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Phylogeography and species delimitation of *Cherax destructor* (Decapoda: Parastacidae) using genome-wide SNPs

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Abstract. *Cherax* is a genus of 58 species of decapod crustaceans that are widespread across Australia and New Guinea. We use single-nucleotide polymorphisms (SNPs) to examine phylogeographic patterns in the most widespread species of *Cherax*, namely, *C. destructor,* and test the distinctiveness of one undescribed species, two *C. destructor* subspecies, previously proposed evolutionarily significant units, and management units. Both the phylogenetic analyses and the analysis of fixed allelic differences between populations support the current species-level taxonomy of *C. setosus*, *C. depressus*, *C. dispar* and *C. destructor*, the distinctiveness of *C. destructor albidus* and *C. d. destructor* and the existence of one undescribed species. The two populations of *C. d. albidus* from the Glenelg and Wimmera rivers were significantly distinct, with eight diagnostic differences (<1% fixed differences, null expectation is four fixed differences), but this low level of divergence is interpreted as within the range that might be expected of management units, that is, among allopatric populations of a single species or subspecies. A southern clade of *C. d. destructor* comprising the Murray River and its tributaries upstream from its confluence with the Darling River is genetically distinct from a northern clade comprising populations from the Lake Eyre Basin, the northern half of the Murray–Darling Basin (Darling River catchment) and the Lower Murray River below the Darling confluence.

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Introduction

Freshwater aquatic animals have fundamentally different biogeographic constraints than do marine and terrestrial animals owing to the limited nature of freshwater and associated connectivity. Rivers, in particular, are one-dimensional dendritic networks where most movement is constrained to upstream or downstream directions. In contrast, marine systems are massively larger in area and volume, have a three-dimensional structure and fewer strong barriers (e.g. land). Organisms residing in marine systems often have population sizes orders of magnitude greater than do animals found in freshwater or terrestrial environments. Terrestrial ecosystems have a more convoluted array of biogeographic constraints driven by differences in local climate, vegetation, geology and elevation, with patterns of animal movement largely being restricted to two dimensions. These differences between environments have important consequences for both within and among population genetic diversity and how it is partitioned across the landscape.

Obligate freshwater animals are constrained by the distribution of freshwater across the landscape. The two fundamental boundaries to their biogeography are drainage divides and the ocean. Ocean boundaries can potentially be crossed during low sea-level stands during glacial periods, which allow rivers to coalesce on the continental shelf or episodic, or large, freshwater flood pulses (which remain largely unstudied as a biogeographic pathway). Drainage divides can be breached by geomorphic processes leading to drainage rearrangement (which includes stream capture, Bishop 1995) or by temporary connections across low drainage divides by overland flow from high local rainfall or seasonal flooding (Rupununi River via Lake Amuku, Guyana, McConnell 1964). In a few unusual cases, otherwise discrete river systems have localised permanent freshwater connections between them (e.g. Two Ocean Pass, Wyoming, USA, Casiquiare River, Brazil, Evermann 1892; Rice 1921).

The landscape of central and south-eastern Australia provides a good setting for examining freshwater biogeography by virtue of two massive river basins, the Lake Eyre Basin (LEB) and Murray–Darling Basin (MDB), each $>1 \times 10^6$ km^2 and the smaller Bulloo–Bancannia Basin (BBB), which sits between them (and typically shares the LEB biota). These two large basins (Fig. 1) provide a potential conduit between the biota of southern and northern Australia as LEB shares a large boundary with the MDB, along with smaller individual boundaries to several river basins draining to northern Australia. The southern and western boundaries of LEB abut regions with high aridity that lack any significant freshwater habitats. LEB has a mix of

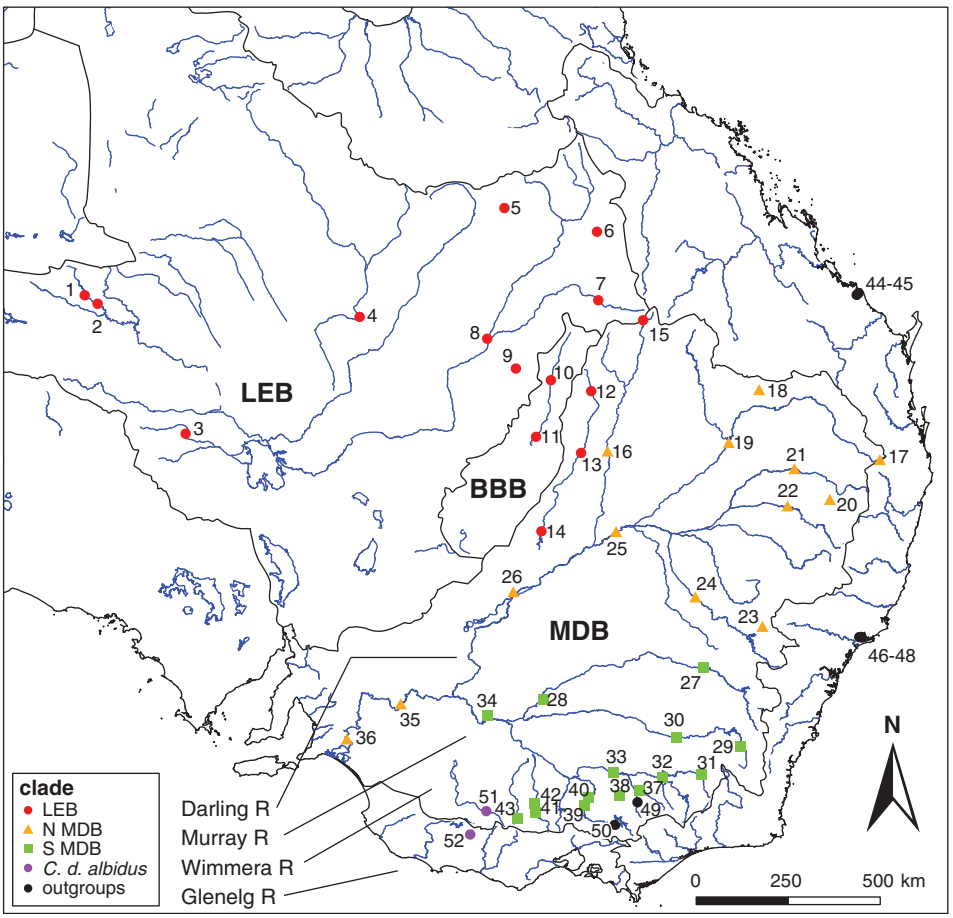

Fig. 1. Locality data for all *Cherax* samples examined. Each colour represents a different lineage, with black representing all the outgroup species. Refer to Table 1 for the corresponding site details.

biota with links to the MDB, northern river basins as well as some endemic species (Unmack 2013). In contrast, the MDB shares drainage divides with 20 smaller coastal river basins to the south and east and, like the LEB, has a mix of species with clear southern links, north-eastern links as well as endemic species (Unmack 2013). The broad geographic spread of these two basins, plus the complex potential for mixing across many separate drainage divides is an unusual setting that provides many biogeographic opportunities for biotic exchange.

Cherax is a genus of decapod crustaceans that are widespread across Australia and New Guinea, with most species diversity being found along the eastern edge and in south-western regions of Australia and across the southern and western portions of New Guinea (Munasinghe *et al.* 2004*b*). Currently, 58 species are recognised in the genus (28 in Australia), plus several known but undescribed species. The most widespread *Cherax* species is *C. destructor*, known as the common yabby and found throughout the LEB, the MDB and the Glenelg and Wimmera rivers of south-western Victoria (Fig. 1). Two subspecies are recognised, namely, the widespread *C. d. destructor* and the more restricted *C. d. albidus* from the Wimmera and Glenelg river basins in western Victoria (Campbell *et al.* 1994; Austin 1996; Austin *et al.* 2003). *Cherax destructor* has broad environmental tolerances, lives in most aquatic habitats from desert waterholes to cooler higher-elevation streams (over 1000 m) and can persist during drought by living in deep, moist burrows. They are fast growing, commonly reach 20 cm in length and have high reproductive output. These attributes have resulted in their widespread use in aquaculture, their cultural importance as a food source to humans and their use as bait by anglers. They can be extremely abundant in small artificial farm dams and bore tanks that are common across otherwise dry landscapes. Unfortunately, the popularity of the species as food, fishing bait and the ubiquitous presence of farm dams has resulted in their human-mediated dispersal to most river basins in eastern, southern and south-western Australia where they are a key threatening process to many other crayfish species (Nguyen 2005; Coughran *et al.* 2009; McCormack 2014).

