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Accessory Publication

Supplementary description of DNA barcodes for fishes in the Murray-Darling River Basin, Australia

Ambassidae

Unlike all other Murray-Darling River Basin (MDB) species, specimens of *Ambassis agassizii* sampled from inside the basin (McNeil *et al.* 2008) were genetically indistinguishable to those in coastal catchments using 18S, 12S rRNA or mtDNA CR sequence gene barcodes.

Atherinidae

The three species of *Craterocephalus* within the MDB (Wedderburn *et al.* 2007) were genetically distinct on the basis of both 18S and 12S rRNA sequences. Small sequence differences between catchments were present in the 12S (1.2%) but not 18S rRNA genes for *C. stercusmuscarum*, the only species with populations outside the MDB, consistent with previous molecular and allozyme data that coastal south-eastern Queensland and MDB populations are currently isolated, but recently separated (McGlashan and Hughes 2001).

Clupeidae

Nematalosa erebi is perhaps the most widespread and abundant species across much of the MDB and northern Australia (Allen *et al.* 2002). Whilst no differences were observed in the 18S rRNA gene barcodes from specimens collected in the lower Murray River and elsewhere, differences between 12S rRNA sequences were present between the MDB and other populations in the Lake Eyre Basin (LEB) (0.8%) and coastal north-eastern Queensland (1.8%).

Eleotridae

This study confirmed the presence of four closely related species of *Hypseleotris* inside the MDB and within-species rRNA sequence variation was small despite sampling widely across the basin. However, other mitochondrial gene markers do reveal significant variation both within and between basins for all four species (Bertozzi *et al.* 2000; Thacker *et al.* 2007). The 12S rRNA sequence for the *H. galii* specimen from coastal south-eastern Queensland appeared closely related to *H.* sp.3 'murray-darling', consistent with morphological and other genetic markers that northern populations of *H. galii* may need to be re-classified as *H.* sp.3 'murray-darling' (Thacker *et al.* 2007). Most individuals of Murray-Darling and east coastal *Hypseleotris* species contained the same 18S rRNA sequence, with the exception of *H.* sp.2 'lake's' which was always genetically distinct (a second 18S rRNA allele appearing in the Barcoo River, LEB and a third in the Victorian section of the Murray-Darling and in coastal New South Wales). In addition, some specimens of *Hypseleotris* were clearly hybrids as they either contained two different 18S rRNA sequences (phenotypic *H.* sp.2 'lake's' from the Queensland section of the MDB, as well as *H. galii* from coastal New

South Wales) or were phenotypic *H.* sp.2 'lake's' with a *H.* sp.1 'midgley's' 12S rRNA sequence (Barcoo River, LEB). In previous studies, *H.* sp.2 'lake's' were reported to frequently share mtDNA sequences and allozyme profiles in common with other *Hypseleotris* species and this led to the suggestion that some, if not all, *H.* sp.2 'lake's' arise through hybridisation between *H.* sp.1 'midgley's', *H.* sp.2 'lake's' and/or *H. galii/* sp.3 'murray-darling' (Bertozzi *et al.* 2000; Thacker *et al.* 2007). The detection of two *H.* sp.2 'lake's' specimens in this study with 12S and 18S rRNA sequences distinct from other sympatric *Hypseleotris* species points to the existence of a non-hybrid form of this species.

No 18S rRNA and few 12S rRNA nucleotide differences were present either within (a single base) or between MDB and coastal *Mogurnda adspersa* (1-2 bases), although mtDNA CR sequences differed by 1.4%. This is consistent with previous genetic evidence for recent population separation (Hurwood and Hughes 1998; Faulks *et al.* 2008).

By contrast, the two *Philypnodon* species showed greater sequence divergence and 12S rRNA differences were of the order of 1.0% for *P. grandiceps* and 0.8 % for *P. macrostomatus* between the MDB and coastal populations. This is consistent with previous studies using cytochrome *b* that supported only localised historical gene flow between east coastal and southern MDB populations in these two species (Thacker *et al.* 2008).

Gadopsidae

Both 18S and 12S rRNA sequences support strong genetic divisions within *Gadopsis*. Specifically, *G. marmoratus* from the MDB and coastal Victoria appear sufficiently different to be considered separate species. Neither of the 18S and 12S rRNA phylogenetic trees find *G. marmoratus* monophyletic, although this is only strongly supported by the 12S rRNA data. This supports earlier claims *G. marmoratus* is actually comprised of two

geographically separated species on the basis of allozyme and sequence data (Miller *et al.* 2004; Kuiter 2009).

