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FRESHWATER MOLLUSCS

Phylogeographic study of the West Australian freshwater mussel, *Westralunio carteri*, uncovers evolutionarily significant units that raise new conservation concerns

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Abstract South-western Australia is isolated from other forested regions of Australia by desert and bounded on southern and western sides by the Southern and Indian Oceans, respectively, with *Westralunio carteri* (Iredale, 1934) as the sole endemic freshwater mussel. Its conservation status is vulnerable. This species has a history of nomenclatural change and its systematic placement and population genetic history are largely unknown. We sampled 46 individuals from 13 sites across *W. carteri*'s distribution and sequenced two mitochondrial genes (16S rDNA and cytochrome c oxidase subunit I) and one nuclear gene (28S rDNA). The mitochondrial haplotype networks and COI phylogenies revealed three evolutionarily significant units (ESUs): "*W. carteri*" I including the west coast populations, "*W. carteri*" II from the south and south-eastern range, and "*W. carteri*" III only occurring in the south-western tip of Australia. Four species delimitation methods identified two molecular operational taxonomic units supporting two distinct species ("*W. carteri*" I and "*W. carteri*" II + III). Phylogeographic patterns revealed herein confirm the historical separation of Western and Southern paleo-basins, also highlighting the isolation of the south-western extremity of the region. This underlines the need for taxonomic revision and will require a re-evaluation of *W. carteri*'s conservation status.

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Introduction

Freshwater mussels (Bivalvia: Unionida) are among the most threatened groups of animals in the world with around 45% of assessed species being threatened or near threatened according to the IUCN Red List of Threatened Species (Lopes-Lima et al., 2018). Freshwater mussels provide a wealth of ecosystem services and given their importance to freshwater ecosystems and local economies, their conservation is crucial (Vaughn, 2018).

Species delimitation is essential because government biodiversity legislation is dependent on species' names as core conservation units (Prié et al., 2012; Ferreira-Rodriguez et al., 2019). Yet, conservation assessments of freshwater mussels are hindered by phylogenetic and taxonomic uncertainties (Walker et al., 2014a; Lopes-Lima et al., 2017; Ferreira-Rodriguez et al., 2019). Many freshwater mussel species are difficult to distinguish based on morphological characters alone (e.g. due to homoplasy, morphological convergence and lack of distinctive synapomorphies) and molecular species delimitation methods have been used with success to identify molecular operational taxonomic units (MOTUs) within cryptic species (Araujo et al., 2018; Johnson et al., 2018; Lopes-Lima et al., 2019).

As adults, freshwater mussels have a limited ability to disperse, owing to their slow movement and relatively confined habitat. However, the larval stage (glochidia in the Margaritiferidae, Unionidae and Hyriidae) is parasitic and attaches to a vertebrate host, generally a fish. Using this strategy, freshwater mussels can disperse their offspring using the host as a vector (Kat, 1984; Strayer, 2008). Not surprisingly, species' distribution among the Unionida closely follows the phylogeographic distribution of their host fishes (Haag, 2012).

Despite their larval dispersal strategy, rates of dispersal remain low among freshwater mussels, leading to substantial genetic structuring within species (Berg et al., 2007; Geist, 2010). To effectively manage conservation of genetic diversity within a freshwater mussel species, it is important to identify evolutionarily significant units (ESUs), because these represent groups of populations with distinct evolutionary trajectories (de Queiroz 2005, 2008; Froufe et al., 2016; Lopes-Lima et al., 2016). The analysis of current biogeographic distribution and phylogenetic structure, along with knowledge of paleo basin formation, tectonic events, and other past landscape modifications can help to explain the evolutionary history of freshwater mussels and thereby contribute to the identification of ESUs (Wilson, 1995; Araujo et al., 2018).

The freshwater mussels of Australia are all members of the Hyriidae, a family with representatives in South America, Australia, New Zealand and New Guinea comprising 90–94 species from 13 to 17 genera (Graf & Cummings, 2007; Marshall et al., 2014; Pereira et al., 2014; Walker et al., 2014a; Graf et al., 2015), with evidence of 'cryptic speciation' having been identified for several undescribed species complexes within the last two decades (Baker et al., 2003; Sheldon 2017). Furthermore, recent molecular phylogenetic analysis has revealed inconsistencies with systematic relationships constructed using traditional morphological characters (Graf et al., 2015). Molecular data are thus necessary as a complement to shell characters, larval forms and internal anatomy to build a solid taxonomic framework for the conservation of Australian freshwater mussels (Walker et al., 2014a). Two Australian hyriids have been assessed as threatened within the last 10 years. *Hyridella glenelgensis* (Dennant, 1898) is listed as critically endangered under State and Commonwealth legislation and is the IUCN Red List (Playford & Walker 2008; Walker et al., 2014a, b). Similarly, *Westralunio carteri* (Iredale, 1934) was assessed as vulnerable by the IUCN Red List of Threatened Species (Klunzinger & Walker, 2014) and subsequently listed as threatened (vulnerable) under State and Commonwealth legislation (Klunzinger et al., 2015).