Early work on *C. destructor* focused on determining species boundaries in the ‘destructor’ species complex. Several species have been described using morphology but consensus on their taxonomy has been slow to establish. Campbell *et al.* (1994) examined *C. destructor* and *C. albidus* and found clear morphological differences, but this contrasted with their estimates of low genetic divergence. They regarded *C. albidus* to be a subspecies of *C. destructor*. Austin (1996) brought several

species recognised (often tentatively) by Riek (1969) into synonymy with *C. destructor*, including *C. albidus*, *C. esculus*, *C. davisi* and *C. rotundus*. Austin's specimens of *C. rotundus* are now considered to be *C. setosus* – their collection location is within the range of *C. setosus,* formerly referred to by Riek (1969) as a subspecies of *C. rotundus* (O'Brien *et al.* 2009). There has been considerable confusion around what exactly *C. rotundus* is and its distribution (reviewed by O'Brien *et al.* 2009). Austin (1996) considered *C. albidus* and *C. rotundus* [= *C. setosus*] to be a subspecies of *C. destructor*.

Several mitochondrial studies have examined the phylogeny of *Cherax*, including *C. destructor* and related species (Austin *et al.* 2003; Munasinghe *et al.* 2004*a*, 2004*b*). Munasinghe *et al.* (2004*a*, 2004*b*) found the distinction between *C. d. destructor* and *C. d. albidus* to be well supported by the mitochondrial data, and considered *C. setosus* and *C. rotundus* to be sister species, together being sister to *C. destructor*. To complicate matters further, the *C. rotundus* examined by Munasinghe *et al.* (2004*a*, 2004*b*) is now considered a new but undescribed species (O'Brien *et al.*, 2009; R. B. McCormack, pers. comm.). Deeper relationships among *Cherax* lineages in eastern Australia have lacked bootstrap support and have varied among studies.

Nguyen *et al.* (2004) examined geographic patterns of mtDNA variation for *C. destructor* (16S rRNA) and identified three lineages. *Cherax d. albidus* was the first branching lineage, with southern *C. d. destructor* (upstream of the Murray and Darling river junction) and northern *C. d. destructor* (lower Murray, Darling catchment, BBB and LEB) forming two distinct lineages. A follow up study based on 11 variable allozyme loci and RAPDs generated similar results (Nguyen and Austin 2005). Hughes and Hillyer (2003) examined Queensland *C. d. destructor* populations (Darling catchment, BBB and LEB) using the mtDNA gene *COI*. They found a separation between LEB and most MDB populations, with the exception that MDB populations in the Paroo and upper Warrego rivers were more closely related to LEB animals than to remaining MDB animals. These results were broadly supported by Nguyen's subsequent results (Nguyen *et al.* 2004; Nguyen 2005). Nguyen *et al.* (2005) used 16S rRNA to investigate the distribution of different *C. destructor* clades primarily in western Victoria, especially relative to the distribution of *C. d. albidus* and to identify any potential translocations in the region. Consistent with Campbell *et al.* (1994) and Nguyen *et al.* (2005), Nguyen (2005) found evidence for mixed populations between *C. d. albidus* and northern *C. d. destructor* at two locations to the west of the Wimmera River. Different populations to the east of Glenelg River in southern Victoria either contained *C. d. albidus* or southern or northern *C. d. destructor*, which suggests there is significant translocation occurring in this region, representing a significant threat to *C. d. albidus* because they appear to readily interbreed and mix with other lineages of *C. d. destructor* (Nguyen 2005).

Here, we provide the first next-generation sequencing dataset based on single-nucleotide polymorphisms (SNPs) for phylogeographic patterns in *C. destructor*, with the most extensive sampling across the range of the species to date (Fig. 1). These data are used to examine biogeographic patterns within *C. destructor* and to identify historical connections across drainage divides. In addition, we test the distinctiveness of the two *C. destructor* subspecies and previously identified within species lineages (Campbell *et al.* 1994; Austin *et al.* 2003; Nguyen *et al.* 2004), as well as examining genetic evidence for translocations of *C. destructor* within its natural range.

Materials and methods

Specimen collection

In total, 367 individuals of *Cherax destructor* were sampled from 45 waterbodies in the Glenelg River, MDB, BBB and LEB of eastern and central Australia (Fig. 1, Table 1). This covers the full native range of the species. Our sampling target for the ingroup taxa was for 10 individuals per population (not always achieved). Outgroup species consisted of *C. setosus* ($n = 7$) from three waterbodies in the Hunter and Karuah river basins (Populations 46–48), *C. depressus* ($n = 5$) from one waterbody in the Baffle Creek Basin (Population 45), *C. dispar* ($n = 4$) from one waterbody in the Baffle Creek Basin (Population 44). Two individuals from an undescribed species (*Cherax* sp. Swamp) collected from two sites in the mid-Murray River (MDB, Populations 49, 50) were also included.

Animals were usually collected with dip nets and snap-frozen in liquid nitrogen in the field and placed in a −80°C freezer on return to Canberra. Muscle samples were taken from the tail with care to avoid including chitin. Samples obtained from third parties were preserved in 95% ethanol and stored at −20°C.

DNA extraction and sequencing

DNA was extracted, sequenced and informative SNP markers were identified by Diversity Arrays Technologies (DArT Pty Ltd, Canberra, ACT, Australia, www.diversityarrays.com, accessed 14 January 2019; Kilian *et al.* 2012). Briefly, sequencing for SNP genotyping was performed using a combination of complexity reduction by using restriction enzymes, implicit fragment-size selection and next-generation sequencing (Kilian *et al.* 2012). Four combinations of restriction enzymes were evaluated for *C. destructor* (*Pst*I enzyme combined with either *Hpa*II, *Sph*I, *Nsp*I and *Mse*I) and the restriction enzyme combination of *Pst*I (recognition sequence 5′-CTGCA|G-3′) and *Sph*I (5′-GCATG|C-3′) was selected for the complexity reduction by double digestion. Sequences were processed using proprietary DArT analytical pipelines (Kilian *et al.* 2012) to yield SNP markers polymorphic within the set of samples. Calling quality was assured by high average read depth per locus (~21×). In addition, approximately one-third of the samples were processed twice from DNA to allelic calls as technical replicates. Scoring consistency (repeatability) was used as the main selection criteria for high-quality and low error-rate markers.

The dataset obtained from DArT Pty Ltd contained the SNP genotypes and various associated metadata, of which CloneID (unique identity of the sequence tag for a locus), repAvg (proportion of technical replicate assay pairs for which the marker score is identical), avgPIC (polymorphism information content averaged over the reference and alternate SNPs), SnpPosition (position in the sequence tag at which the defined SNP variant base occurs), and TrimmedSequence (sequence tag with adaptor sequence removed) were used in additional filtering and analyses. The SNP data and r scripts that were used in the

Table 1. Locality data for all *Cherax* populations examined

Site refers to the localities shown in Fig. 1. Tree label refers to operational taxonomic unit (OTU) codes in Fig. 5 and S1. State abbreviations include: NSW, New South Wales; NT, Northern Territory; Qld, Queensland; SA, South Australia; Vic., Victoria. Sample sizes (*n*) and latitude and longitude are provided in decimal degrees and station codes can be used to track references to genetic material deposited in the South Australian Museum and Robert McCormack's sample collection (Populations 47–50)