Galaxiidae

All *Galaxias* species posessed unique 12S rRNA barcodes. The data support closer evolutionary ties between *G. maculatus* and *G. rostratus* than the other *Galaxias* species since their 18S rRNA sequences were identical.

Melanotaenidae

All three *Melanotaenia* species examined share identical 18S rRNA sequences, whereas differences are present between the 12S rRNA of *M. fluviatilis*, *M. splendida* and *M. douboulayi*. Hybrid *Melanotaenia* are known to occur (Lintermans 2007) and at least some populations in the north of the MDB are likely to possess mixed mitochondrial haplotypes.

Percichthyidae

The sequence data presented here confirms the existence of closely related and geographically separated populations of *Macquaria* species (Musyl and Keenan 1992; Faulks *et al.* 2010*a*, 2010*b*). In *M. australasica*, distinct MDB and coastal New South Wales forms have been described. Both populations share identical 18S and 12S rRNA sequences, whereas their mtDNA CR sequences differ by 2-3%. Likewise, *M. ambigua* from the MDB, LEB and Fitroy-Dawson River basin (FDB) were confirmed as geographically separate populations based on mtDNA CR sequences. Only a single nucleotide polymorphism was present in the

12S rRNA barcodes of *M. ambigua* from the MDB/LEB and FDB, whereas mtDNA CR differences were more pronounced (2% between MDB and LEB strains and 5-6% between MDB or LEB and the FDB strain). Previous allozyme (Musyl and Keenan 1992) and comprehensive mtDNA CR data (Faulks *et al.* 2010*b*) have been used to propose at least sub-species level differences in *M. ambigua* from the MDB, LEB and FDB populations. Whilst we did not detect sufficient differences in the rRNA sequences to support species-level separation, the genome structure of the *M. ambigua* specimen from LEB differs from the MDB and FDB strains as it harbours variant 18S rDNA sequences, similar to those we observed in *Tinca tinca* and *Aldrichetta forsteri*.

The two MDB *Maccullochella* species *M. peelii peelii* and *M. macquariensis* (Douglas *et al.* 1995; Nock and Baverstock 2008; Nock *et al.* 2010) are closely related and share identical 18S rRNA, but distinct 12S rRNA, barcodes. A single nucleotide base difference was present between the *M. peelii peelii* 12S rRNA sequence and those for the two species present in east coast drainages, *M. peelii mariensis* and *M. ikei*. A previous study using the more variable mtDNA CR showed differences of the order of 6-7% between *M. peelii peelii* and east coast *M. peelii mariensis* and *M. ikei* and only 1% between *M. peelii mariensis* and *M. ikei* (Bearlin and Tikel 2003).

Nannopercidae

N. australis 12S rRNA sequences are quite similar to *N. obscura* with *N. variegata* more distant, whereas *N. australis* appeared more divergent according to the 18S rRNA. These results are taken to indicate that mitochondrial DNA “capture” is likely to have occurred by *N. australis* from *N. obscura* in the recent past. Furthermore, the existence of two species of *N. australis* has recently been proposed (Kuiter 2008). One is present in the

MDB, western Victoria and northern Tasmania, whereas the other, differing in the mtDNA CR (4.0%), 12S (0.8%) and 18S (0.2%) rRNA sequences, occurs in south-eastern Victoria, extreme north-eastern Tasmania and Flinders Island. Interestingly, *N. australis* individuals from the Macquarie River catchment in north-eastern Tasmania shared identical 18S and 12S rRNA sequences and the mtDNA CR differed by only 0.1% to a specimen from the Finniss River in South Australia, supporting recent gene flow between some populations, whereas modern populations are increasingly fragmented (Cook *et al.* 2006; Cook *et al.* 2007).

Plotosidae

Neosilurus hyrtlii and *Porochilus rendahli* are both wide-spread species and relatively low genetic differentiation has been reported between populations of *N. hyrtlii* (Huey *et al.* 2006). We also found relatively little 18S and 12S rRNA sequence differentiation between MDB, coastal and LEB populations, although mtDNA CR sequences were more informative. *Neosilurus hyrtlii* from the MDB differed to specimens from coastal south-eastern Queensland and LEB catchments by 0.5 % and 0.8% respectively and *P. rendahli* differed to specimens from coastal south-eastern and north-eastern Queensland catchments by 0.5% and 2.4% respectively.