Westralunio carteri is a regional endemic, restricted to south-western Western Australia. It is listed as vulnerable due to a contraction of its former range primarily from secondary salinization of its freshwater habitats (Klunzinger et al., 2015). The genus *Westralunio* was described by Iredale (1934) and included *Westralunio ambiguus* and the subspecies *Westralunio ambiguus carteri*. McMichael & Hisock (1958) later consolidated these names to a

single species, *W. carteri*. The taxonomy of the species was based almost exclusively on shell morphology. Given that shells can vary with habitat and locality (Balla & Walker, 1991; Baker et al., 2003) and the conflicting taxonomy between Iredale (1934) and McMichael & Hiscock (1958), we herein examine the patterns of genetic diversity and phylogeographic structure of *W. carteri* to identify ESUs for conservation and delineate MOTUs to help clarify the taxonomy.

In this study, we aim to (a) describe the genetic structure of *Westralunio carteri* across the endpoints of its distribution, (b) reveal phylogeographic patterns and evolutionary history, (c) evaluate hidden cryptic diversity using a combination of species delimitation methods and (d) discuss the conservation implications of these results.

Materials and methods

Tissue sampling, DNA extraction, sequencing and alignment

A total of 46 individual specimens of *W. carteri* were collected from 13 sites (hydrology follows AWRC, 1976) across the distribution of the species (Table 1). In the field, a small sample from the foot was collected (following Naimo et al., 1998) and placed in 99% ethanol. Genomic DNA was extracted from the tissue samples, using a standard high-salt protocol (Sambrook et al., 1989) and amplified for one nuclear and two mitochondrial markers: the F-type mtDNA cytochrome oxidase subunit 1 gene (COI; ca. 700 bp fragment), with LCO_22me and HCO_700dy primers (Walker et al., 2006, 2007); the mtDNA 16Sr DNA (16S rRNA; ca. 500 bp fragment), with 16SL and 16SH primers (Palumbi et al., 1991); and the nuclear 28SrDNA (ca. 800 bp fragment), with 28S-RD1.3f and 28S-rD4b primers (Whiting, 2002). PCR conditions followed Froufe et al. (2014), with annealing temperatures ranging from 48 °C (16S and COI) to 54 °C (28S). Sequences were obtained by Macrogen Inc., Korea. Individual gene alignments were built for each marker with ClustalW, in Bioedit 7.2.5 (Hall, 1999), including sequences from GenBank (Table 1, phylogeny specimens).

Genetic diversity of *Westralunio carteri*

COI and 16S individual alignments were joined in unrooted networks using TCS 1.21 (Clement et al., 2000). Uncorrected p-distances among and within haplogroups were calculated using MEGAX (Kumar et al., 2018). For COI, genetic diversity indices (i.e. haplotype and nucleotide diversity) and indices of neutrality (Tjima's and Fu's) were calculated using DnaSp6 v6.11 (Rozas et al., 2017) and pairwise Fst values were calculated in Arlequin 3.5.2.2 (Excoffier & Lischer, 2010).

Phylogeny and species delimitation in *Westralunio carteri*

A COI alignment was constructed with GUIDANCE2 (Sela et al., 2015) following Fonseca et al. (2016). It included all newly sequenced individuals in addition to outgroups. Outgroups included two other Australian Hyriidae species which Graf et al. (2015) confirmed as non-*Westralunio* (Velesunioninae: *Velesunio ambiguus* (Philippi, 1847) and Hyriinae: Hyridellini: *Cucumerunio novaehollandiae* (Gray, 1834)) and one representative each from each of the other two freshwater mussel families which possess glochidia: one Unionidae (*Unio pictorum* (Linnaeus, 1758)) and one Margaritiferidae (*Margaritifera margaritifera* Linnaeus, 1758) (Table 1). The COI alignment was then analysed with maximum likelihood (ML) and Bayesian Inference (BI) methods using IQ-TREE v 1.6.10 (Nguyen et al., 2015) and MrBayes 3.2.7a (Ronquist et al., 2012), respectively. For the BI analyses, the best-fit models of nucleotide substitution and partition scheme were selected using PartitionFinder 2 (Lanfear et al., 2016) under the Bayesian Information Criterion. Two independent runs of 10×10^6 generations were sampled at intervals of 1,000 generations producing a total of 10,000 trees. Burn-in was determined upon convergence of log likelihood and parameter values using Tracer 1.7.1 (Rambaut et al., 2018). For the ML analysis, the best-fit models of nucleotide substitution and partition scheme were selected using ModelFinder (Kalyaanamoorthy et al., 2017). Maximum-likelihood searches were then conducted with an initial tree search followed by 10 independent runs and 10,000 ultrafast bootstrap replicates.