Site number	Tree label	Location	State	*n*	Latitude	Longitude	Station code
1	LEB Fink Henb	Finke River, Running Waters, Henbury Station	NT	2	−24.30810000	132.90300000	MH13-21
2	LEB Fink Henb	Finke River, Three Mile Waterhole, Henbury Station	NT	4	−24.51412000	133.22173000	MH13-16
3	LEB Neal Stew	Neales River, Stewart Waterhole	SA	5	−27.68916667	135.37277778	ABTC77820-4
4	LEB Geor Eyre	Eyre Creek, Glengyle	Qld	2	−24.83446700	139.62570000	n.a.
5	LEB Diam Oond	Mills Creek, Oondooroo	Qld	3	−22.17477900	143.16570200	n.a.
6	LEB Coop Edgb	Edgbaston	Qld	1	−22.75198000	145.42735000	n.a.
7	LEB Coop Barc	Barcoo River, Blackall	Qld	10	−24.42651290	145.45946528	PU14-33
8	LEB Coop Wind	Cooper Creek, Currareva Waterhole, Windorah	Qld	5	−25.37004409	142.74455861	PU14-27
9	LEB Coop Kyab	Kyabra Creek	Qld	4	−26.09739163	143.44454292	PU14-28
10	BUL Bull Como	Bulloo River, Como Waterhole, Como Station	Qld	10	−26.38415771	144.29851095	PU14-26
11	BUL Bull Cowa	Bulloo River, Cowarra Waterhole, Autumnvale Station	Qld	10	−27.76764597	143.93715703	PU14-25
12	MDB Paro Yala	Paroo River, Yalamurra	Qld	10	−26.64909000	145.28788000	PU14-29
13	MDB Paro Eulo	Paroo River, Eulo	Qld	9	−28.16167000	145.03684000	PU14-24
14	MDB Paro Toon	Paroo River, semi-permanent waterhole on Toonborough Station	NSW	10	−30.07705400	144.06703700	PU13-20
15	MDB Warr Nive	Nive River, upper, Tambo	Qld	10	−24.91816000	146.55490000	PU14-31
16	MDB Warr Cunn	Warrego River, Cunnamulla Weir (above)	Qld	10	−28.11697420	145.68612491	PU14-23
17	MDB Cond Kill	Condamine River upstream of Killarney	Qld	8	−28.32314902	152.34175090	PU14-12
18	MDB Cond Yule	Yulebah Creek, Yulebah	Qld	1	−26.61473600	149.39015300	n.a.
19	MDB Cond Bear	Balonne River below Beardmore Dam	Qld	10	−27.91045335	148.64883180	PU14-138
20	MDB Maci Seve	Severn River, north of Ashford	NSW	10	−29.29831200	151.11975400	PU13-33
21	MDB Maci Goon	McIntyre River, below weir, Goondiwindi	NSW	10	−28.54720700	150.25272300	PU13-34
22	MDB Gwyd Pall	Gwydir River, Gum Flat Public Reserve downstream of Pallamallawa	NSW	10	−29.45701800	150.08212300	PU13-28
23	MDB Macq Cudg	Cudgegong River near Gulgong	NSW	10	−32.40345116	149.47178703	PU14-09
24	MDB Macq Warr	Macquarie River below Warren Weir	NSW	10	−31.68469800	147.83505145	PU14-20
25	MDB Darl Bour	Darling River below Bourke Weir	NSW	10	−30.08688000	145.89360400	PU13-25
26	MDB Darl Will	Wilcannia, Darling River, below weir	NSW	10	−31.55778100	143.38320100	PU13-19
27	MDB Lach Forb	Lachlan River, Forbes	NSW	7	−33.40110000	148.01820000	PU14-19
28	MDB Lach Oxle	Lachlan River, Oxley	NSW	10	−34.19775800	144.10842900	PU13-37
29	MDB Mbid Cott	Cotter River immediately upstream of Paddys River	ACT	10	−35.32853200	148.93789100	PU14-83
30	MDB Mbid Wagg	Wollundry Lagoon in Wagga Wagga	NSW	10	−35.10978400	147.36330700	PU13-42A
31	MDB Murr Tink	Upper Murray, Billabong ~5.5 km east of Tintaldra	NSW	10	−36.03753100	147.97289700	PU13-38B
32	MDB Murr Albu	Murray River below Hume Dam	NSW	10	−36.09941111	147.02256017	PU14-81
33	MDB Murr Yarr	Murray River, Bourkes Beach #1, anabranch by Murray Track	Vic.	10	−35.98284865	145.83399548	PU14-08
34	MDB Murr Eust	Small creek on floodplain by Euston cemetary	NSW	9	−34.58563600	142.73807919	PU14-72
35	MDB Murr Berr	Murray River, Berri, Martins Bend	SA	10	−34.29151344	140.63175333	PU14-74
36	MDB Murr Swan	Murray River, Swanport	SA	8	−35.15222050	139.31324580	PU14-76
37	MDB Oven Tarr	Ovens River, Tarrawingee	Vic.	10	−36.41323500	146.45560600	PU13-60
38	MDB Brok Bena	Broken River, Benalla Weir	Vic.	1	−36.54816200	145.97600600	n.a.
39	MDB Goul Naga	Lake Nagambie	Vic.	10	−36.78553100	145.13377000	PU13-70
40	MDB Goul Murc	Goulburn River, downstream of Murchison	Vic.	10	−36.59682677	145.22860748	PU14-80

(*Continued*)

Table 1. (Continued)

Site number	Tree label	Location	State	*n*	Latitude	Longitude	Station code
41	MDB Lodd Laan	Loddon River, Barringhup	Vic.	10	−36.94243800	143.93432100	PU13-84
42	MDB Lodd Newb	Loddon River, Newbridge	Vic.	10	−36.74101471	143.90109357	PU14-79
43	MDB Avoc Avoc	Avoca River, Avoca	Vic.	8	−37.08998200	143.47130200	PU13-82
44	BAF *C. dispar*	Round Hill Creek	Qld	4	−24.25364216	151.81911231	PU15-16
45	BAF *C. depressus*	Oyster Creek	Qld	5	−24.29610751	151.77782655	PU15-21
46	HUN *C. setosus*	Wallaroo State Forest, beside Pacific Highway	NSW	5	−32.66525000	151.84043330	n.a.
47	KAR *C. setosus*	12 Mile Creek, Wallaroo State Forest, Swan Bay (Port Stephens)	NSW	1	−32.66750000	151.89300000	2605
48	KAR *C. setosus*	Reedy Swamp, off Haynes Road, Wallaroo State Forest (Port Stephens)	NSW	1	−32.66148000	151.88430000	2649
49	MDB *C.* sp. Swamp	Drain along Wangaratta–Whitfield road	Vic.	1	−36.70129000	146.41674000	2759
50	MDB *C.* sp. Swamp	538 Goulburn Valley Highway, Snobs Creek	Vic.	1	−37.25876000	145.86470000	2867
51	MDB *C. d. albidus*	Wimmera River east of Glenorchy	Vic.	10	−36.91827241	142.72009974	PU14-77
52	GLE *C. d. albidus*	Dwyers Creek	Vic.	10	−37.49070061	142.32801999	PU14-78

analysis have been deposited in Dryad (see https://doi.org/10.5061/dryad.21mb45j).

Additional SNP Filtering

The SNP data and associated locus metadata were read into a genlight object ({adegenet}, see http://adegenet.r-forge.r-project.org/, accessed 6 February 2019; Jombart 2008) to facilitate processing with package dartR (ver. 1.0.5, see http://github.com/green-striped-gecko/dartR, accessed 14 January 2019; Gruber *et al.* 2018). Only loci with 100% repeatability (repAvg) were chosen for subsequent analysis. Further filtering was undertaken on the basis of call rate (≤90% unless otherwise specified). Because of the high read depth (~21×), most 'missing data' are not being called during genotyping because of a mutation at one or both of the restriction-enzyme recognition sites. Finally, we filtered out secondary SNPs where they occurred in a single sequenced tag, retaining only the SNP with the highest information content (highest AvgPIC). Any monomorphic loci arising as a result of the removal of individuals or populations were also deleted. Given the low within-population sample sizes ($n < 10$), we did not filter loci for departures from Hardy–Weinberg equilibrium (HWE) or linkage disequilibrium. The data remaining after this additional filtering are regarded as highly reliable and were used in all of our data analyses.

Visualisation

Genetic similarity of individuals and populations was visualised using ordination (principal coordinate analysis or PCoA, Gower 1966). A scree plot of eigenvalues (Cattell 1966) provided an indication of the number of informative axes to examine, taken in the context of the average percentage variation explained by the original variables.

Fixed-difference analysis

A fixed difference between two populations at a locus occurs when the populations share no alleles at that locus. A lack of fixed differences between populations in proximity indicates that they have either diverged very recently, or that there is gene flow, perhaps episodic, between them. Either way, they are either not on independent evolutionary trajectories, or they have only recently embarked on such a trajectory. In contrast, accumulation of fixed differences between two populations is an indication of a lack of gene flow. The fixed-difference analysis was undertaken using the scripts gl.collapse.recursive and gl.collapse.pval in dartR (see http://github.com/green-striped-gecko/dartR; Gruber *et al.* 2018). In brief, fixed differences were summed over populations (that is, field sites) taken pairwise (that is, two at a time), and when two populations had no fixed differences, the populations were amalgamated; the process was repeated until there was no further reduction (Georges and Adams 1996; Georges *et al.* 2018). The impact of missing genotype values on the rate of false detection of fixed differences was constrained by filtering on call rate by locus ($t = 0.90$) and individual ($t = 0.70$). False positives for fixed differences can occur when sample sizes are low; for this reason, we set as a target, 10 individuals ($2n = 20$) per site. Where such sample sizes were low ($n < 5$; $2n < 10$) and the samples were from the same subdrainage, we amalgamated them manually before the fixed-difference analysis. The set of putative operational taxonomic units (OTUs) arising from the above fixed-difference analysis were then tested for significance (using the test = TRUE option in gl.collapse.recursive.r in dartR, Georges *et al.* 2018), and pairs for which the number of fixed differences was not statistically significant were further amalgamated. The final set of OTUs are, by definition, diagnosable at one or more SNP loci.