The evolutionary position of the third catfish species within the MDB (*Tandanus tandanus*) appears complex and is complicated by repeated translocations of this species both within and outside the basin (Musyl and Keenan 1996). Here some central east coastal Australian populations share identical 18S and 12S rRNA sequences with their MDB counterparts, whereas other east coastal populations (*T.* sp.1, Bellinger River, New South Wales) and northern (*T.* sp.2, Mulgrave River, Queensland) are distinct according to 12S (2.6, 6.2%) rRNA sequences. Nevertheless, consistent differences are present between the

mtDNA CR sequences of MDB and east coastal populations (Jerry 2008). In addition, the northern *T.* sp.2 18S rRNA sequence is divergent (0.1%) to the southern populations. Our data further supports the presence of at least two geographically separated and morphologically cryptic *Tandanus* species in addition to *T. tandanus* in coastal catchments (Jerry 2005; Jerry 2008; Rourke *et al.* 2010).

Retropinnidae

The 18S and 12S rRNA sequences confirm large biogeographic genetic boundaries within *Retropinna semoni* and the differences are sufficiently large to support species-level separation between the more closely related MDB and LEB populations (0.1, 0.5 % difference for 18S and 12S respectively) and those from east coastal rivers (0.2, 5.1 %) as has previously been proposed (Hammer *et al.* 2007). The phylogenetic trees place the two *R. semoni* groups separated by *R. tasmanica* lending strong support for separation into at least two species.

Terapontidae

Bidyanus bidyanus and *B. welchi* were the only described species examined in this study with identical 18S and 12S rRNA barcodes. Their recent separation is supported by the relatively low level of sequence divergence in the mtDNA CR (5.4%) which is comparable to the differences present between other closely related species such as *Maccullochella peelii mariensis* and *M. ikei* (2.5%), *M. peelii peelii* and *M. peelii mariensis* (7.5%) (Bearlin and Tikel 2003).

The second terapontid species in the MDB, *Leiopotherapon unicolor*, is one of the most widespread of the Australian native freshwater fish species (Allen *et al.* 2002). A previous comprehensive genetic study using allozyme and mitochondrial large subunit (16S) rRNA gene data revealed little genetic structure across the entire continent that was attributed to the ability of this species to rapidly disperse under favourable conditions (Bostock *et al.* 2006), although MDB specimens were not examined. As expected, we detected no rRNA genetic differentiation between the MDB and LEB and only a single 12S rRNA nuclotide difference to a specimen sourced from an aquaculture facility in coastal Queensland, whereas mtDNA CR differences ranged from 0.4% (LEB) to 3.1% (presumably coastal Queensland).

Introduced species

Unique 18S and 12S rRNA sequences were obtained for the 12 exotic species in 5 families (Cobitidae, Cyprinidae, Percidae, Poeciliidae, Salmonidae) now established in the MDB, including Crucian Carp (*Carassius carassius*), recently reported as present in the Campaspe River, Victoria (Davies *et al.* 2008). Sequence barcodes for an additional 3 native species thought to have been translocated to the MDB (*Galaxias truttaceus*, *Macquaria novemaculeata* and *Oxyeleotris lineolata*) were also obtained, although only *Galaxias truttaceus* appears to have established self-sustaining populations.

Estuarine species

Samples of the remaining species, which included 3 catadromous species *Anguilla australis* and *Anguilla reinhardtii* (Anguillidae) and *Pseudaphritis urvillii* (Bovichtidae) and 11 marine species in 6 families (Gobiidae, Hemiramphidae, Mugilidae, Pleuronectidae,

Sciaenidae and Sparidae) frequently encountered in the estuarine or lower freshwater reaches of the lower MDB, possessed unique 18S and 12S rRNA sequences. Whilst some specimens were sourced through the seafood trade rather than from the MDB estuary, relatively low levels of rRNA gene divergence within catadromous and marine species were observed, even between specimens collected several thousand kilometres apart, comparable to the results obtained with more variable mitochondrial genetic markers such as the COI barcode (Ward *et al.* 2009).

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