Table 1 List of taxa used for phylogenetic analyses: GenBank accession codes and collection sites data

Taxon	COI	16S	28S	Basin/Locality	Waterbody	Voucher/source
Unionidae *Unio pictorum*	AF156499	DQ060163	AF305383	Europe	N/A	Graf & Ó Foighil (2000a, b), Källersjö et al. (2005)
Margaritiferidae *Margaritifera margaritifera*	JN243891	AF303281	JN243869	Europe	N/A	Machordom et al. (2003), Whelan et al. (2011)
Hyriidae: Hyriinae *Cucumerunio novaehollandiae*	KP184901	KP184853	KP184877	NSW, Australia-MANNING	Glochester R	UMMZ 304501, Graf et al. (2015)
Hyriidae: Velesunioninae *Velesunio ambiguus*	KP184915	KP184868	KP184892	NSW, Australia-HAWKSBERRY	Napean R	FMNH 337195, Graf et al. (2015)
Hyriidae "*Westralunio carteri*" I	MT040666	–	–	WA. Australia-MOORE-HILL	Gingin Bk	WAM S82791
"	MT040670	MT040067	MT040076	WA. Australia-SWAN COAST	Lk Leschenaultia	WAM S82739
"	KP184918	KP184871	KP184895	"	"	UMMZ 304517
"	MT040671	–	MT040074	"	Marbling Bk	WAM S82790
"	KP184917	KP184870	KP184894	"	Neerigen Bk	UMMZ 304516
"	MT040628	–	–	WA. Australia-COLLIE	Collie R	WAM S56210
"	MT040629	–	–	"	"	WAM S56211
"	MT040630	–	–	"	"	WAM S56212
"	MT040631	–	–	"	"	WAM S56213
"	MT040632	–	–	"	"	WAM S56214
"	MT040665	MT040066	MT040075	"	"	WAM S82777
"	MT040646	–	–	WA. Australia-PRESTON	Preston R	WAM S56215
"	MT040647	–	–	"	"	WAM S56216
"	MT040648	–	–	"	"	WAM S56217
"	MT040649	–	–	"	"	WAM S56218
"	MT040650	–	–	"	"	WAM S56219
"	MT040651	–	–	WA. Australia-MURRAY	Serpentine R	WAM S56220
"	MT040652	–	–	"	"	WAM S56221
"	MT040653	–	–	"	"	WAM S56222
"	MT040654	–	–	"	"	WAM S56223
"	MT040655	–	–	"	"	WAM S56224
"	MT040664	MT040065	–	"	"	WAM S82779
"	MT040656	–	–	WA. Australia-SWAN COAST	Wungong Bk	WAM S56225
"	MT040657	–	–	"	"	WAM S56226
"	MT040658	–	–	"	"	WAM S56229
"*Westralunio carteri*" II	MT040669	–	–	WA. Australia-ALBANY COAST	Goodga R	WAM S82756

Table 1 continued

Taxon	COI	16S	28S	Basin/Locality	Waterbody	Voucher/source
”	MT040633	–	–	”	”	WAM S56200
”	MT040634	–	–	”	”	WAM S56202
”	MT040635	–	–	”	”	WAM S56203
”	MT040636	MT040058	MT040068	WA. Australia-KENT COAST	Kent R	WAM S56205
”	MT040637	MT040059	MT040069	”	”	WAM S56206
”	MT040638	–	–	”	”	WAM S56207
”	MT040639	–	–	”	”	WAM S56208
”	MT040640	–	–	”	”	WAM S56209
”	MT040667	–	–	”	”	WAM S82758.1
”	MT040668	–	–	”	”	WAM S82758.2
”	MT040659	–	–	WA. Australia-BLACKWOOD	St. John Bk	WAM S82773
”	MT040660	MT040062	–	”	”	WAM S66164
”	MT040661	–	–	”	”	WAM S66165
”	MT040662	MT040063	MT040072	”	Waychinicup R	WAM S66127
”	MT040663	MT040064	MT040073	”	”	WAM S66128
“*Westralunio carteri*” III	MT040641	MT040060	MT040070	WA. Australia-BUSSELTON COAST	Margaret R	WAM S56235
”	MT040642	MT040060	MT040071	”	”	WAM S56236
”	MT040643	–	–	”	”	WAM S56237
”	MT040644	–	–	”	”	WAM S56238
”	MT040645	–	–	”	”	WAM S56239

GenBank accession codes for mitochondrial protein-coding cytochrome C subunit I (COI), mitochondrial ribosomal subunit (16S), nuclear ribosomal subunit (28S) and collection sites data. *Bk* Brook, *FMNH* Field Museum of Natural History, Chicago, *Lk* Lake, *NSW* New South Wales, *R* River, *UMMZ* University of Michigan Museum of Zoology, *WA* Western Australia, *WAM* Western Australian Museum. Coordinate system WGS 1984