Phylogeny

We took two approaches to estimate the phylogeny of *Cherax*. First, we conducted a Fitch–Margoliash distance analysis, as implemented in Phylip (ver. 3.695, see http://evolution.genetics.washington.edu/phylip.html/, accessed 6 February 2019; Felsenstein 1989), applied to Euclidean distances between the final OTUs identified by the fixed-difference analysis. Bootstrap estimates of node reliability were obtained by randomly

resampling loci and recalculating the distance matrix, with 1000 replicates.

In the second approach, SNP genotypes were constructed for each individual by concatenating the sequence tags containing the SNP loci, after trimming off the adaptors, into a single partition. Including the full sequence tags (differing by one SNP) allowed calculation of base frequencies and transition and transversion ratios. Some loci had the SNP removed with the adaptor, because of chance matching of the adaptor sequence to the terminal region containing the SNP. These loci were removed before concatenation. Heterozygous SNP positions were represented by standard ambiguity codes (see Felsenstein 2004, p. 255). Two datasets of 476 221 base pairs per OTU were analysed, one including all 381 individuals, the other consisting of one individual per population (51 individuals total, both Finke populations were treated as one). Maximum likelihood was conducted on both datasets, but only the latter dataset was bootstrapped. Analyses were conducted with RAxML (ver. 8.2.9, see https://cme.h-its.org/exelixis/web/software/raxml/, accessed 6 February 2019; Stamatakis 2014) on the CIPRES cluster (Miller *et al.* 2010), using the GTRCAT model and searching for the best-scoring maximum-likelihood tree by using the model GTRGAMMA in a single program run, with bootstrapping set to finish based on the autoMRE majority-rule criterion. Trees were rooted with *Cherax setosus*, *C. depressus*, *C. dispar* and *Cherax* sp. Swamp (Table 1).

Results

SNP datasets

The full dataset, which includes all samples, comprised 9140 polymorphic SNP loci from the sample set comprising samples from 45 sites for the ingroup taxa ($n = 1$–10, $N = 367$) and seven sites for the four outgroup species ($n = 2$–7, $N = 18$). This full dataset was derived by applying filters to the 50 125 polymorphic SNP loci scored for 347 individuals of *C. d. destructor*, 20 individuals of *C. d. albidus*, seven individuals of outgroup taxon *C. setosus*, five individuals of *C. depressus*, four individuals of *C. dispar* and two individuals of *Cherax* sp. Swamp. After filtering on repeatability (repAvg = 1.0) and call rate by locus (≥0.90), the number of SNP loci in the dataset dropped to 26 947 and then 10 976 respectively. Four specimens, including one individual each from the Macintyre, Macquarie, Condamine and Paroo rivers (Populations 21, 23, 17 and 13 respectively) had individual call rates of less than 50% (a threshold set taking into account the presence of the outgroups) and were removed from the dataset. The 1836 SNPs that co-occurred with another SNP at the same locus were filtered, with only the SNP with the highest AvgPIC being retained. This left 9140 polymorphic loci in the full dataset.

The ingroup dataset (*C. d. destructor* and *C. d. albidus*) comprised 7429 polymorphic SNP loci from 45 sites ($n = 1$–10, $N = 358$). The initial 43 364 SNP loci were polymorphic for the 367 individuals, of which 20 189 had a repeatability of 1.0. Further filtering on locus call rate (≥0.90) reduced the number of loci to 8532. Nine specimens (three individuals each from the Paroo (Populations 12, 13) and the Macquarie rivers (Populations 23, 24), and one individual each from the Macintyre (Population 21), Condamine (Population 17) and Warrego (Population 16) rivers) had an individual call rate of less than 70% and were removed from the dataset. The removal of 1087 secondary SNPs at a locus and 16 monomorphic SNPs yielded the final 7429 polymorphic SNP loci for the ingroup dataset.

Last, the *C. d. destructor* dataset was obtained by subsetting the ingroup dataset to include only individuals of *C. d. destructor* and removing resultant monomorphic loci to yield 6924 polymorphic SNP loci from the 43 sites ($n = 1$–10, $N = 338$).

Visualisation

The PCoA plots show considerable structure within the sampled *Cherax* (Fig. 2). *Cherax destructor* samples are responsible for most of the variation represented in Axes 1 (22.3%) and 2

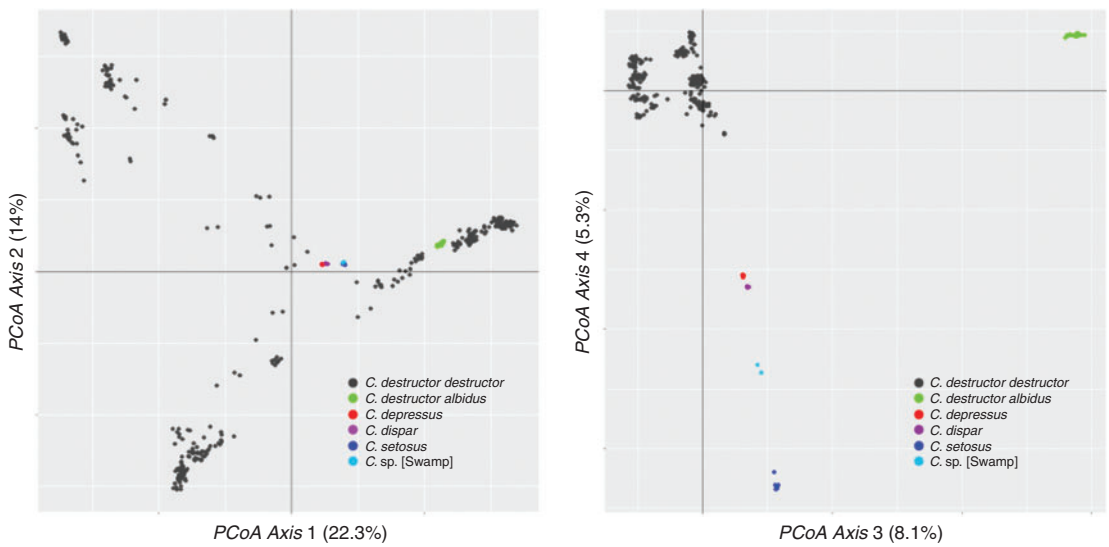

Fig. 2. Graphical representation of genetic similarity among *Cherax destructor destructor*, *C. d. albidus*, *C. depressus*, *C. dispar*, *C. setosus* and an undescribed species assigned the name *Cherax* sp. Swamp, using principal coordinate analysis. Axes are not to scale.

(14.0%). *Cherax setosus*, *C. dispar*–*C. depressus* form distinct clusters with their relationship to *C. d. destructor* and *C. d. albidus* evident on the plot of Axes 3 (8.1% and 4 (5.3%). The distinction between *C. d. destructor* and *C. d. albidus* is again strongly supported. The undescribed taxon (*Cherax* sp. Swamp) is also supported as distinct in this preliminary analysis. Examination of a plot of the ingroup taxa only (Fig. 3) shows differentiation of the LEB, BBB and Paroo River population from the MDB, and differentiation of the southern basin of the MDB from the northern basin, which includes sites in the Darling River and lower Murray River. Examination of structure in deeper dimensions by further subsetting the populations (not shown) showed that the populations in the Avoca and Loddon rivers (Populations 41–43) were genetically distinct from the remaining populations of the southern MDB, and there was a stronger association between the upper Lachlan River population (Population 27) with those of the Murrumbidgee (Populations 29, 30) than would be expected on the basis of river distance proximity. Similarly, the Nive River population in the upper Warrego (Population 15, MDB) is most similar to that of Barcoo River (Population 7) in Cooper Creek (LEB).

