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RESEARCH ARTICLE

Range-wide population genetics study informs on conservation translocations and reintroductions for the endangered Murray hardyhead (*Craterocephalus fluviatilis*)

Samantha Thiele[1] | Mark Adams[2,3] | Michael Hammer[4] | Scotte Wedderburn[3] | Nick S. Whiterod[5] | Peter J. Unmack[2] | Minami Sasaki[1] | Luciano B. Beheregaray[1]

[1]Molecular Ecology Laboratory, College of Science and Engineering, Flinders University, Adelaide, SA, Australia

[2]Centre for Applied Water Science, Institute for Applied Ecology, University of Canberra, Canberra, ACT, Australia

[3]School of Biological Sciences, The University of Adelaide, Adelaide, SA, Australia

[4]Natural Sciences, Museum and Art Gallery of the Northern Territory, Darwin, NT, Australia

[5]Aquasave–Nature Glenelg Trust, Hindmarsh Valley, SA, Australia

Correspondence
Luciano B. Beheregaray, Molecular Ecology Laboratory, College of Science and Engineering, Flinders University, Adelaide, SA 5001, Australia.
Email: luciano.beheregaray@flinders.edu.au

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Abstract

1. Freshwater ecosystems worldwide harbour disproportionately high numbers of endemic species under threat from human activity, particularly accelerated habitat fragmentation. The Murray–Darling Basin in south-eastern Australia, one of the country's largest and arguably its most vulnerable freshwater ecosystem, is inhabited by a number of small-bodied fishes that are threatened with imminent extinction.
2. Here an extensive microsatellite dataset was used, supplemented by additional allozyme and mitochondrial DNA analyses, to assess the genetic diversity, population structure and contemporary migration patterns in the Murray hardyhead *Craterocephalus fluviatilis*, one of Australia's most threatened fishes.
3. Genetic diversity estimates, primarily based on 413 fish collected during the latter period of intense drought (1997–2010) from 23 sites and genotyped at 14 microsatellite loci, were higher than those previously detected for other regionally co-occurring small-bodied freshwater fishes.
4. Population structure analyses identified a subtle primary split between 'lower Murray' (lower river reaches) versus 'upstream Murray' (upper river reaches) and a total of nine genetically similar sub-populations. This includes unexpected sub-population differentiation in the Lower Lakes, a region at the terminus of the Murray–Darling Basin that most often has inter-connected habitat.
5. Very low levels of contemporary migration were detected between most inferred populations (<2%) during the drought, with all exceptions involving moderate levels of migration from an upstream sub-population into an adjacent downstream sub-population.
6. This article describes how these genetic data have guided translocation and reintroduction efforts in recent years. We advocate the use of assisted gene flow as a central component of continuing efforts to rescue this species from imminent extinction.

KEYWORDS
assisted gene flow, conservation biology, genetic rescue, habitat fragmentation, microsatellite DNA, teleost

1 | INTRODUCTION

Freshwater ecosystems are of crucial ecological importance to the planet and its biota (Carpenter, Stanley, & Vander Zanden, 2011; Dudgeon et al., 2006; Foley et al., 2005). Freshwater ecosystems occupy less than 4% of the Earth's surface and contain only ~1.4% of the world's fresh water (Carpenter et al., 2011; Zedler & Kercher, 2005). Nevertheless, they harbour disproportionately high levels of biodiversity (~10% of all known species, including around one-third of all vertebrates; Balian, Segers, Lévèque, & Martens, 2007; Strayer & Dudgeon, 2010), provide essential support to other ecosystems (Balian et al., 2007; Dudgeon et al., 2006), and have huge economic, cultural, and social significance to the human societies that depend upon them (Dudgeon et al., 2006; Vörösmarty et al., 2010).

Freshwater ecosystems are also among the most threatened in the world, with few remaining unaffected by a range of all-pervading pressures from human activity (MEA, 2005; Vörösmarty et al., 2010). Although efforts have been made to mitigate these adverse effects in many countries, such efforts often focus on water security for immediate human needs and thus either ignore or sacrifice freshwater biodiversity (Vörösmarty et al., 2010). As a result, freshwater ecosystems around the world have become increasingly degraded and fragmented, leaving many obligate freshwater species vulnerable to regional extirpation or overall extinction (Abell et al., 2008; Arthington, Dulvy, Gladstone, & Winfield, 2016).

Habitat fragmentation can be a natural feature of freshwater ecosystems and need not automatically result in negative consequences (Fahrig, 2003). Indeed, it is often proposed as a primary factor behind the observed high levels of freshwater biodiversity and endemism (Dudgeon et al., 2006; Magurran, 2009). However, when coupled with the combined effects of over-extraction of water, pollution, landscape modification, invasive species, changes to water flow regimes, overharvesting of apex species, and climate change (Balcombe et al., 2011; Collares-Pereira & Cowx, 2004; Crook et al., 2015; Maceda-Veiga, 2013), many freshwater ecosystems and their biota are unable to cope with the current pace and scale of human-mediated fragmentation (Carpenter et al., 2011; Lennox, Crook, Moyle, Struthers, & Cooke, 2019; Nilsson, Reidy, Dynesius, & Revenga, 2005). This is particularly applicable to obligate freshwater species because, unlike many terrestrial taxa, they become geographically fixed and cannot readily disperse between adjacent disconnected habitats (Abell et al., 2008).

The pressures on freshwater ecosystems have seen freshwater fishes, alongside amphibians, become some of the world's most threatened vertebrates (e.g. 46% of freshwater fish species in North America are currently imperilled; Jelks et al., 2008). Unfortunately, despite being as species-rich as their marine counterparts (Arthington et al., 2016) and far more diverse than amphibians (Balian et al., 2007; Zhang, 2011), small-bodied freshwater fishes are often under represented or overlooked in biodiversity assessments (Adams, Wedderburn, Unmack, Hammer, & Johnson, 2011; Lundberg, Kottelat, Smith, Stiassny, & Gill, 2000), and those considered vulnerable to extinction often receive comparatively little attention from ecologists and conservation biologists (Di Marco et al., 2017; Mota, Sousa, Araújo, Braga, & Antunes, 2014). This neglect is even more problematic given that a high proportion of freshwater fishes are endemic to relatively small and isolated habitats (Mota et al., 2014), while many display life history traits (e.g. low fecundity, short lifespan, migratory behaviour; Olden, Hogan, & Zanden, 2007; Growns, Rourke, & Gilligan, 2013; Rolls & Sternberg, 2015) that leave them highly exposed to habitat disturbance and drought (Chessman, 2013; Lennox et al., 2019).

As with all developed countries, Australia has its own share of threatened freshwater ecosystems and associated species. Prominent among these is the heavily regulated Murray–Darling Basin (MDB) in south-eastern Australia (Figure 1), the country's largest and arguably most human-impaired river system (Laurance et al., 2011). Bedevilled by the burden of underpinning much of Australia's agricultural activity despite being a relatively low rainfall region, the MDB also recently experienced a major, basin-wide 'Millennium Drought' between 1997 and 2010 (Dijk et al., 2013). Human water use and low rainfall resulted in a critical water shortage, particularly in the latter 3 years of the drought, and the consequent decline of many native species (Hammer et al., 2013; Wedderburn, Hammer, & Bice, 2012). Reflecting these combined pressures, almost half of the 45 freshwater fish species in the MDB have a conservation listing under state and federal legislation (Koehn & Lintermans, 2012). Climate change models also predict a gradual reduction in average rainfall, and more extreme events, over the coming decades (Balcombe et al., 2011), thus placing further stress on MDB species. The population declines, regional extirpations and isolation of several fishes prompted considerations of genetic issues associated with wild and captive populations. This led to a series of microsatellite-based conservation or riverscape genetic studies on Yarra pygmy perch *Nannoperca obscura* (Brauer, Unmack, Hammer, Adams, & Beheregaray, 2013), southern pygmy perch *Nannoperca australis* (Cole et al., 2016), southern purple-spotted gudgeon *Mogurnda adspersa* (Sasaki, Hammer, Unmack, Adams, & Beheregaray, 2016), river blackfish *Gadopsis marmoratus* (Lean, Hammer, Unmack, Adams, & Beheregaray, 2017), and the large-bodied Macquarie perch *Macquaria australasica* (Pavlova et al., 2017).