Four species delimitation methods were applied to the COI dataset (excluding outgroups) to determine the number of molecular operational taxonomic units (MOTUs). Two distance-based methods were implemented; the BOLD BIN system (Ratnasingham & Hebert, 2013) and the Automatic Barcode Gap Discovery (ABGD) (Puillandre et al., 2012). For BOLD, the COI dataset was analysed with an online cluster sequences tool implemented in BOLD4 (Ratnasingham & Hebert, 2013). The ABGD method was also applied (COI and 16S) using its online version (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) with default settings and the Kimura-2-parameter distance matrix (Puillandre et al., 2012). An additional statistical parsimony method was implemented with TCS 1.21 (Clement et al., 2000), with a 95% connection limit following Lopes-Lima et al. (2019). Finally, we applied a coalescent-based molecular species delimitation method, using Bayesian implementation of the Poisson Tree Processes model (bPTP) (Zhang et al., 2013). A BI phylogenetic tree (data not shown) was obtained (COI Codons Models: HKY+I; F81; HKY) and used as an input tree in the bPTP Web server (available at: http://species.h-its.org/) with 1×10^6 iterations of MCMC and 20% burn-in.

Divergence time estimates

At present, no internally calibrated molecular clock is available for the family Hyriidae (using the fossil record). Therefore, divergence times among lineages were estimated from COI sequences using BEAST2 v2.6.1 (Bouckaert et al., 2014), and the substitution

rate of 0.265 ± 0.06% per million years recently estimated for *Unio* spp. (Froufe et al., 2016) was applied with normal distribution prior. The dataset was run under the HKY+I substitution model according to results from PartitionFinder2 v2.1.1 (Lanfear et al., 2016). An uncorrelated lognormal relaxed clock (Drummond et al., 2006) and the two-parameter birth–death model (Nee et al., 1994; Gernhard, 2008) were used. Other parameters used default settings. The random seed was 1,572,354,798,200. The analysis ran for 10^7 generations, sampling every 1000 generations. The quality of the runs was assessed through parameter convergence using Tracer 1.7 (Rambaut et al., 2018). The maximum credibility tree of mean heights was constructed using TreeAnnotator and discarding 2000 trees as burn-in.

Results

Dataset parameters

From the COI alignment with 559 nucleotides (nt) in length, 46 sequences, 24 haplotypes, and 47 polymorphic and 40 parsimony-informative sites were retrieved. No insertions, deletions or stop codons were observed after translating all sequences to amino acids. From the 16S rRNA alignment with 491 nt in length, 11 sequences, 6 haplotypes, and 18 polymorphic and 16 parsimony-informative sites were retrieved. A single haplotype was detected in 28S rDNA with no variation across the sampling range. Full genetic datasets were deposited in GenBank (see Table 1 for accession numbers and specimen provenance details).

Genetic diversity, phylogeny and species delimitation

A single haplotype network based on COI mtDNA was obtained and three haplogroups (red, green and blue: Fig. 1) were retrieved (corresponding to the three obtained MOTUs, see below) separated by a minimum of 12 mutations. The haplogroup "*W. carteri*" I (red) presents the higher number of haplotypes (12) followed by "*W. carteri*" II (blue) with 9 and "*W. carteri*" III (green) with 3 (Fig. 1). "*Westralunio carteri*" I is separated from "*W.* carteri" II by a minimum of 24 mutations and from "*W. carteri*" III by at least 26 mutations, with a minimum of 10 mutations separating "*W. carteri*" II and "*W. carteri*" III (Fig. 1). The haplotype network of the 16S rRNA fragment also shows the same number of haplogroups, with "*W. carteri*" I separated from "*W. carteri*" II by 13 mutations and from "*W. carteri*" III by 16 mutations, and two mutations separating "*W. carteri*" II and "*W. carteri*" III.

Genetic distances among haplogroups are shown in Table 2. For the COI gene, the mean pairwise genetic distances varied from 2.5% between "*W. carteri*" II and "*W. carteri*" III, to 5.4% between "*W. carteri*" I and "*W. carteri*" III. For the 16S rRNA gene, genetic distances varied from 0.6% between "*W. carteri*" II and "*W. carteri*" III, to 3.4% between "*W. carteri*" I and "*W. carteri*" III.

Nucleotide and haplotype diversity were greatest for "*W. carteri*" II and least for "*W. carteri*" III (Table 3). Neutrality indices were negative for all haplogroups (suggesting an excess number of alleles at low frequency), although this was significant only for Fu's F in "*W. carteri*" I. All pairwise *F*st values among haplogroups were similar and high (Table 4).

Both phylogenetic trees showed similar topologies in the main nodes, with the BI topology shown in Fig. 2. Within the ingroup, three clades were obtained, corresponding to the same three haplogroups, i.e. "*W. carteri*" I, II and III, with "*W. carteri*" I being sister to another clade formed by "*W. carteri*" II + *W. carteri* III (Fig. 2).