Fixed-difference analysis

The analysis of fixed differences confirmed the diagnosability (= significant number of fixed differences) of each of the following seven OTUs corresponding broadly to the existing classification of the group: *Cherax setosus* (Populations 46–48, $n = 7$), *C. depressus* (Population 45, $n = 5$), *C. dispar* (Population 44, $n = 4$), *Cherax* sp. Swamp (Populations 49, 50, $n = 2$), *C. d. destructor* ($n = 344$ from 38 locations), *C. d. albidus* (Wimmera, Population 51, $n = 10$) and *C. d. albidus* (Glenelg, Population 52, $n = 10$; Table 2). Divergences ranged in pairwise comparisons from 229 and 1,978 fixed differences between *C. destructor* and the remaining OTUs. *Cherax d. destructor* differed from *C. d. albidus* by 117–133 fixed differences, and the two *C. d. albidus* OTUs differed from each other by only eight fixed differences (null expectation 4, $P < 0.005$). Divergence of populations within *C. d. destructor* ranged from 0 to 1224 fixed differences, but these did not survive the recursive fixed-difference analysis because they were linked by populations with intermediate allele profiles.

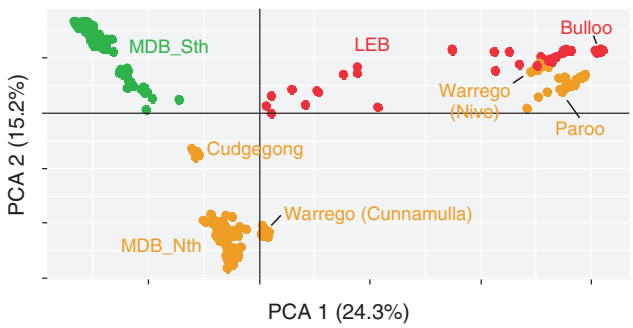

Fig. 3. Graphical representation of genetic similarity between *Cherax destructor* individuals from the Murray–Darling Basin (MDB), Lake Eyre Basin (LEB) and Bulloo Basin, using principal coordinate analysis. Colour scheme is consistent with other figures. Axes are not to scale.

Phylogeny

The distance tree generated for the seven OTUs identified in the fixed-difference analysis had a topology with 100% bootstrap support for all nodes (Fig. 4). As expected, *C. d. destructor* was sister to the two *C. d. albidus* populations (Glenelg and Wimmera). The undescribed species *Cherax* sp. Swamp was the first branching lineage to the ingroup defined by outgroup taxon *C. setosus*.

For the one per population dataset, excluding outgroups, 472 634 of the 476 221 bp were constant, 1937 variable characters were parsimony uninformative and 5059 characters were parsimony informative. Maximum likelihood recovered one tree with a –ln score of −749 146.027260 (Fig. 5). Support across most of the deeper nodes of the tree was strong except for the node separating the southern and northern *C. d. destructor*

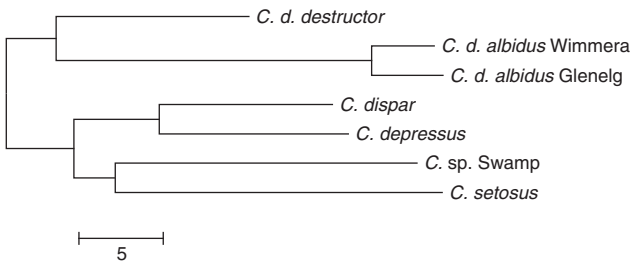

Fig. 4. Phylogeny for *Cherax* based on Fitch–Margoliash maximum likelihood applied to distances between the diagnosable units identified by the fixed-difference analysis. Bootstrap values on all nodes were 100%.

Table 2. Counts of fixed allelic differences (lower matrix) and percentage fixed differences (upper matrix) for the operational taxonomic units (OTUs) identified in this study

Species	*Cherax d. destructor*	*C. d. albidus* Wimmera	*C. d. albidus* Glenelg	*C.* sp. Swamp	*C. setosus*	*C. depressus*	*C. dispar*
Cherax d. destructor		2	1	9	10	5	5
C. d. albidus Wimmera	133		0	27	29	24	23
C. d. albidus Glenelg	117	8		26	27	22	21
C. sp. Swamp	644	1865	1781		14	14	12
C. setosus	732	1978	1871	921		14	12
C. depressus	229	1031	962	564	575		3
C. dispar	239	1143	1060	564	587	108	

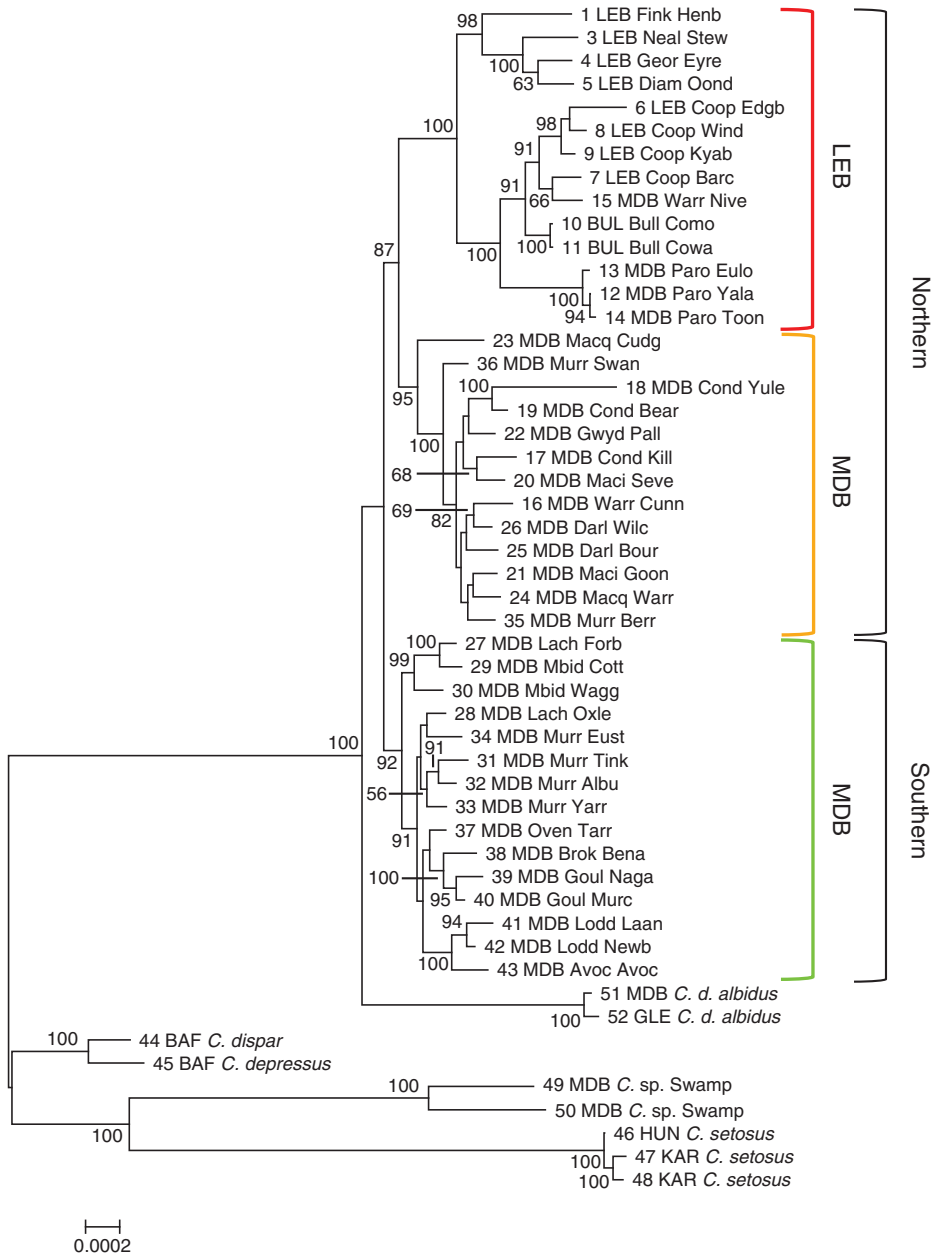

Fig. 5. Maximum likelihood tree for 51 *Cherax* populations based on an analysis of 9140 concatenated sequence fragments. The major lineage branches are labelled the northern and southern lineages, and within the northern lineage, we also defined the Lake Eyre Basin (LEB) and Murray–Darling Basin (MDB) clades. Each operational taxonomic unit (OTU) code is based on the site number and locality as per Table 1 and Fig. 1. The geographic distribution of lineages is shown in Fig. 1. Bootstrap values shown were based on 408 replicates.

clades. *Cherax d. albidus* was recovered as the sister lineage to *C. d. destructor*, whereas the southern and northern *C. d. destructor* clades were sister, but lacking bootstrap support. The northern clade separated into MDB populations from the Lower Murray River (below the Darling River junction), plus most of the Darling River catchment populations which were sister to all the LEB and BBB populations, plus the three Paroo River populations (Populations 12–14) and the upper Warrego (Nive River, Population 15). Bootstrapping ceased after 408 replicates because the autoMRE majority-rule criterion was met.

