05 (3; 0.00-0.11)	1.80 (3; 1-2.7)	5/8	1/14 (7)
Western	6	0.28 (2; 0.18-0.36)	1.85 (2; 1-2.7)	4/6	2/15 (13)
Mitchell		1.1*	(0.07)		
Northern		1.1	1.1*	(0.11)	
Western					
<i>Hannia greenwayi</i> I + <i>Hannia greenwayi</i> II *					
Eastern					
Mitchell					
Northern				(0.00)	
Western				1.0*	(0.36)
<i>Syncomistes bonapartensis</i>					
Eastern		(1.04)/(0.13**)			
Mitchell					
Northern					
Western					

Table 3

Figure Captions

Figure 1. North-western Australia showing the hypothesised low sea-level (a) and high sea-level (b) regions, and the distribution of sample sites (numbered dots). In both panels rivers are denoted by blue lines and major catchment boundaries are outlined in black. In Fig. 1a, low sea-level drainage patterns are shown in the blue region, which shows bathymetry down to 135 m below present-day sea-level. The dashed lines mark the low sea-level boundaries to dispersal and river catchments in our study area are coloured according to their region. In Fig. 1b, river catchments are coloured according to their high (current) sea-level region. Inset is a map of Australia and the state boundaries with a square highlighting the study region. Rivers are denoted by capital letters and are as follows: (A) Fitzroy, (B) Isdell, (C) Charnley, (D) Calder, (E) Sale, (F) Glenelg, (G) Prince Regent, (H) Roe, (I) Mitchell, (J) King Edward, (K) Drysdale, (L) King George, (M) Berkley, (N) Durack, (O) Pentecost, (P) Ord, (Q) Victoria, (R) Daly. Sample site numbers refer to the full sample locality details supplied

in Table S1.

Figure 2. Maximum likelihood (ML) tree branches, inferred from the full mitochondrial cytochrome b gene dataset, for: a) *Hephaestus jenkinsi*, b) *Syncomistes trigonicus* and *S. wunambal*, c) *Hannia greenwayi* I and *Hannia greenwayi* II, and d) *Syncomistes bonapartensis*. The hypothesised low sea-level region that each lineage was found in is indicated by coloured lines. Samples are labelled by the catchment they are from (see Fig. 1, Table S1). Nodal support is indicated by bootstrap values (estimated from 1000 replications). Only bootstrap values >50 are presented.

Figure 1

Figure 2

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