Molecular species delimitation methods (BOLD, ABGD, TCS (95%) and bPTP) applied in our study recovered, by consensus, two of these lineages as molecular operational taxonomic units (MOTUs), i.e. "*W. carteri*" I and "*W. carteri*" II + "*W. carteri*" III. Also, bPTP recovered "*W. carteri*" III as an additional MOTU, separate from "*W. carteri*" II. (Figure 2). The geographical distributions of these three lineages are shown in Fig. 3, colour-coded by the three lineages, as follows:

(A) "*W. carteri*" I found in north-westerly hydrographic basins to the north of and including the Preston River Basin, with a northern limit of Gin Gin Brook in the Moore-Hill Basin;

(B) "*W. carteri*" II in south-westerly and southerly flowing river basins extending from the Blackwood Basin, eastward to the Albany Coast Basin, with an eastern limit of Waychinicup River;

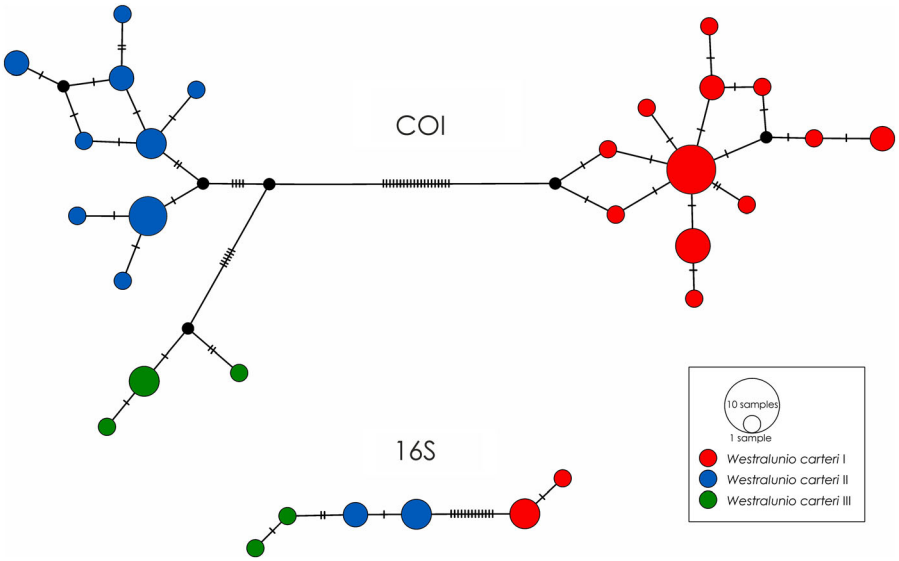

Fig. 1 Haplotype (TCS) networks showing the relationships of "*Westralunio carteri*" individuals sequenced for COI and 16S (Table 1). Circle size is proportional to the observed haplotype frequencies and black points represent unobserved haplotypes and potential intermediates. Colours represent the three lineages detected in the obtained phylogeny; *Westralunio carteri* I (red), "*Westralunio carteri*" II (blue) and "*Westralunio carteri*" III (green)

Table 2 Pairwise genetic distance matrices of Western Australia *Westralunio* lineages, as recognized in the present study

	Within lineages		Among lineages		
	COI	16S	"*Westralunio carteri*"I	"*Westralunio carteri*" II	"*Westralunio carteri*" III
"*Westralunio carteri*" I	0.006	0.001	–	0.028[b]	0.0342[b]
"*Westralunio carteri*" II	0.003	0.002	0.052[a]	–	0.0063[b]
"*Westralunio carteri*" III	0.003	0.002	0.054[a]	0.025[a]	–

Left column: mean uncorrected *p*-distance within lineages for cytochrome oxidase subunit I (COI) and for 16S rRNA gene fragment. Right column: [a]mean uncorrected *p*-distance among lineages of COI (below the diagonal) and [b]16S (above the diagonal) genes

(C) "*W. carteri*" III from Margaret River in the Busselton Coast Basin.

Divergence time estimates

All effective sample size (ESS) values accessed in Tracer v.1.7 were above 1000. The average estimated time divergence for the crown ages for the three "*W. carteri*" lineages was between 0.98 and 1.96 Mya (Fig. 4). The estimated age of the most recent common ancestor (MRCA) of all three "*W. carteri*" lineages was around the mid-Miocene, 11.0 Mya (Fig. 4), while that of "*W. carteri*" lineages II and III was during the late Miocene, 4.9 Mya (Fig. 4).

Discussion

Although *W. carteri* has been included in a broader phylogeny of the Hyriidae (Graf et al., 2015), this is the first study to investigate the genetic diversity within this nominal taxon. The "*W. carteri*" phylogenies reveal two major allopatric clades: one ("*W. carteri*" I) in the drainages of the west coast, draining to the Indian Ocean, and the other ("*W. carteri*" II + "*W. carteri*" III) in the south coast, draining to the Southern Ocean of south-western Australia. This Southern Ocean clade is further divided into two subclades: one ("*W. carteri*" III) occurring in the Margaret River in the south-west and the other ("*W. carteri*" II) in southern drainages to the south and east of Blackwood River to Waychinicup River in the

Table 3 Summary of the indices of genetic diversity estimated from the COI sequencing data for each "*Westralunio carteri*" lineage