For the complete dataset, excluding outgroups, 1084 variable characters were parsimony uninformative and 7116 characters were parsimony informative. Maximum likelihood recovered one tree with a –ln score of –904 206.777300 (Fig. S1, available as Supplementary material to this paper). Deeper relationships were all consistent with the one-per-population dataset. Within-clade patterns were usually similar, except within the northern

MDB clade, which was mostly poorly supported by bootstrap values in the one-per-population tree. Most individuals within a population grouped together except for the following: southern clade, lower Lachlan River [28] and the mid-Murray [34]; northern clade, MDB, had several mixed populations, namely Warrego River (Population 16), lower Macquarie River (Population 24), Darling River (Populations 25, 26) and lower Murray River (Populations 35, 36); in northern clade, LEB, Neales River (Population 3) was placed differently and neither it nor Diamantina River (Population 5) was monophyletic.

Discussion

Both the phylogenetic analyses applied to individual SNP genotypes and the fixed-difference analysis supported the current taxonomy of *Cherax setosus*, *C. depressus*, *C. dispar* and *C. destructor*. Support was also given for the status of the undescribed species *Cherax* sp. Swamp (O'Brien *et al.* 2009; R. B. McCormack, pers. comm.). *Cherax d. albidus* is distinct from *C. d. destructor* (1–2% fixed differences), which does not challenge the current taxonomy that has these as subspecies. The two populations of *C. d. albidus* from the Glenelg and Wimmera rivers were significantly distinct with eight diagnostic differences (<1%, null expectation, 4); however, this low level of divergences is interpreted as within the range that might be expected among allopatric populations of a single species or subspecies.

Cherax destructor displays a series of divergences across its range that only partially follow drainage-basin boundaries. The primary divergence was between the two subspecies, namely, *C. d. destructor* and *C. d. albidus*. The original distribution of *C. d. albidus* beyond the Wimmera–Glenelg basins remains uncertain owing to extensive translocation of *C. destructor* in western Victoria (Nguyen 2005). Wimmera Basin is traditionally considered part of the MDB, but it is likely to have been isolated for 700 000 years after Lake Bungunnia started to dry (Stephenson 1986; McLaren *et al.* 2009). Highlighting the isolation of the Wimmera Basin is the fact that most fish species in the Wimmera–Glenelg basins are more closely related to each other, rather than to populations elsewhere in the MDB, with either identical or similar genetic signatures (Hammer *et al.* 2014), which is a pattern similar to that found in previous studies on *C. destructor* (Austin *et al.* 2003; Nguyen *et al.* 2004, 2005). In contrast to these mtDNA and RAPD results, the genetic divergence for SNPs between the Wimmera and Glenelg basins is higher, but SNPs appear to be more capable of distinguishing between closely related populations than are mtDNA comparisons. Historical movement of the aquatic biota between the Wimmera and Glenelg basins is likely to have been facilitated by low drainage divides, such as along the chain of lakes from Balmoral north towards Dimboola, geomorphically referred to as the Douglas Depression, which may represent a former southern outlet of the Murray River (Brownbill *et al.* 1995; McLaren *et al.* 2011), or in the low-elevation drainage divide region between Cherrypool and Wartook.

The separation between the southern and northern *C. d. destructor* clades of the MDB, LEB and intervening drainages is quite different from that found so far in most aquatic organisms studied in the region. In all but one taxon studied (*Hypseleotris klunzingeri*, Thacker *et al.* 2007), the LEB is genetically distinct from MDB populations (Hughes and Hillyer 2003; Carini and Hughes 2004; Hughes *et al.* 2004; Beheregaray *et al.* 2017; overview for fish, see Unmack 2013), with populations within the MDB having either no or a minimal structure in most cases (Unmack 2013; Attard *et al.* 2018). By contrast, the first branching lineage within *C. d. destructor* separates populations in the Murray River system upstream of the Darling River confluence from those in the lower Murray River, Darling catchment and the LEB and BBB. The downstream limit of the southern clade is at least Euston (Population 34), with animals sampled at Wentworth near the Murray and Darling River junction being from the northern clade (Nguyen *et al.* 2004). Clearly, further sampling between these locations is necessary to get a more complete picture of where the boundary is and how much mixing between clades is occurring (as suggested by the fixed-difference analysis) as well as whether that boundary is changing over time. We can only speculate on what may drive the divergence across this boundary between the two clades, but perhaps it could be a result of distance coupled with local adaptation between populations from the cooler, wetter portion of their range (the southern clade) *v.* those from the more arid portions (northern clade).

The northern clade contains a complex series of lineages, which indicates multiple movements at different times and places between the northern MDB and LEB–BBB. The primary split in the northern lineage is between the most northern MDB and lower Murray River populations *v.* those in the LEB–BBB (and Paroo). A relationship between MDB and LEB populations is not an uncommon biogeographic pattern, with many fish species (Unmack 2013), *Macrobrachium* shrimp (Carini and Hughes 2004) and *Velesunio* mussels (Hughes *et al.* 2004) having sister species or sister clade relationships between MDB and LEB–BBB (keeping in mind that, at the species level, it is the whole of the MDB vs LEB, not just part of the MDB as per the case in *C. d. destructor*). A few studies have tried this separation and have come up with values such as 150 thousand years ago (*Nematalosa erebi*) and 1.2 million years ago (*Retropinna semoni*, Hughes and Hillyer 2003), 58–280 thousand years ago (*Macquaria ambigua*, Faulks *et al.* 2010), 800 thousand years ago (*Macrobrachium*, Carini and Hughes 2004) and 500 000 generations (*Velesunio*, Hughes *et al.* 2004). There is little similarity in age estimates across these species, but this could be simply a result of the difficulty in accurately estimating time of divergence from molecular data. Thus, it remains unclear as to whether these all represent independent biogeographic exchanges, nor is it clear where the exchanges occurred.

In *C. d. destructor,* there appear to have been two additional movements into the MDB, one from the LEB via BBB into the Paroo River (Populations 12–14), the other from the upper Barcoo River into the upper Warrego River (from Population 7 into Population 15). LEB populations have a sister relationship between an eastern lineage (Cooper Creek), BBB, Paroo River (MDB), which is sister to a western lineage, and all the remaining LEB populations. Within the eastern lineage, the Paroo River (MDB) is the first branching lineage, followed by the Bulloo River (BBB), which is sister to Cooper Creek (LEB). We interpret this as representing a series of isolations, with Bulloo

River and Cooper Creek having more recent connections to each other than to the Paroo River. There is a clear opportunity for the biota to mix between the Bulloo and Paroo rivers today, although such movements would still be difficult. But under slightly wetter climates (which have occurred over the last glacial cycle), movement would have been potentially easier. Between the Bulloo and Paroo rivers, just east of the township of Thargomindah, is a small semi-isolated basin that drains into Lake Bindegolly. There is only a very slight elevational difference between the outflow during floods on Bundilla Creek (a northern tributary of Lake Bindegolly) and Bulloo River, which could easily connect by floodwaters if both systems were flooding at the same time (e.g. in the vicinity of −27.785356, 144.215713). Although the Lake Bindegolly system is usually isolated from Paroo River, if sufficiently wet, floodwaters would extend to lakes Wyara and Numalla, which regularly connect to the Paroo River when that river floods. Given the overall general tectonic stability of that region (Senior *et al.* 1978; Moussavi-Harami and Alexander 1998), it is likely that this avenue for exchange between the Bulloo and Paroo rivers has existed for a long time, spanning the divergences mentioned above.

The second LEB–MDB connection is between the upper Barcoo River (Population 7, an upper Cooper Creek tributary, LEB) and the Nive River (Population 15, an upper Warrego River tributary, MDB). No other aquatic species examined has so far shown this same or similar pattern, although few studies have included samples from the upper Warrego Basin. Warrego Basin samples usually group with the rest of the MDB, with no relationship to the LEB. Our next sampling site downstream is Cunnamulla (Population 16), which contained MDB type animals. Hughes and Hillyer (2003) sampled *Cherax* from across the Warrego Basin and the only evidence they found was that the upper-most populations from Sandford Park Lagoon and Quilberry contained a mtDNA type different from that of downstream populations (their next downstream site was Cunnamulla, which contained typical MDB-related haplotypes). These waterholes are ~150 km upstream from Cunnamulla. There do not appear to be any obvious places where connections by low-drainage divides might exist between the headwaters of the Barcoo and Nive rivers. It is also possible that animals may have been translocated, although we would expect to see evidence of introgression, given the short number of generations since translocation, rather than of replacement, given that *C. destructor* readily hybridise at least between the northern lineage and *C. d. albidus* (Nguyen and Austin 2004). The boundary between these lineages in the Warrego River should be determined and monitored to see whether and how it is changing over time.