The present study adds yet another species to this series, the Murray hardyhead *Craterocephalus fluviatilis*. Murray hardyhead is listed as critically endangered by multiple bodies, including the

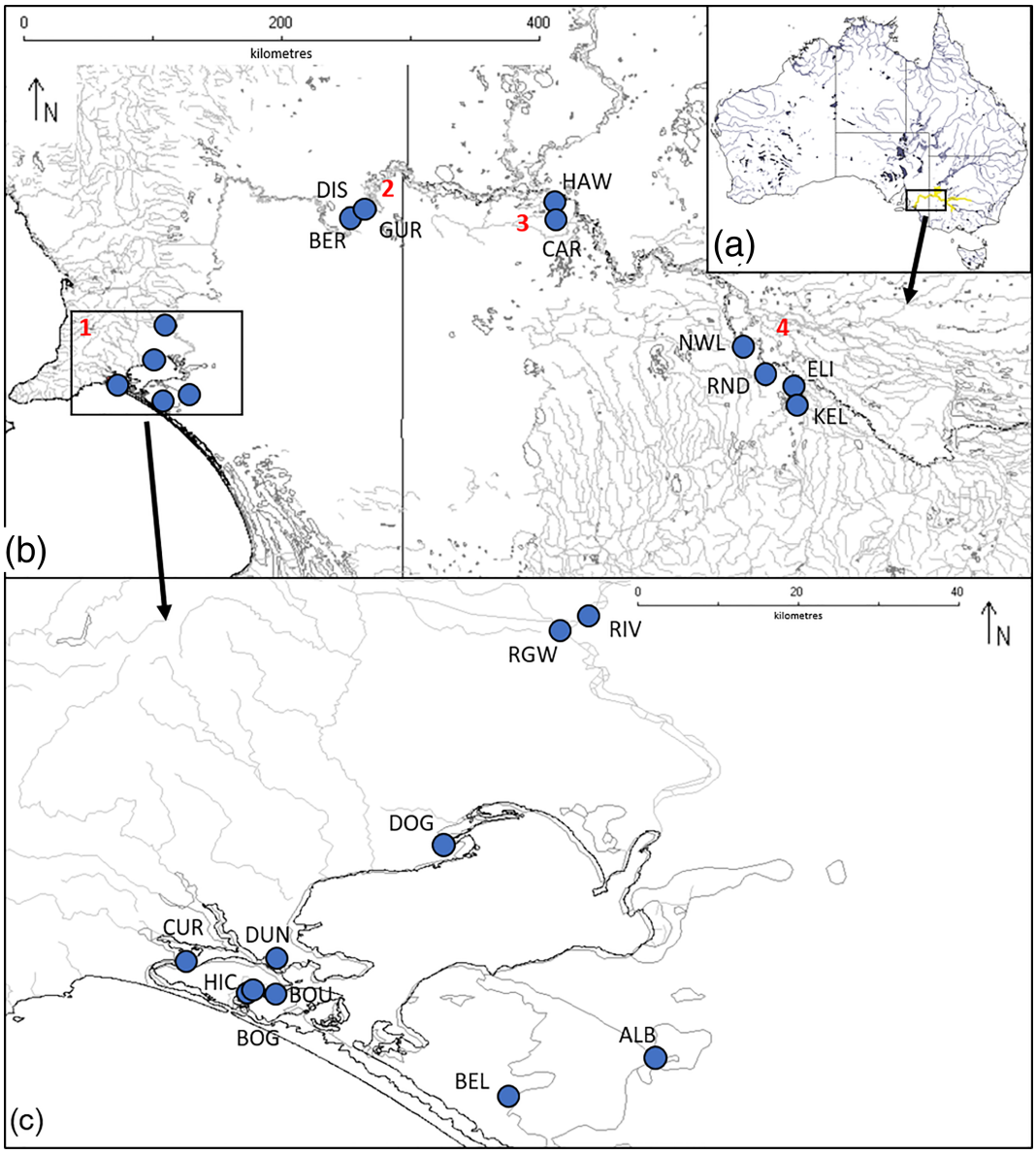

FIGURE 1 Maps depicting sampling locations for Murray hardyhead *Craterocephalus fluviatilis*. (a) Map of Australia showing the presumed former distribution of the species (in yellow). (b) Expanded inset of the location of all extant populations. (c) Expanded inset of 'lower Murray' sites. Sites are labelled with site codes as listed in Table 1 and grouped into four regions, labelled 1–4: the lower Murray (1), Riverland (2), Mildura Lakes (3) and Kerang Lakes (4)

International Union for Conservation of Nature and as endangered by Australia's Environment Protection and Biodiversity Conservation Act 1999 owing to population decline and regional extinctions. Murray hardyhead is a small-bodied (<10 cm), schooling and wetland-dependent freshwater fish with a largely annual lifespan and a high dependence on floodplain connectivity for dispersal. It is also tolerant of a wide range of conditions, including euryhaline waters, that are considered to provide a competitive advantage, at times, over less salt-tolerant species (Ebner, Raadik, & Ivantsoff, 2003; Ellis et al., 2013; Wedderburn, Walker, & Zampatti, 2007; Wedderburn, Walker, & Zampatti, 2008). The species is endemic to lowland floodplain wetlands of the southern MDB where it was considered historically abundant (Backhouse, Lyon, & Cant, 2008; Lintermans, 2007) (Figure 1). However, its distribution has been greatly reduced in recent decades; populations presumably extirpated during the Millennium Drought placed the species in imminent risk of regional extinction (Ellis et al., 2013; Hammer et al., 2013). At present, Murray hardyhead has a patchy distribution spanning approximately 1,400 km of river, occupying wetlands associated with the Murray River between Kerang in Victoria to Lake Alexandrina in South Australia. Natural recovery and management actions just before the end of, and following, the Millennium Drought, have afforded some improvement in the status of the species (Hammer et al., 2013), yet management actions focus at present on maintaining fragmented populations in largely isolated sites, with limited consideration of dispersal mechanisms (Ellis et al., 2013; Stoessel et al., 2020).