	N	h	Hd	π	Fu's Fs	Tajima's D
"*Westralunio carteri*" I	25	12	0.873	0.00740	**− 6.924***	− 1.424
"*Westralunio carteri*" II	13	7	0.846	0.00606	− 2.185	−0.172
"*Westralunio carteri*" III	5	3	0.700	0.00286	− 1.113	− 1.094

N Number of individuals, *h* haplotypes, *Hd* haplotype diversity and π nucleotide diversity. Tests of population growth within each "*W. carteri*" lineage, i.e. the results of Tajima's D and Fu's Fs neutrality tests are also shown. Statistically significant ($P < 0.05$) values are shown in bold with an asterisk

Table 4 Pairwise F_{ST} values (below diagonal) and p-values (above diagonal) among "*Westralunio carteri*" lineages

	"*Westralunio carteri*" I	"*Westralunio carteri*" II	"W*estralunio carteri*" III
"*Westralunio carteri*" I	–	< 0.001	< 0.001
"*Westralunio carteri*" II	0.862	–	< 0.001
"*Westralunio carteri*" III	0.876	0.802	–

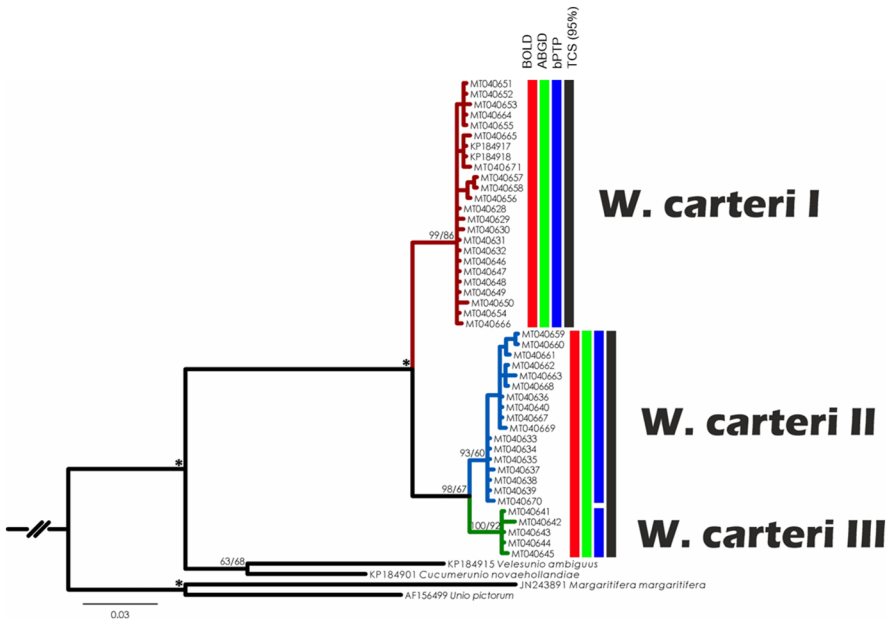

Fig. 2 Phylogenetic tree obtained by Bayesian Inference (BI) analysis of "*Westralunio carteri*" individuals (COI + 16S + 28S). For the major nodes support values (%) are given as Bayesian posterior probability/maximum likelihood bootstrap support. Species delimitation methods applied in this study are represented by colour-coded bars to the right of each "*W. carteri*" lineage: BOLD-red, ABGD-green, bPTP-blue and TCS-black. Species delimitation separation between "*W. carteri*" II and III was only observed by bPTP, which is represented here as a white line

Albany Coast Basin. Moreover, these two groups are also evident in both 16S and COI networks and supported by the large pairwise F_{ST} and *p*-distance values. A signal of demographic expansion was only observed in "*W. carteri*" I as shown by the star-shaped topology of the COI network and the low, significant values of Fu's F. No variation was detected for the nuclear marker 28S. This lack of 28S diversity has