Within the MDB clade of the northern lineage, there was overall lower levels of geographic structure among populations than there was among populations from the southern lineage. This implies more recent mixing among populations in the northern MDB clade than the southern lineage. Within the MDB clade of the northern lineage, both of the tree-based analyses and PCoA (Fig. 3–5) showed the upper Macquarie population (Population 23) as the most divergent. Their more intermediate position mid-way towards the southern lineage in the PCoA could be the result of gene flow with the southern lineage. Most other populations show only limited correspondance to geography in the phylogenetic trees, but most are separated only by short internodal branch lengths in the maximum-likelihood results (Fig. 5). In the southern lineage, the first branching lineage consists of the Murrumbidgee River populations (Populations 29, 30) grouping with the upper Lachlan River (Population 27), possibly suggesting that there has been between-catchment gene flow between the upper reaches of the drainages. One potential natural connection between these drainages is via the presently endorheic Lake George catchment. Lake George has spilled via Geary's Gap into the Yass River (a tributary to the Murrumbidgee River) on many occasions during the Quaternary (Coventry 1976; Singh and Geissler 1985). When the lake is full, the Lake George–Lachlan catchment divide is characterised by a chain of lakes and swamps only a few metres above lake high stand; thus, very slight changes in topography could shift where the lake drains. Several other groups of populations formed cohesive geographic groups, including the following: Murray River at Euston (Population 34) with nearby lower Lachlan River (Population 28); upper Murray River populations (Populations 31–33; but not matched with the Ovens catchment (Population 37)), mid-Murray Victorian tributaries (Populations 38–40) and western Victorian Murray River tributaries (Loddon and Avoca (Populations 41–43)). Thus, with the exception of the Ovens and upper Lachlan rivers, populations were always grouped geographically.

Our samples sizes for some populations within the LEB clade were low and our geographic coverage was more limited, with several major rivers being represented only by a single population (e.g. Diamantina, Georgina, Finke). Our phylogenetic analysis found the first split within the clade between the easternmost rivers (Cooper Creek, Bulloo and Paroo rivers) and the rivers to the west. The Finke River (Populations 1, 2) was the first branching lineage in the western rivers. It is currently the most isolated major river in the LEB because it has not reached Lake Eyre for a considerable time period (>10 thousand years ago, Kotwicki 1989) and it is probably quite a long time earlier when there were regular connections. Neales River (Population 3) is the next branching lineage; it is a small somewhat isolated western tributary to Lake Eyre, whereas the Georgina (Population 4) and Diamantina (Population 5) rivers connect during major flooding, thus explaining their closer relationship. Biogeographic patterns in the eastern rivers were discussed above under movements between catchments.

One issue affecting any studies on *C. destructor* is the fact that they have been widely translocated (Nguyen 2005; Coughran *et al.* 2009; McCormack 2014). It is likely that this has been facilitated by the formation and increase in the number of farm dams on most agricultural land. Catching *C. destructor* in farm dams for human consumption has a long history in Australia and, in more recent decades, they have been strongly promoted for aquaculture. They are also commonly used as bait for angling and are available in the aquarium trade. This results in many potential routes for translocation to occur (McCormack 2014). Today, *C. destructor* has been translocated to natural waters in Western Australia, Tasmania and coastal rivers of

Victoria, New South Wales and Queensland (Nguyen 2005; Coughran *et al.* 2009). Within coastal New South Wales, new records increased from 20 sites reported in 2009 (Coughran *et al.* 2009) to 52 in 2013 (McCormack 2014). These translocations have important conservation implications because of the impact of introduced *C. destructor* on other crayfish and amphibian species (Coughran *et al.* 2009; Coughran and Daly 2012). Although new translocations outside of the natural range of *C. destructor* are easy to detect, translocations within their natural range are difficult to identify because any translocations will occur into already established populations in most cases. Nguyen (2005) used genetic data to identify several populations in artificial habitats within the natural range of *C. destructor* that have experienced translocations, e.g. from the northern clade into the range of *C. d. albidus*.

Our current study sampled *C. destructor* only from natural habitats (Table 1), which is likely to have reduced our chances of finding evidence of translocations. Most individuals examined grouped by population, and those that did not, were usually geographically proximate to each other (Fig. S1), which suggests that there is little evidence of broad-scale translocation and mixing with natural populations. The main exception to this was various populations from the Lower Murray River up through the Darling River, which, although proximate, extends across a vast distance. Three populations had geographically anomalous results. The first is upper Warrego River (Population 15), which grouped with upper Barcoo River (Population 7); however, there was no evidence of any introgression within Warrego River, suggesting that this is most likely to be a natural pattern. The second is the upper Macquarie River (Cudgegong, Population 23), which was the first branching lineage within northern MDB clade populations (Fig. 5). It was also the most distinct population in the PCoA plot within the northern MDB, with populations being placed intermediate between the populations of northern and southern clades (Fig. 3), which is indicative of mixing between these clades. It is unclear whether this is due to recent natural gene flow or translocation. The third example is the upper Lachlan River (Population 27), which groups with the adjacent upper Murrumbidgee River (Population 29) in all analyses. They also are intermediate between upper Murrumbidgee River (Population 29) and lower Lachlan River (Population 28), which suggests that they contain a mix of alleles from both populations. Again, it is not currently possible to determine whether this is a result of translocation or natural gene flow.

Conflicts of interest

A. Kilian declares a potential conflict of interest in that he is the Director of Diversity Arrays Technology Pty Ltd, the company that generated the SNP data on a cost recovery basis. The remaining authors declare that they have no conflicts of interest.

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Supplementary material

Phylogeography and species delimitation of *Cherax destructor* (Decapoda: Parastacidae) using genome-wide SNPs