In the past, recovery and conservation plans for threatened fishes have tended to focus on active cornerstone issues such as habitat restoration, establishing captive populations, and undertaking re-introductions, while limiting genetic and ecological monitoring to 'passive' assessments of stock structure, population health, and the choice of source populations (Arthington et al., 2016; Collares-Pereira & Cowx, 2004; Godet & Devictor, 2018). However, faced with so many freshwater fishes under the threat of extinction globally, many are now arguing the need for genetic and ecological insights to play a more central and active role in recovery plans (Frankham et al., 2017; Grummer et al., 2019; Pavlova et al., 2017). In advocating this new perspective, the present study serves two primary purposes. First, it presents a comprehensive population genetic study, centring on microsatellite markers, for all extant and recently extirpated populations of the endangered Murray hardyhead. These data extend and expand on previous genetic studies, based on allozymes and mitochondrial DNA, which identified four conservation units for this species throughout the southern MDB and nominated the lower Murray as deserving of special conservation significance by virtue of its elevated levels of genetic variability via historic introgression with its upstream congener, the Darling hardyhead, *Craterocephalus amniculus* (Adams et al., 2011; Unmack & Dowling, 2010). Second, it

TABLE 1 Sampling details for all sites surveyed for the microsatellite study of *Craterocephalus fluviatilis*

Region	Sub-population code	Site	Site code	*n*	Latitude	Longitude	Year
1	LLAKE	Lake Albert	ALB	2	−35.608	139.376	2003
		Lake Albert	ALB*	10	−35.608	139.376	2006
		Belcanoe, Lake Albert	BEL	2	−35.653	139.205	2006
		Mundoo	MND	3	−35.546	138.929	2012 (Recapture)
		Mundoo east	MNE	8	−35.549	138.926	2013 (Recapture)
		Old Clayton	OLD	7	−35.495	138.911	2013 (Recapture)
		Finniss Junction	FIN	27	−35.485	138.887	2013 (Recapture)
		Currency Creek, Lake Alexandrina	CUR	27	−35.493	138.823	2010
		Hindmarsh Island Channel	HIC	10	−35.528	138.898	2001
		Hindmarsh Island Channel	HIC*	8	−35.528	138.898	2006
		Boundary Creek, Hindmarsh Island	BOU	5	−35.530	138.93	2003
		Dunn's Lagoon, Clayton	DUN	4	−35.493	138.933	2003
		Dunn's Lagoon, Clayton	DUN*	7	−35.493	138.933	2013 (Recapture)
		Dog Lake, Lake Alexandrina	DOG	2	−35.365	139.128	2010
	BOGGY	Boggy Creek	BOG	33	−35.530	138.91	2009–2010
	RGRIV	Rocky Gully wetland	RGW	20	−35.112	139.267	2006
		Rocky Gully wetland	RGW *	28	−35.112	139.267	2009–2010
		Riverglades (near Murray Bridge)	RIV	3	−35.097	139.301	2006
2	BERRI	Berri Evaporation Basin	BER	10	−34.307	140.576	2006
		Berri Evaporation Basin	BER*	17	−34.307	140.576	2010
		Disher Creek Evaporation Basin	DIS	15	−34.258	140.699	2006
		Disher Creek Evaporation Basin	DIS*	9	−34.258	140.699	2009
		Gurra wetland	GUR	24	−34.290	140.638	2010
3	CARDR	Cardross Lakes	CAR	16	−34.311	142.089	2006
	HAWTH	Lake Hawthorn	HAW	12	−34.208	142.096	2006
		Lake Hawthorn	HAW*	13	−34.208	142.096	2007
4	NWLAK	North Woorinen Lake	NWL	10	−35.243	143.435	2002
		North Woorinen Lake	NWL*	15	−35.243	143.435	2006
		North Woorinen Lake	NWL**	5	−35.243	143.435	2009
	ROUND	Round Lake	RND	10	−35.472	143.612	2006
		Round Lake	RND*	13	−35.472	143.612	2010
	KELIZ	Lake Kelly	KEL	29	−35.550	143.817	2011
		Lake Elizabeth	ELI	9	−35.696	143.815	2002

Note: Sites listed multiple times are temporal replicates, with follow up sampling indicated by an asterisk. Sites are designated using the codes and regions shown in Figure 1 and are grouped into their final sub-populations.
Abbreviation: N/A, not available.

reveals how these range-wide genetic and ecological data have featured prominently in guiding recent translocations and reintroductions, as well as informing forthcoming conservation efforts both to establish new populations (natural and captive) and to refresh extant but declining populations. In doing so, this study may encourage other researchers to adopt similar, more assertive applications of their own genetic and ecological datasets.

2 | METHODS

2.1 | Sites and sample collection

This study was based on 413 specimens ethically collected under valid state permits between 2001 and 2013 (Table 1) linked to intensive conservation efforts (Ellis et al., 2013; Hammer et al., 2013; Wedderburn, Hillyard, & Shiel, 2013). All tissues were sourced from the South Australian Museum from specimens previously collected by various contributors using seine nets, and either euthanized and stored in liquid nitrogen as detailed in Adams et al. (2011) or released after taking a pectoral or caudal lobe fin clip and placing this clip into 100% ethanol. Every known extant population during the study period was sampled, with the sampling region encompassing much of the Murray River, from the Kerang area in Victoria to the Lower Lakes in South Australia (Figure 1). Of the 23 sites sampled, eight were opportunistically resampled in a subsequent year, and one (North Woorinen Lake) was sampled three times (Table 1). Most sites were unable to be sampled near the end of the Millennium Drought owing to their imperilled state, while several populations became extirpated during the drought itself.

2.2 | Allozyme and mitochondrial DNA profiling of Lake Kelly population

Whereas the microsatellite-based analyses included several sites not previously profiled by the conservation genetic study of Adams et al. (2011), the frozen tissues required for allozyme analysis were only available for the Lake Kelly site (KEL; Figure 1). Therefore, the allozyme and mitochondrial DNA (mtDNA) sequence datasets were extended to include Lake Kelly fish (n = 15 for allozymes; n = 7 for mtDNA), thus ensuring that all three genetic datasets have maximal geographical concordance. The procedures, analyses, and nomenclature used for allozyme and mtDNA profiling all follow Adams et al. (2011).

2.3 | Laboratory protocols for microsatellite genotyping

DNA extractions were generated from frozen caudal muscle or fin clips using a modified salting-out method (Sunnucks & Hales, 1996), with DNA pellets washed twice with 70% ethanol. Fifteen species-specific microsatellite loci developed by Rodriguez-Zarate, Carvalho, Hammer, and Beheregaray (2014) were used to generate the data: Cf1, Cf2, Cf3, Cf5, Cf6, Cf7, Cf8, Cf9, Cf11, Cf13, Cf15, Cf16, Cf18, Cf19, and Cf20. These loci were amplified using the polymerase chain reaction (PCR) in batches that share the same M13 tag. Details about PCR reagents and concentration, as well as annealing temperatures for each locus are given in Rodriguez-Zarate et al. (2014). Thermal cycling conditions follow touchdown PCRs developed by Beheregaray, Moller, Schwartz, Chao, and Caccone (2004). Amplification success was tested by electrophoresis, PCR products were resolved in an automated DNA sequencer ABI 3130 (Applied Biosystems) and the microsatellite peaks were analysed using Genemapper 4.0 (Applied Biosystems).

2.4 | Statistical analyses of microsatellite data

All data were run through MicroChecker (van Oosterhout, Hutchinson, Wills, & Shipley, 2004) prior to analysis in order to identify null alleles and scoring errors. Deviations from Hardy–Weinberg equilibrium (HWE) were assessed using GenAlex 6.503 (Peakall & Smouse, 2006; Peakall & Smouse, 2012) and GENEPOP 4.7 (Rousset, 2008). One locus coded as Cf9 was removed from further analysis due to consistently returning statistically significant values for both deviation from HWE and null alleles across the majority of populations assessed. Such results retained significance after the application of Bonferroni corrections. While two other loci, Cf19 and Cf15, showed significant deviations from HWE and evidence for null alleles in several populations, they were not significant when Bonferroni corrections were applied and were thus kept for most analyses. These loci were only removed from some STRUCTURE analyses when assessing the significance of the genetic heterogeneity observed in the Lower Lakes. All analyses required the removal of locus Cf13 owing to it being monomorphic across all populations studied.