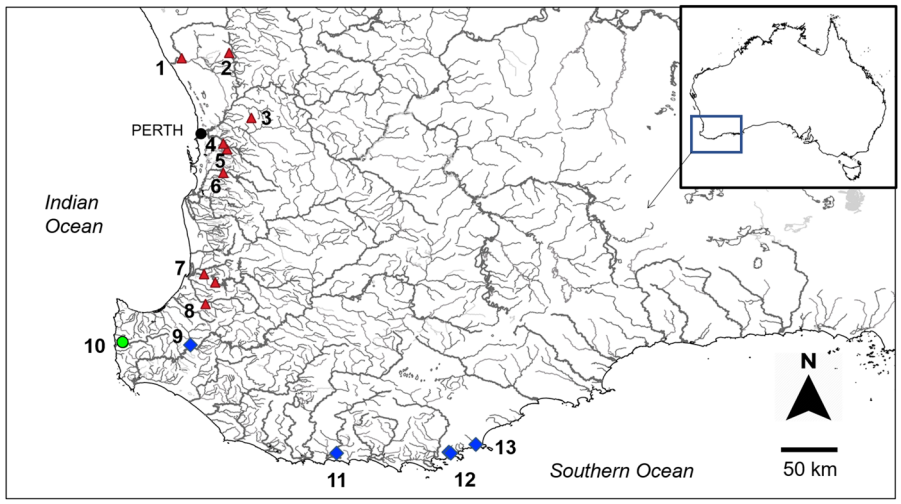

Fig. 3 Map of freshwater mussel populations sampled for phylogenetic analysis: **a** red triangles "*Westralunio carteri*" I—(1) Gin Gin Brook, (2) Marbling Brook, (3) Lake Leschenaultia, (4) Canning River, (5) Neerigen Brook, (6) Wungong Brook, (7) Collie River, (8) Preston River; **b** blue diamonds "*Westralunio carteri*" II—(9) Blackwood River, (11) Kent River, (12) Goodga River, (13) Waychinicup River; **c** green circle "*Westralunio carteri*" III—(10) Margaret River. Refer to Table 1 for river basin and sample site details

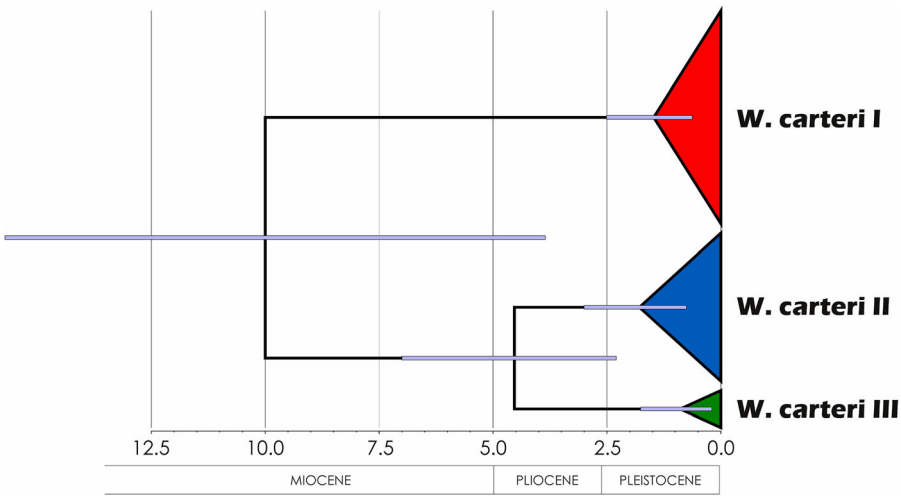

Fig. 4 BEAST maximum clade credibility tree for "*Westralunio carteri*" lineages. Time scale is in million years. The grey horizontal bars indicate the height 95% HPD interval for the crown-age estimates. The size of the triangles is proportional to the number of haplotypes

been reported for other congeneric species of freshwater mussels, due to its low substitution rate (Froufe et al., 2016; Araujo et al., 2018).

According to Bayesian analysis, "*W. carteri*" II + III populations diverged during the mid-late Miocene, which is consistent with divergence timing for a number of south-west Australian terrestrial taxa (Rix et al., 2014) and other freshwater taxa (Gouws et al., 2006, 2010; Unmack et al., 2011; Morgan et al., 2014). These studies suggest that vicariant events due to increased aridity periods acted as the main driving force reducing genetic connectivity and dispersion across river basins.

Furthermore, the separation of the "*W. carteri*" clades mirrors previous phylogenetic patterns of freshwater taxa in the region, including fishes (e.g.

Unmack et al., 2011; Galeotti et al., 2015) and crayfishes (e.g. Gouws et al., 2006, 2010), and is likely a consequence of geological division of the south-west coast drainage division during the Eocene. Beard (1999) shows that geological formation appeared to create two distinct watersheds, or drainage subdivisions in south-western Australia: one on the south coast to the south of the "Jarrahwood Axis" and one on the west coast, to the west of the Yilgarn Craton and the Darling Scarp.

Our study estimates the separation of the "*W. carteri*" II and "*W. carteri*" III lineages took place between during the late Miocene/Pliocene. During these periods, Western Australian palaeodrainages suffered from cycles of intense aridification (Unmack, 2001; Hopper & Gioia, 2004) that might have confined the common ancestral populations to refuge areas promoting their separation. We speculate that increasing aridity would have decreased host fish movement among drainages, particularly given that freshwater fishes of south-western Australia do not undertake ocean migration and rely on flooding events for basin connectivity and dispersal (Morgan et al., 2014).

The south and western Australian drainages were free from considerable readjustments since the late Pliocene (Unmack, 2001; Murphy & Austin, 2004), justifying the separation of the three lineages. However, the presumed wet and humid climate over the past two million years (Hopper, 1979; Hopper & Gioia, 2004) may have provided more favourable conditions for regional inter-basin dispersal within each lineage. In fact, although unique haplotypes were found in some populations, we could not find a clear geographic structure within each lineage, indicating recent gene-flow or connectivity events. However, a higher number of individuals per population are required to confirm this apparent lack of structure.