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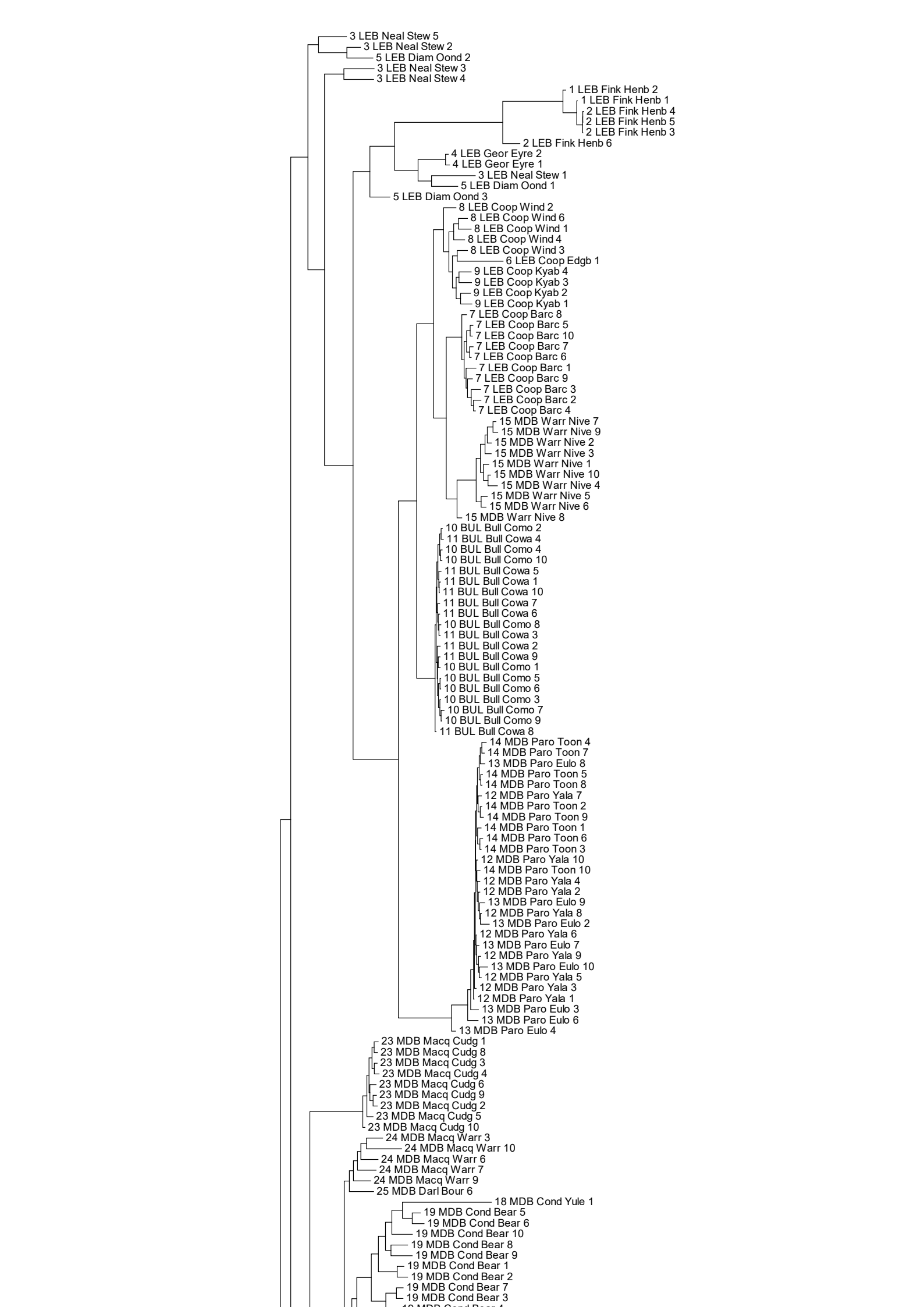
Fig. S1. Maximum-likelihood tree for 381 *Cherax* individuals (see following pages), based on an analysis of 9140 concatenated sequence fragments. Each operational taxonomic unit (OTU) code is based on the site number and locality as per Table 1 and Fig. 1.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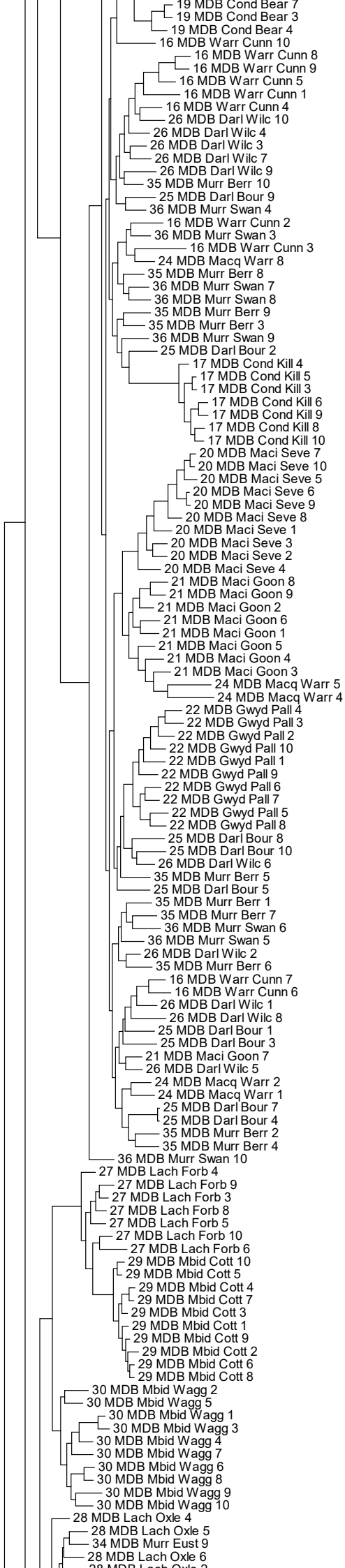
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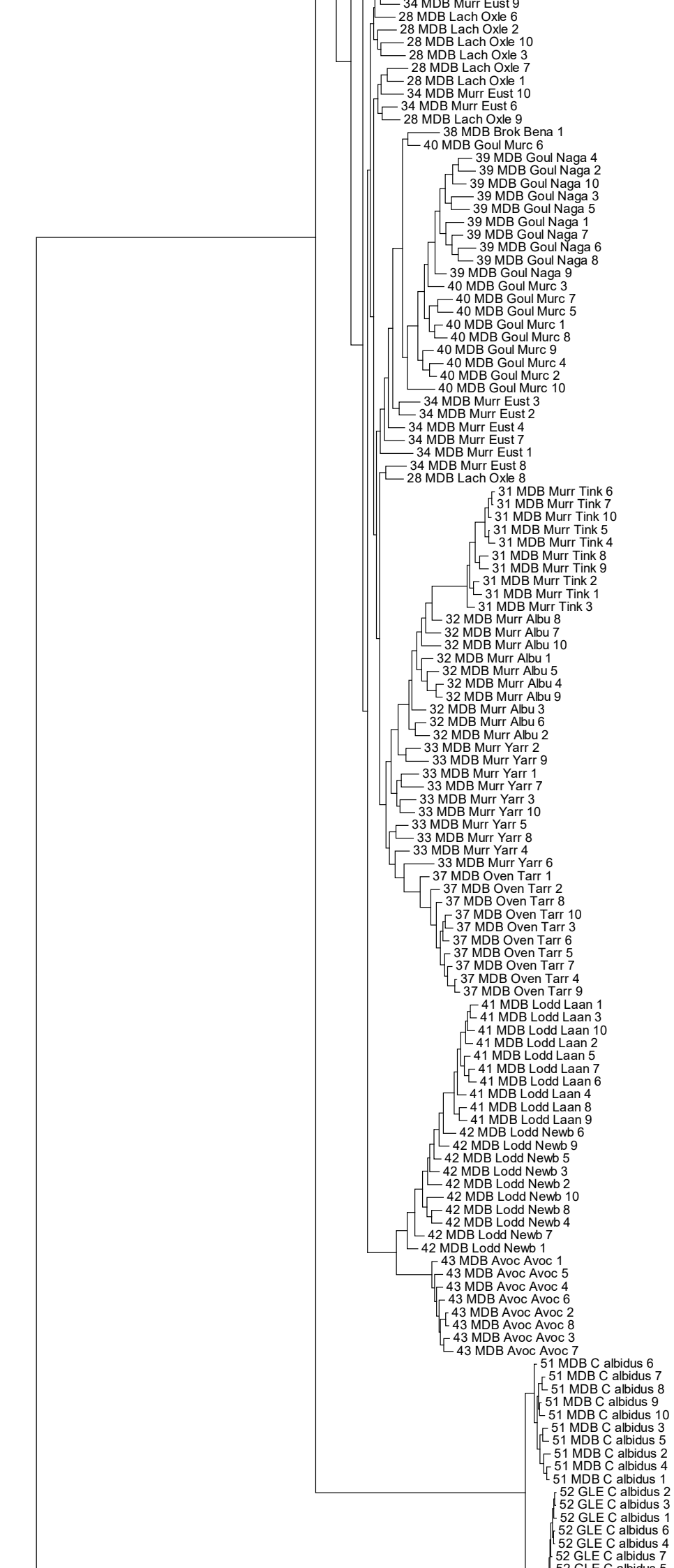
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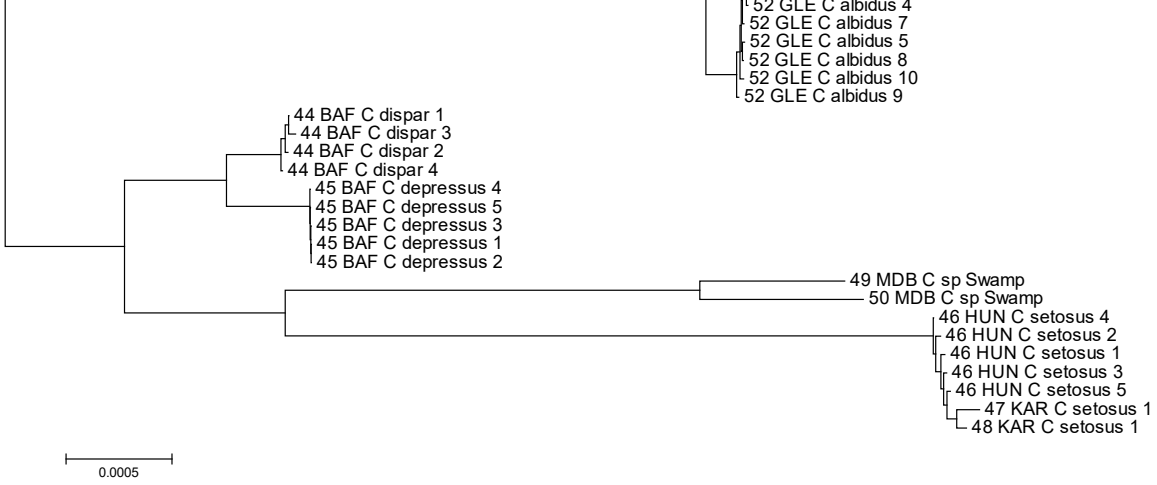
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