The number of effective alleles, observed heterozygosity, expected heterozygosity, and number of private alleles were calculated using GenAlex 6.503 (Peakall & Smouse, 2006; Peakall & Smouse, 2012). A population-level inbreeding coefficient for each locality (F_{IS}) was obtained with FSTAT 2.9.3 (Goudet, 1995; Goudet, 2001), and allelic richness (A_R) for localities where the sample size was at least 10 was calculated with HP-Rare 1.1 (Kalinowski, 2004).

Population structure was assessed in STRUCTURE 2.3.4 (Pritchard, Stephens, & Donnelly, 2000) using a burn-in value of 10^4 and a Markov chain Monte Carlo of 10^5, with 10 replicate runs and possible genetic clusters (K) ranging from 1 to 33. The resulting genetic populations were further assessed using the same settings and a maximum K of 18 in the lower Murray metapopulation and 15 in the upstream Murray metapopulation. The results of each analysis were uploaded to Structure Harvester (Earl & vonHoldt, 2012) to determine the most likely K based on deltaK values. This relies on the Evanno method (Evanno, Regnaut, & Goudet, 2005) to determine the

most likely number of genetic clusters returned by STRUCTURE analyses.

As the arrangement of sites along the river channel is largely linear, the analytical methods often used for dendritic river systems (Brauer, Unmack, Smith, Bernatchez, & Beheregaray, 2018) were not required in this study. Levels of population differentiation were assessed by calculating pairwise F_{ST} across all localities and carrying out an analysis of molecular variance (AMOVA) based on F_{ST} values using Arlequin 3.5.2.2 (Excoffier & Lischer, 2010). This was done both with all localities placed into one group in Arlequin to ensure that pairwise F_{ST} analyses can be conducted and with localities grouped based on geographical location. Significance levels for these analyses were calculated using 10^4 permutations.

Principal coordinates analyses (PCoAs) were conducted on the microsatellite data and visualized using the package Adegenet in R (Jombart, 2008). The resulting PCoA plots were generated both with and without scaling and centring, with the scaled plus centred plot proving to explain the greatest amount of variance.

Migration analyses were completed in BayesAss 3.0 (Wilson & Rannala, 2003), with the sampled localities grouped into populations as per the results of the STRUCTURE analyses. This was done with a burn-in of 10^6 and a Markov chain Monte Carlo of 10^7. Delta values for migration rates, allele frequencies, and inbreeding coefficients were set to 0.55, 0.4 and 0.15 respectively to ensure that the acceptance rates are between 20 and 60% (Wilson & Rannala, 2003). Convergence rates were checked by outputting log likelihoods from the analysis to be visualized in Tracer 1.6 (Rambaut, Drummond, Xie, Baele, & Suchard, 2018).

3 | RESULTS

3.1 | Allozyme and mtDNA profiling of Lake Kelly

The addition of 15 Lake Kelly fish to the allozyme dataset of Adams et al. (2011) resulted in an 'extended allozyme study' for Murray hardyhead comprising the genotypes of 154 individuals at 25 polymorphic loci (Adams, 2011; site-specific allozyme frequencies in Table S1). The Lake Kelly population showed comparable levels of genetic diversity to other upstream Murray sites (Table S1) and there were no indications that allozyme frequencies violated HWE expectations. Both analyses of population structure (Geneland and GENEPOP) used by Adams et al. (2011) found statistically significant evidence for the same four primary sub-populations as previously identified in the original dataset: lower Murray, middle Murray, North Woorinen (after correcting for a labelling mixup in Adams et al., 2011; see Adams, 2011) and upper Kerang, the latter comprising Lake Kelly and its two closest neighbours (RND and ELI). These four sub-populations are also evident on the neighbour-joining tree among all sites (Figure S1).

Only one of the four mtDNA haplotypes detected in Lake Kelly (haplotype d15; cytochrome b and ATPase 6/8) had not previously been detected in Murray hardyhead (Table S2). All four haplotypes belong to the 'd' clade (*sensu* Adams et al., 2011), the only clade among four that occurs in Murray hardyhead but not its sister species Darling hardyhead (Figure S2). A comparison of haplotype frequencies among the three upper Kerang sites (Table S2) showed statistically significant differences in haplotype proportions between Lake Kelly and each of the other two sites ($P < 0.01$ after Bonferroni correction), a result also supported by statistically significant differences in allozyme frequencies for the *Est2* locus among all three sites (Table S1).

3.2 | Site-specific summary statistics for microsatellites

The final microsatellite dataset comprised the genotypes of 413 fish at 13 polymorphic loci. Genetic diversity was similar across all populations studied (Table 2). In particular, none of the measures of genetic diversity displayed any substantial differences between replicates for the nine sites sampled more than once, 1–10 years apart. Observed heterozygosity values were generally similar to those expected under HWE and ranged from 0.45 to 0.75. Allelic richness in each population was consistently low (range 3.09–4.78; Table 2). There was no strong evidence of particularly high F_{IS} at any site, with values ranging from −0.1 to 0.19 (Table 2). Notably, four of the populations with $F_{IS} > 0.1$ had small sample sizes, suggesting that the results for these localities may reflect sampling error.

3.3 | Population structure

Based on the deltaK values from STRUCTURE, there are two primary populations present in Murray hardyheads (Figures 2 and S3), here referred to as 'lower Murray' and 'upstream Murray' (region 1 versus regions 2–4; Figure 1). Comparisons of these populations for a range of basic measures of within-population genetic variability (allele counts for each locus; Na, Ne, Ho, and A_R) showed that, as for both the allozyme and mtDNA analyses (Tables S1 and S2), the lower Murray population displays elevated levels of genetic diversity, although only one measure (Ho) was statistically significant (Table S3).

In contrast to the more conservative deltaK analysis (Figure S3), the maximum likelihood analysis suggested the presence of additional sub-populations (optimum K = 7–9; Figure S4). This was strongly supported by separate STRUCTURE analyses on each primary population, both of which revealed additional, sub-structure in each region. Together, these additional analyses identified a total of nine sub-populations, consistent with the maximum likelihood results.

Three sub-populations were delineated in the lower Murray (Figure 2b); BOGGY (the Boggy Creek site), LLAKE (all other 'Lower Lakes' sites excluding BOG), and RGRIV (sites RGW + RIV, ~40 km upstream from the Lower Lakes; Figure 1). Of these, BOGGY was identified as a distinctive sub-population, both with and without the inclusion of the two loci most likely to skew the results (Cf19 and Cf12). In contrast, the RGRIV sub-population was only delineated in the analysis when these two loci were removed. A separate

TABLE 2 Summary statistics of the microsatellite dataset by locality of Murray hardyhead