Nevertheless, in the "*W. carteri*" I lineage, we identified signs of a bottleneck followed by a fast demographic expansion by the mid Pleistocene, as mirrored by the star-shaped topology of the "*W. carteri*" I lineage COI network, corroborated by its negative Fu's F and Tajimas D values. Conversely, *W. carteri* II does not seem to show any evident demographic process.

Our results support the separation of "*W. carteri*" into two species: "*W. carteri*" I and "*W. carteri*" II + "*W. carteri*" III based on most of the employed species delimitation methods. The bPTP method further separates "*W. carteri*" II and "*W. carteri*" III, which is not surprising given that this method has been shown to overestimate the number of MOTUs (e.g. Dellicour & Flot, 2018). Future morphometric analyses combined with the present genetic results might support putative new species.

The third MOTU lineage ("*W. carteri*" III) revealed only by bPTP modelling suggests that a subspecies rank may be warranted, although further population sampling within the Busselton Coast Basin is required to determine the extent of this apparent MOTU. We hypothesize that populations within Busselton Coast to the north and west of Blackwood River are "*W. carteri*" I and that populations to the west of the Naturaliste Ridge are "*W. carteri*" III. Populations to the east of Blackwood River within the Donnelly, Warren and Shannon Basins, as well as other populations along the South Coast are likely "*W. carteri*" II.

The present results have major conservation implications for the *Westralunio* taxa in south-west Western Australia. The high divergence level revealed between "*W. carteri*" I and "*W. carteri*" II + "*W. carteri*" III supported by all species delimitation methods supports the separation into two species. Given that species are generally the taxonomic units used in conservation status assessment and legislation/policy, the taxonomic status of these two taxa needs urgent confirmation. Moreover, the three lineages "*W. carteri*" I, "*W. carteri*" II and "*W. carteri*" III correspond to distinct ESUs (as defined by Moritz, 1994); similarly, ESUs are recurrently observed in a number of other freshwater mussels (e.g. Froufe et al., 2016; Lopes-Lima et al., 2016; Sousa et al., 2018). These ESUs should be conserved and managed independently, given that they represent genetically unique populations and are geographically isolated.

Klunzinger et al. (2015) elucidated the conservation status of *W. carteri* by modelling historical and contemporary distributional records with environmental data. The authors revealed that salinity, flow permanency and total nitrogen were the variables most critical in limiting the species' occurrence. Reduction in the species' extent of occurrence was due primarily to secondary salinization of formerly freshwater habitats, which resulted in the species being assessed as vulnerable by the IUCN Red List (Klunzinger & Walker, 2014). Therefore, the taxonomic split, suggested here, implies that the individual conservation

status of each MOTU, i.e. "*W. carteri*" I and "*W. carteri*" II + "*W. carteri*" III, should be re-assessed by the IUCN to inform protection management under State and Commonwealth conservation legislation.

Using distribution data from Klunzinger et al. (2015), the extent of occurrence (EOO) for "*W. carteri*" I is estimated to be 8814 km^2, with an historic EOO of 31,559 km^2, a reduction in EOO of approximately 72% which might qualify the MOTU as endangered under criterion A2c of the IUCN Red List. The EOO for "*W. carteri*" II is estimated to be 8660 km^2 with an historic EOO of 10,070 km^2, a reduction of approximately 14%, not qualifying for any threatened category under Criterion A. Due to an EOO of $< 20{,}000$ km^2, "*W. carteri*" II is close to qualifying as vulnerable status under Criterion B. However, because there is no available evidence for severe habitat fragmentation, or number of locations ≤ 10, or extreme fluctuations, this rank cannot be attributed. Although some populations of "*W. carteri*" II are suggested to be increasing in size (Benson et al., 2017, 2019) and a large proportion of habitats and populations occur in national parks and specially protected areas for conservation (Klunzinger et al., 2015), it is unknown whether similar trends are true across this MOTU's range. In the absence of these data, we suggest that "*W. carteri*" II + "*W. carteri*" III be listed as near threatened on the IUCN Red List but is likely to qualify for the vulnerable category in the near future.

Besides the two MOTUs or putative species here described, the three lineages or ESUs warrant independent conservation actions and management. For example, the brood stock for potential propagation programs on each ESU should consider the original geographic distribution. The same is true when considering eventual translocations and reintroductions. Biological traits important for conservation planning, such as the reproductive physiology and habitat requirements, as well as biotic interactions, should also be investigated independently for each ESU, especially the range of host fish use (and see Klunzinger et al., 2012), that is critical for the maintenance and viability of freshwater mussel populations.

Given the threatening processes outlined in recent publications (Klunzinger et al., 2015; Benson et al., 2017, 2019; Ferreira-Rodriguez et al., 2019), maintaining riparian vegetation and habitats for host fishes, environmental flows in regulated rivers, the flow of freshwater (and mitigation of salinity) and reductions in nutrient pollution should be conservation priorities for both species/MOTUs revealed in this study.

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