Location	Na (± SE)	% Polymorphic loci	H_O	H_E	F_{IS}	A_R	Pa
ALB	2.92 (±0.211)	92.9	0.750	0.545	–0.05	N/A	0.077
ALB*	6.77 (1.057)	92.9	0.679	0.625	–0.033	4.52	0.231
BEL	2.92 (0.265)	85.7	0.643	0.536	0.143	N/A	0.000
MNE	3.31 (0.365)	78.6	0.619	0.516	0	N/A	0.000
MND	5.39 (0.747)	92.9	0.607	0.597	0.049	N/A	0.000
OLD	5.85 (0.732)	92.9	0.633	0.657	0.113	N/A	0.000
FIN	10.62 (2.049)	92.9	0.682	0.693	0.035	4.78	0.308
CUR	9.46 (1.842)	92.9	0.647	0.678	0.067	4.61	0.231
HIC	7.00 (1.000)	92.9	0.736	0.639	–0.1	4.58	0.154
HIC*	5.62 (0.764)	92.9	0.625	0.634	0.082	N/A	0.077
BOG	6.31 (0.796)	92.9	0.610	0.616	0.026	3.81	0.077
BOU	4.92 (0.548)	92.9	0.700	0.659	0.049	N/A	0.000
DUN	4.00 (0.439)	92.9	0.589	0.547	0.066	N/A	0.231
DUN*	5.69 (0.779)	92.9	0.684	0.651	0.027	N/A	0.000
DOG	2.31 (0.347)	71.4	0.536	0.411	0.185	N/A	0.077
RGW	7.92 (1.456)	92.9	0.646	0.647	0.027	4.30	0.077
RGW*	7.39 (1.253)	92.9	0.600	0.632	0.069	4.08	0.308
RIV	3.31 (0.429)	78.6	0.619	0.512	–0.01	N/A	0.000
BER	4.39 (0.513)	85.7	0.557	0.529	–0.001	3.50	0.000
BER*	5.23 (0.802)	85.7	0.509	0.541	0.097	3.73	0.000
DIS	5.77 (0.928)	85.7	0.531	0.576	0.113	3.87	0.000
DIS*	3.92 (0.684)	85.7	0.448	0.488	0.154	N/A	0.000
GUR	5.84 (0.861)	92.9	0.522	0.567	0.104	3.64	0.154
CAR	5.46 (0.781)	85.7	0.560	0.566	0.042	3.60	0.077
HAW	6.00 (1.209)	85.7	0.605	0.584	0.007	4.07	0.000
HAW*	5.77 (1.033)	85.7	0.549	0.588	0.112	3.98	0.077
NWL	3.69 (0.536)	85.7	0.479	0.468	0.029	3.09	0.000
NWL*	3.92 (0.665)	85.7	0.538	0.512	–0.016	3.18	0.000
NWL**	3.15 (0.451)	85.7	0.505	0.455	0.089	N/A	0.000
RND	5.15 (0.783)	92.9	0.571	0.556	0.027	3.80	0.000
RND*	5.23 (0.818)	85.7	0.489	0.553	0.162	3.75	0.077
ELI	4.62 (0.874)	92.9	0.548	0.525	0.016	N/A	0.385
KEL	7.54 (1.233)	92.9	0.560	0.608	0.096	4.12	0.615

Note: Number of alleles (Na), percentage of polymorphic loci, observed (H_O) and expected (H_E) heterozygosity, population inbreeding coefficient (F_{IS}), allelic richness (A_R) and percentage of private alleles (Pa). An asterisk beside the site code denotes a temporal replicate. Explanations of site codes are given in Table 1.

Abbreviation: N/A, not applicable.

STRUCTURE analysis on the upstream Murray population found evidence for six sub-populations (Figure 2c); BERRI in the middle Murray (sites BER + DIS + GUR in region 2 of Figure 1), the two 'region 3' lakes CARDR and HAWTH, and three among the 'region 4' lakes in the uppermost part of the present range of the species (NWLAK, ROUND, and KELIZ, the latter comprising sites KEL + ELI). All but two of these sub-populations (BERRI and KELIZ) were readily distinguished in the STRUCTURE plot and showed little evidence of admixture (Figure 2c).

3.4 | Genetic differentiation among sites and sub-populations

Pairwise F_{ST} analyses showed variable levels of differentiation between sites, with F_{ST} values less than 0.05 between many Lower Lakes sites and as high as 0.25 between some lower Murray and upstream Murray sites (Figure 3). Overall, upstream Murray localities showed greater differentiation than lower Murray sites. Pairwise F_{ST} values were no larger than 0.15 when re-calculated for the nine sub-

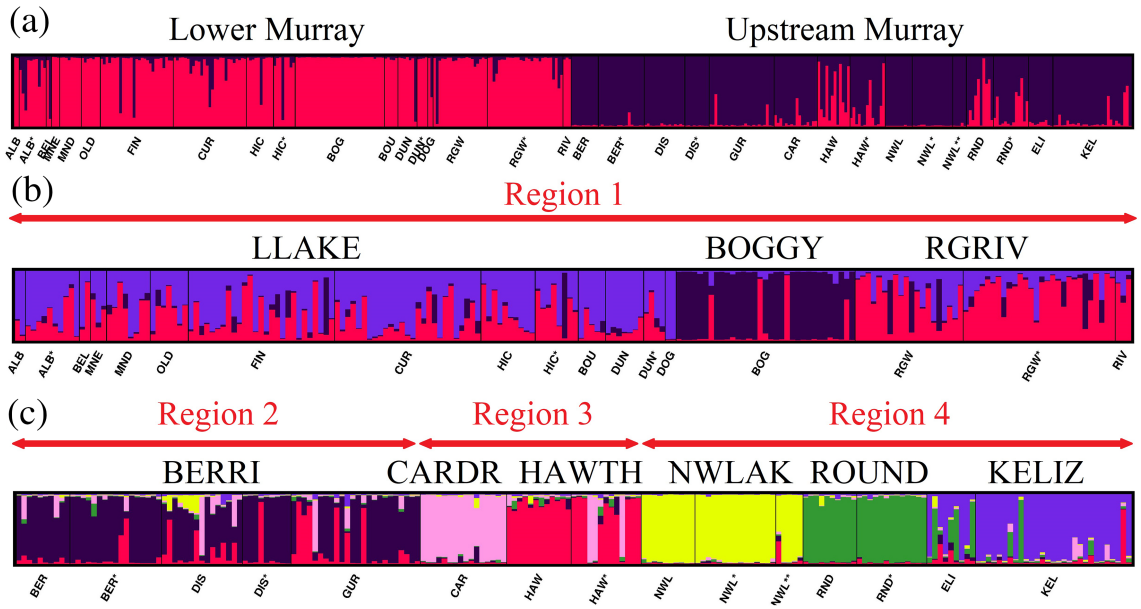

FIGURE 2 (a) The results of the initial STRUCTURE analysis of all localities of Murray hardyhead *Craterocephalus fluviatilis*, showing K = 2. (b) STRUCTURE analysis of the lower Murray population (region 1; Figure 1), showing K = 3. (c) STRUCTURE analysis of the upstream Murray population (regions 2–4; Figure 1), showing K = 6. Sub-populations are labelled as listed in Table 1

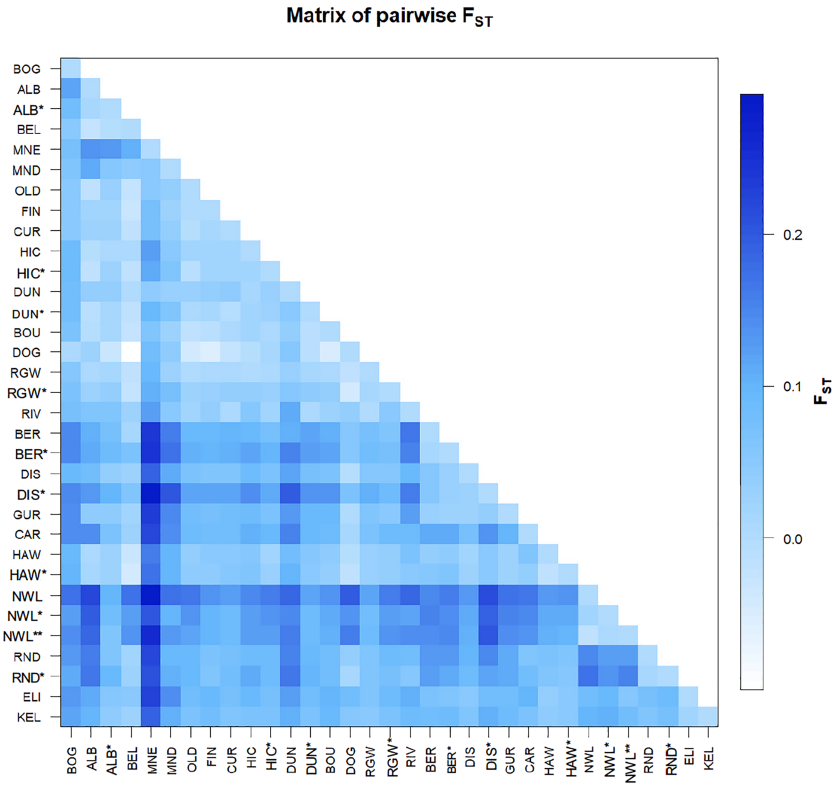

FIGURE 3 Heatmap showing pairwise FST values between all sampled localities of Murray hardyhead *Craterocephalus fluviatilis*. The localities are ordered spatially from downstream to upstream and are labelled as listed in Table 1

populations inferred from STRUCTURE, with most pairwise values >0.1 involving either the BOGGY or NWLAK sub-populations (Figure 4). Nevertheless, most between-sub-population F_{ST} values were statistically significant at the $P < 0.05$ level. The lower versus upstream dichotomy only exhibited a low F_{ST} value of 0.04, implying that comparatively little of the genetic diversity present in the metapopulation reflects genetic divergence between these two primary populations. This is further supported by the AMOVA results, which indicated that ~90% of the overall genetic heterogeneity occurred within individuals, regardless of whether they were partitioned into two primary populations or nine sub-populations (Table 3; $P < 0.001$).

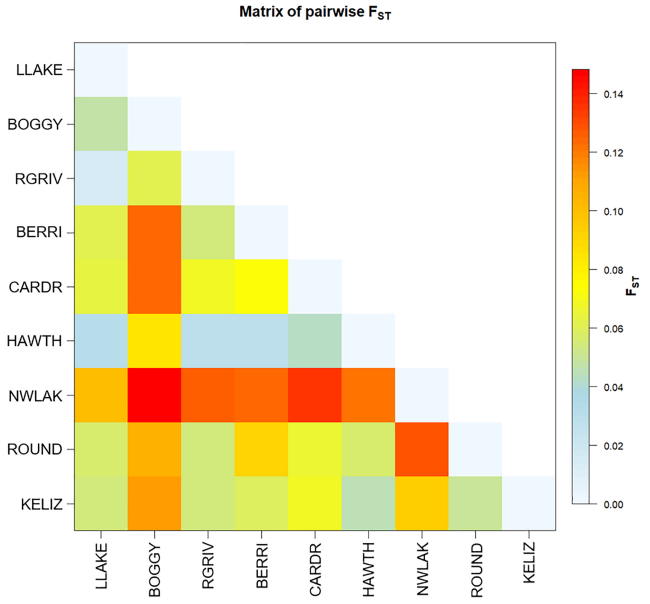

FIGURE 4 Heatmap showing the pairwise FST values among each of the genetic sub-populations identified of Murray hardyhead *Craterocephalus fluviatilis*. The clusters are ordered by geographical location from downstream to upstream

TABLE 3 AMOVA results for Murray hardyhead considering the (a) nine inferred sub-populations and (b) lower versus upstream Murray primary dichotomy

Source of variation	d.f.	Percentage of variation	F-statistics	*P*-value
(a)				
Among clusters	8	6.39	0.06	<0.001
Among populations within clusters	24	1.92	0.02	<0.001
Among individuals within populations	380	1.01	0.01	0.13
Within individuals	413	90.67	0.09	<0.001
(b)				
Among clusters	1	3.60	0.03	<0.001
Among populations within clusters	31	5.56	0.06	<0.001
Among individuals within populations	380	1.00	0.01	0.14
Within individuals	413	89.84	0.01	<0.001

Further support for the presence of relatively low levels of genetic heterogeneity between sub-populations was evident in the PCoA plot (Figure S5), which showed little or no differentiation between most sites and sub-populations. Four weakly-separated clusters were evident, one each for sub-populations BOGGY and NWLAK, as well as two overlapping clusters representing either lower Murray (sub-populations LLAKE and RGRIV) or upstream Murray (sub-populations BERRI, CARDR, HAWTH, ROUND, and KELIZ) sub-populations. As with all other analyses, temporal replicates were not distinguishable on the PCoA plot.

3.5 | Quantifying admixture and migration

STRUCTURE analyses indicated the presence of patchy and in some cases apparently bidirectional gene flow between the populations and sub-populations delineated (Figure 2); however, such analyses are often confounded by their inability to distinguish genuine admixture from the presence of shared ancestral polymorphism (Lawson, van Dorp, & Falush, 2018). Dedicated BayesAss migration analyses found little overall evidence of recent widespread migration, with > 85% of individuals remaining in their population of birth at most localities, with all values >2% reflecting unidirectional migration events from upstream sources (Figure 5). All three instances of significant migration proportions (>20%; HAWTH → BERRI, CARDR → BERRI, RGRIV → LLAKE) occurred from an upstream sub-population, via the main river channel, to the nearest downstream sub-population (Figure 5).

3.6 | Temporal changes during the Millennium Drought

As discussed earlier, F_{IS} values provided little indication of excess of homozygosity at individual sites (Table 2). Nevertheless, most locations for which temporal replicates were available showed some increase in F_{IS} as the Millennium Drought progressed, with increases of around 0.1 or more recorded at five of the nine replicated sites over <5 years (sites HIC, BER, HAW, NWL, and RND; Tables 1 and 2). Furthermore, all but three sites recorded a non-significant reduction in levels of observed heterozygosity over time (Table 2), a pattern mirrored when genotypes for all nine sites are pooled into 'initial sampling' versus 'subsequent sampling' aggregations (non-significant reduction in variability evident at most measures as the drought progressed, e.g. H_O = 0.640 ± 0.048 versus H_O = 0.607 ± 0.049; Table S4). Qualitative support for some but not all of these drought-associated changes at individual sites was evident in the various STRUCTURE analyses (Figure 2).

4 | DISCUSSION

The primary aims of this study were to assess genetic diversity, population structure, and contemporary dispersal patterns in the endangered Murray hardyhead throughout its present range. This work currently represents the greatest resolution of delineated populations analysed for this species and includes data for several now-extirpated

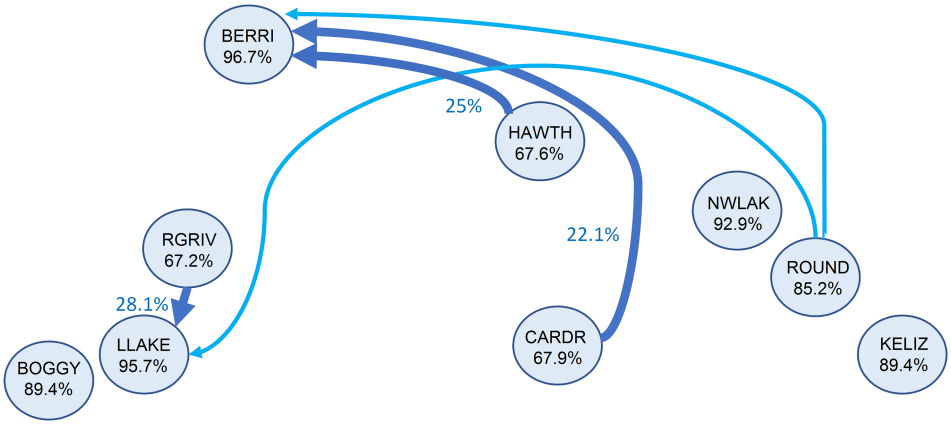

FIGURE 5 The results of BayesAss migration analyses for the nine sub-populations identified for the Murray hardyhead *Craterocephalus fluviatilis*. The proportion of individuals remaining in their locality of birth is shown within the circles. Thick dark arrows indicate the greatest amount of migration with the exact proportion displayed alongside the arrows. Migration of <10% is displayed as thin light arrows. Migration of <2% is not shown

populations (Table 4). Detailed analysis of the microsatellite data indicated the existence of nine, partially isolated sub-populations, and these showed comparatively moderate levels of genetic diversity and little contemporary connectivity. These findings are broadly consistent with previous studies on the population genetic structure of this species based on allozymes and mtDNA (Adams et al., 2011) and with other population genetic surveys of small-bodied freshwater fishes in the MDB (e.g. southern pygmy perch, Brauer, Hammer, & Beheregaray, 2016; Cole et al., 2016; purple-spotted gudgeon, Sasaki et al., 2016; river blackfish, Lean et al., 2017). The availability of multiple classes of genetic marker to infer population structure further bolsters the rigour of the conclusions and their role in formulating sound conservation and management strategies (Rodríguez-Peña et al., 2018; Sunnucks, 2000). Here the conservation implications of these findings and their impact on recent management efforts to mitigate extinction of the species are discussed, and recommendations are made for continuing management options into the future.

4.1 | Genetic diversity

It is generally well-recognized that low genetic diversity and high levels of inbreeding can compromise the adaptive potential of a population because of the associated consequences of deleterious fitness and thus increase the risk of local extinction (Hedrick & Garcia-Dorado, 2016; Pavlova et al., 2017; Willi, Van Buskirk, & Hoffmann, 2006). The average microsatellite allelic richness of Murray hardyhead (A_R = 3.95, range from 3.09 to 4.78; Table 2) was low compared with values reported in a global review of freshwater fishes (average A_R = 9.10; DeWoody & Avise, 2000), but higher than other small-bodied fishes within the MDB (range 1.92–2.80 for four other regionally co-occurring species; Brauer et al., 2016; Cole et al., 2016; Lean et al., 2017; Sasaki et al., 2016; Brauer et al., 2013). Values of the population-level inbreeding coefficient (F_{IS}) were mostly positive in Murray hardyhead but relatively low at the deme level (Table 2). This finding, albeit tentative owing to the relatively small sample sizes available, could initially suggest that inbreeding is yet to affect sub-populations. Nonetheless, in the absence of historical baseline measures for the species and of direct molecular-based measures of individual-level inbreeding, and, given that most populations for which temporal replicates were taken showed increasing values of F_{IS} over time, this inference cannot be validated by the current data. Indeed, the commonly observed impact of genetic factors on species decline (Frankham et al., 2017; Spielman, Brook, & Frankham, 2004) argues for the likelihood that Murray hardyhead has already experienced steady reductions in levels of genetic diversity since its geographical range began to decline after the 1980s (Lintermans, 2007).

At a regional level, the microsatellite analyses found that the lower Murray population harbours higher levels of genetic variability than the upstream Murray population, perfectly mirroring the results from both the allozyme and mtDNA datasets (Adams et al., 2011). This strongly corroborates a previous assertion that lower Murray sub-populations (LLAKE, BOGGY, and RGRIV) should be assigned a relatively high conservation value, although there is currently insufficient comparative microsatellite data to test whether the extra allozyme and mtDNA diversity present at lower Murray sites reflects alleles/haplotypes derived via natural introgression from Darling hardyhead *C. amniculus*, an upstream sister species.

Murray hardyheads display several life history traits that have presumably facilitated their ability to retain relatively high levels of genetic diversity throughout the Millennium Drought. First, the resilience of this species to conditions in isolated bodies of water such as extreme salinity levels (Ellis et al., 2013; Wedderburn et al., 2008) has probably enabled individuals to survive and reproduce for longer periods of time under such normally adverse conditions. Second, its relatively high vagility in the same basin compared with other small-bodied fishes (e.g. pygmy perches; Brauer et al., 2013; Cole et al., 2016; Brauer et al., 2016; rainbowfish; Brauer et al., 2018) would probably have enhanced the retention of genetic diversity via admixture during sporadic periods of connectivity, as evidenced by the overall low levels of genetic divergence among sub-populations (Figures 2–5). Third, the species' reproductive potential has, in some cases (e.g. DIS and BER), facilitated rapid recovery after short-term demographic bottlenecks associated with critical water shortage and habitat deterioration. Fourth, the tendency of this species to form schools may have allowed larger Murray hardyhead populations to persist in relatively small habitats by maximizing the effective size of the breeding population and therefore reducing genetic drift (Ebner

TABLE 4 Summary of the status of all Murray hardyhead populations surveyed in this study, as well as of the translocation and stocking efforts undertaken since genetic data were first available

				Conservation actions	
Region	Subpop	Site	Present status (in 2019)	Backup maintenance and breeding	Reintroduction
Lower Lakes	LLAKE	ALB	Extirpated (last recorded in 2009)	-	14,200 fish to three nearby sites over 2016–19
		BEL	Extirpated (last recorded in 2005)	-	
		MND	Uncertain (last recorded in 2015)	-	
		MNE	Uncertain (last recorded in 2014)	-	7,000 fish (from surrogate refuge and captively bred at Flinders University) in 2012
		OLD	Extant (recorded in 2018)	-	-
		FIN	Extant	-	-
		CUR	Extant	-	-
		BOU	Extant (recorded in 2018)	-	-
		DUN	Uncertain (last recorded in 2016)	-	-
		DOG	Extant	-	-
	BOGGY	BOG	Extant	2009–11 then established surrogate refuge	-
Lower Murray	RGRIV	RGW	Reintroduced (following extirpation in 2011)	2010–11, then established surrogate refuge	3,250 fish (from surrogate refuge) in 2016
		RIV	Extirpated	-	-
Riverland	BERRI	BER	Extant	-	-
		DIS	Extant	-	-
		GUR	Rediscovered in 2019 (extirpated in 2015)	-	135 fish (adults and captive-bred from BER and DIS) reintroduced in 2010; persisted for some time but unsuccessful
	NOOR	Noora Basin	Newly discovered in 2017	-	-
	CARDR	CAR	Extirpated (2014)	2007–12	-
	HAWTH	HAW	Extirpated	2007–12	600 captive-bred fish (from KOOR) released in 2018, but unsuccessful
	KOOR	Koorlong Lake	Reintroduced (in 2009 and 2013), recorded in 2019	2018	Reintroduction of 390 adult and juvenile captive-bred fish (from CARDR and HAWTH) in 2009 and 2013
	BRICK	Brickworks Billabong	Reintroduced (in 2014 and 2015), recorded in 2019	-	Reintroduction of 70 fish from KOOR in 2014; 2,500 fish from DIS in 2015
NSW	LFC	Little Frenchman's Creek, New South Wales	Reintroduced (in 2018), recorded in 2019	Small surrogate refuge also established in 2018	Reintroduction of 800 fish (from NOOR) in 2018
Kerang	NWLAK	NWL	Extirpated	2009–11	-
	ROUND	RND	Extant	2009–12	-
	KELIZ	KEL	Extirpated	-	-
		ELI	Reintroduced (following extirpation), recorded in 2019	-	Reintroduction of 50 fish in 2015

(Continues)

TABLE 4 (Continued)

Region	Subpop	Site	Present status (in 2019)	Conservation actions	
				Backup maintenance and breeding	Reintroduction
Surrogate refuge	MUND	Munday Dam	Assisted colonization	Established with 221 fish (from BOGGY and RGW) in 2010 and 2011	-
	BEYO	Beyond Wetlands	Assisted colonization	Established with 300 fish (from MUND) in 2018	-

Note: The listed sites represent all wetlands and lakes where Murray hardyhead has been recorded since 2004. Explanations of site codes are given in Table 1.

et al., 2003). All these traits may have helped alleviate the effects of habitat fragmentation on genetic diversity in Murray hardyhead, although clearly demographic stochasticity has reduced the number of local and regional extirpations during the Millennium Drought (Table 4).

4.2 | Population structure

When the results of all genetic analyses are considered, there is good evidence for the presence of nine sub-populations of Murray hardyhead throughout its range: three in the lower Murray and six in the upstream Murray. The greater resolving power of the hypervariable microsatellite markers allowed three of the four sub-populations identified by the allozyme and mtDNA datasets to be additionally split: three in the 'lower Murray' (including sub-population BOGGY, for which only microsatellite data are available), three in the 'middle Murray', and two in the 'upper Kerang'. Importantly, however, while all three genetic datasets revealed the presence of multiple sub-populations, they also all indicated that sub-populations only display modest levels of genetic divergence, with most of the species-level genetic diversity occurring among individuals. Thus, all available genetic analyses support the notion that genetic fragmentation in this species is a recent (i.e. post-European settlement) phenomenon and likely to be related to contemporary human influences and climatic changes in the southern MDB (Cole et al., 2016; Walker & Thoms, 1993; Wedderburn et al., 2017).

Threatened small-bodied fishes of the MDB typically show strong population structure and low dispersal throughout their range (Brauer et al., 2013; Cole et al., 2016; Lean et al., 2017; Thacker, Unmack, Matsui, Duong, & Huang, 2008), as opposed to large species that show high dispersal (Beheregaray et al., 2017; Faulks, Gilligan, & Beheregaray, 2010; Harrisson et al., 2017). In this respect, Murray hardyhead showed patterns of population structure that are somewhat intermediate between these two modal patterns, displaying only low to moderate levels of genetic divergence between sub-populations but also low levels of gene flow, at least during the Millennium Drought. Together these results suggest that sub-populations of Murray hardyhead have presumably been capable of experiencing gene flow during less regulated and more favourable environmental conditions or have only become isolated relatively recently when compared with other small-bodied fish species in the MDB.

An unexpected finding was the presence of two distinct sub-populations in the Lower Lakes, one widespread (LLAKE) and the other (BOGGY) at a single restricted site that would normally be hydrologically connected to other LLAKE sites (e.g. BOG versus HIC and BOU; Figure 1). Such genetic distinction may have resulted from a disconnection of Boggy Creek for approximately 3 years during the latter stages of the Millennium Drought when the site was a managed refuge for Murray hardyhead (Hammer et al., 2013; Wedderburn et al., 2013). As for any largely annual species, there is always the potential for an isolated population to become genetically distinct in only a few generations although the combined effects of inbreeding, selection, and genetic drift (Frankham et al., 2017). Recent isolation presumably also explains its current diversity levels, as this sub-population appears to have been isolated for fewer than 10 generations at the time of sampling (Ellis et al., 2013; Wedderburn et al., 2013).

4.3 | Gene flow

Although overall levels of migration were low, most migration inferred in this species occurred from upstream populations to areas further downstream, as is typical for riverine fauna (Brauer et al., 2018; Whiterod, Zukowski, Asmus, Gilligan, & Miller, 2017) and for other fish species in the MDB (Faulks, Gilligan, & Beheregaray, 2011; Lean et al., 2017). All three major exceptions, two in the middle Murray (regions 2 and 3; Figure 1) and one in the lower Murray, involved moderate migration rates (~22–28%) from a proximate upstream sub-population. The MDB is a highly variable dryland flood-pulse system, and the most recent opportunity for flood-driven connectivity at this scale before the study period was a small flood in 2000–2001 (Bureau of Meteorology, Australian Government unpublished river discharge data, e.g. station A4261001). When viewed alongside the successful use of environmental water to maintain populations at several sites during the drought (Ellis et al., 2013), such natural, albeit occasional, gene flow further demonstrates the potential resilience of this species provided that its sub-populations can persist until habitat connectivity

is re-established. Such opportunities are becoming less frequent and increasingly uncertain as the natural frequency, magnitude, duration, and timing of flooding has been greatly altered by river regulation (Baumgartner, Zampatti, Jones, Stuart, & Mallen-Cooper, 2014; Berney & Hosking, 2016).

4.4 | Genetic management to inform conservation translocations to aid recovery

Conservation translocations have been important to arrest the population decline of Murray hardyhead experienced during the Millennium Drought. Initially, emergency rescue from seven sites across four sub-populations occurred at the end of the drought, which facilitated captive maintenance and breeding and the establishment of surrogate refuges (Table 4: Ellis et al., 2013; Hammer et al., 2013). These varied backup populations have been the source for reintroduction that have successfully re-established five recently (Rocky Gully Wetland) or historically (Brickworks Billabong, Lake Elizabeth, and Little Frenchman's Creek) extirpated sites. However, reintroductions are not always successful, and this is the case for some former sites, particularly those across Lake Albert. For pragmatic reasons, translocations have relied on the meta-population distinction rather than ensuring that individuals are obtained from several spatial scales (e.g. region, sub-population, or site). There has been limited wild to wild translocation of the species, which has involved temporarily holding adults before subsequent release. There is a recognition that increased translocations will be required to allow the recovery of the species to continue.

Thus, the outcomes of this study are timely as they inform the genetic management of future translocations, as well as of reintroductions. Reflecting concerns about the potential for outbreeding depression and loss of adaptive potential, conservation biologists have traditionally been reluctant to employ 'assisted gene-flow' – the deliberate mixing of different genetic sub-populations – as part of the genetic rescue of a threatened species (Edmands & Timmerman, 2003; Frankham, Ballou, & Briscoe, 2010; Pavlova et al., 2017; Tallmon, Luikart, & Waples, 2004). Recent meta-analyses, however, have consistently demonstrated that the risk of outbreeding depression is usually negligible for genetically similar sub-populations of taxonomically validated species (i.e. where there is no genetic evidence that different populations reflect cryptic species) and, even when encountered, rarely last more than a few generations or has an impact on population persistence (Frankham, 2015; Frankham et al., 2017). A recent assessment of the literature concludes that genetic rescue should be carried out more often when proposed translocations conform to current guidelines (Bell et al., 2019). That seems to be especially true for small populations that are influenced by strong genetic drift and are therefore less likely to display localized adaptations (Bell et al., 2019), as might be the case for Murray hardyhead. We contend that all the conservation genetic insights available for Murray hardyhead point to this being a prime candidate where assisted gene flow should be a major component of conservation attempts, whether for individual populations or the species as a whole. We therefore recommend that managers strongly consider the deliberate mixing of any combination of sub-populations within the two primary populations (e.g. mix sub-populations in the lower Murray; mix sub-populations in upstream Murray) for which suitable source individuals are available, particularly when attempting to return the species to regions where they were formerly known to occur. In the event that individual numbers are not ideal, mixing between the two primary populations is an option also supported by all the results from this and previous studies. It is also necessary for genetic monitoring of wild and translocation sites to become a routine component of the conservation of the species (*sensu* Attard et al., 2016). In conclusion, we also advocate a similar approach for a wide range of other highly fragmented freshwater species as we stand on the brink of a major new extinction crisis in fresh waters (Darwall et al., 2018). To quote the words of Frankham et al. (2017) …. "doing nothing is a choice that is often harmful to the persistence of populations and species."

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

ORCID

Nick S. Whiterod https://orcid.org/0000-0002-7356-9834
Peter J. Unmack https://orcid.org/0000-0003-1175-1152
Luciano B. Beheregaray https://orcid.org/0000-0003-0944-3003

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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