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Identification of the Rainbowfish in Lake Eacham Using DNA Sequencing

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KEYWORDS

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ABSTRACT

The Lake Eacham rainbowfish (Melanotaenia eachamensis) was once thought to be confined to its type locality within the Lake Eacham World Heritage National Park. M. eachamensis disappeared from the lake following the translocation of several species into the lake and the species was pronounced extinct in the wild in 1987. In a 2007 survey we noticed that rainbowfish were present in the lake once again. We used a molecular marker to identify these fish and the likely source population. Analysis of the D-loop region of mitochondrial DNA revealed that the species now present in the lake is Melanotaenia splendida, and is most closely related to several M. splendida populations in the immediate vicinity. Here we explore a range of scenarios that may have led to this colonisation event and highlight the dangers associated with translocation.

Introduction

The tale of the Lake Eacham rainbowfish (*Melanotaenia eachamensis* Allen & Cross) is something of an Australian conservation biology saga. This species was once thought to be confined to its type locality, Lake Eacham, an isolated crater lake on the Atherton Tablelands in the hinterlands of Cairns. Prior to the 1980s the species was highly abundant and commonly observed along the fringe of the lake shore (Allen and Cross 1982). The lake itself has a raised surround and the catchment has been entirely isolated from the neighbouring Barron, Mulgrave and North Johnstone River systems for at least 6000 years (Timms 1979). The only other fish species present in the lake at the time of the survey were the fly-specked hardyhead (*Craterocephalus stercusmuscarum* Gunther) and the northern trout gudgeon (*Mogurnda mogurnda* Richardson) (Allen and Cross 1982).

Sometime between 1983 and 1987 several other species were translocated into the lake, including mouth almighty (*Glossamia aprion*), banded grunter (*Amniataba percoides*), bony bream (*Nematalosa erebi*) and the archer fish (*Toxotes chatareus*), all of which are abundant in the surrounding streams (Barlow *et al*. 1987). Shortly thereafter the Lake Eacham rainbowfish vanished entirely; the first reported extinction of a vertebrate from within a World Heritage National Park in Australian history (Wager 1993). It was also the first freshwater fish to be pronounced extinct in Australia. The northern trout gudgeon and yabby (*Cherax cairnsensis*) were also reportedly missing from the survey (Barlow *et al*. 1987). The most popular

explanation for the disappearance was that the rainbowfish was predator naïve because of its long history in isolation and thus succumbed to predation principally by the mouth almighty. Later comparative examinations of the species' antipredator behaviour supported this notion (Brown and Warburton 1997).

Following its disappearance from the lake, *M. eachamensis* was listed as extinct in the wild. Fortunately, aquarium enthusiasts had unofficially collected the fish and established breeding colonies in captivity (Barlow *et al*. 1987). These populations were later to become the centre of attempts to reintroduce the fish to the lake in 1989 and the early 1990s, all of which failed in spectacular fashion despite the fact that the captive populations retained a significant level of genetic variation (Zhu *et al*. 1994). In a single restocking event, 3000 fish were released into the lake and none were resighted just three months later. Several studies have since shown that rainbowfish populations vary dramatically in their predator recognition and antipredator behaviour (Brown and Warburton 1997, 1999; Brown 2003) and that captive-reared populations show a loss of shoaling behaviour (Kydd and Brown 2009) and atypical stress responses (Zuberi *et al*. 2011). No attempt was made to train the fish to recognise and respond to predators before reintroduction, or to remove the translocated species, which is now recognised as best practice (Brown and Laland 2001; Brown and Day 2002).

Following these failures there was some reluctance to continue supporting the attempts to manage the species and questions were raised about its distinctiveness from the surrounding populations of *M. splendida* (Wager 1993). Crowley and Ivantsoff (1991), for example, found that the morphometrics of *M. eachamensis* overlapped with *M. splendida* in every criterion and they found no differences at allozyme loci. Zhu *et al*. (1994), however, showed significant divergence between the two species in the nucleotide sequences of segments of the *cytochrome b* gene and the tRNA (Pro) control region. This new information led to a rapid reevaluation of the species as a discrete evolutionarily significant unit worthy of further protection (Zhu *et al*. 1998).

Allen (1989) reported a population of fish that seemed to closely resemble *M. eachamensis* in the nearby Dirran Creek, a tributary of the North Johnstone River. Follow up surveys and analysis based on morphometrics and meristics identified that *M. eachamensis* was alive and well in the wild (Pusey *et al*. 1997), but at least some of these samples were incorrectly identified, probably owing to the high degree of phenotypic plasticity in rainbowfish (McGuigan *et al*. 2003) and the fact that most meristic characters show a high degree of overlap within the family (Allen and Cross 1982; Crowley and Ivantsoff 1991). Further analysis using molecular tools showed that the species was isolated to just a few small populations, including those at Dirran Creek and Lake Euramoo (Zhu *et al*. 1998; McGuigan *et al*. 2000). Analysis using mtDNA, nuclear microsatellites, and morphometric characters showed that *M. eachamensis* populations were entirely distinct from the surrounding *M. splendida* populations and were more closely aligned with *M. australis* (Zhu *et al*. 1998; McGuigan *et al*. 2000). Despite its re-emergence from the ashes, *M. eachamensis* is still listed as critically endangered by the IUCN and little is known about its current distribution and abundance.

Sometime between 2000 and 2007, rainbowfish reappeared in Lake Eacham (Fig. 1*a*). The visual appearance of these fish resembled *M. splendida*, which is common in many of the surrounding waterways. The purpose of the present paper was to confidently identify the fish and determine the likely source population using molecular tools. Molecular techniques are particularly useful in the present context because the traditional morphometric and meristic analyses are too unreliable for identification of rainbowfish species (Crowley and Ivantsoff 1991; Zhu *et al*. 1998; McGuigan *et al*. 2000). Moreover, molecular analysis has repeatedly revealed cryptic species diversity in the Australian freshwater fauna (e.g. Page *et al*. 2004; Faulks *et al*. 2010) and is rapidly becoming a vital tool for identifying and conserving biodiversity.

Fig. 1. (*a*) A male rainbowfish specimen captured in Lake Eacham in 2007 and identified as *M. splendida* using mtDNA sequencing. (*b*) A male *M. eachamensis* from Dirran Creek.

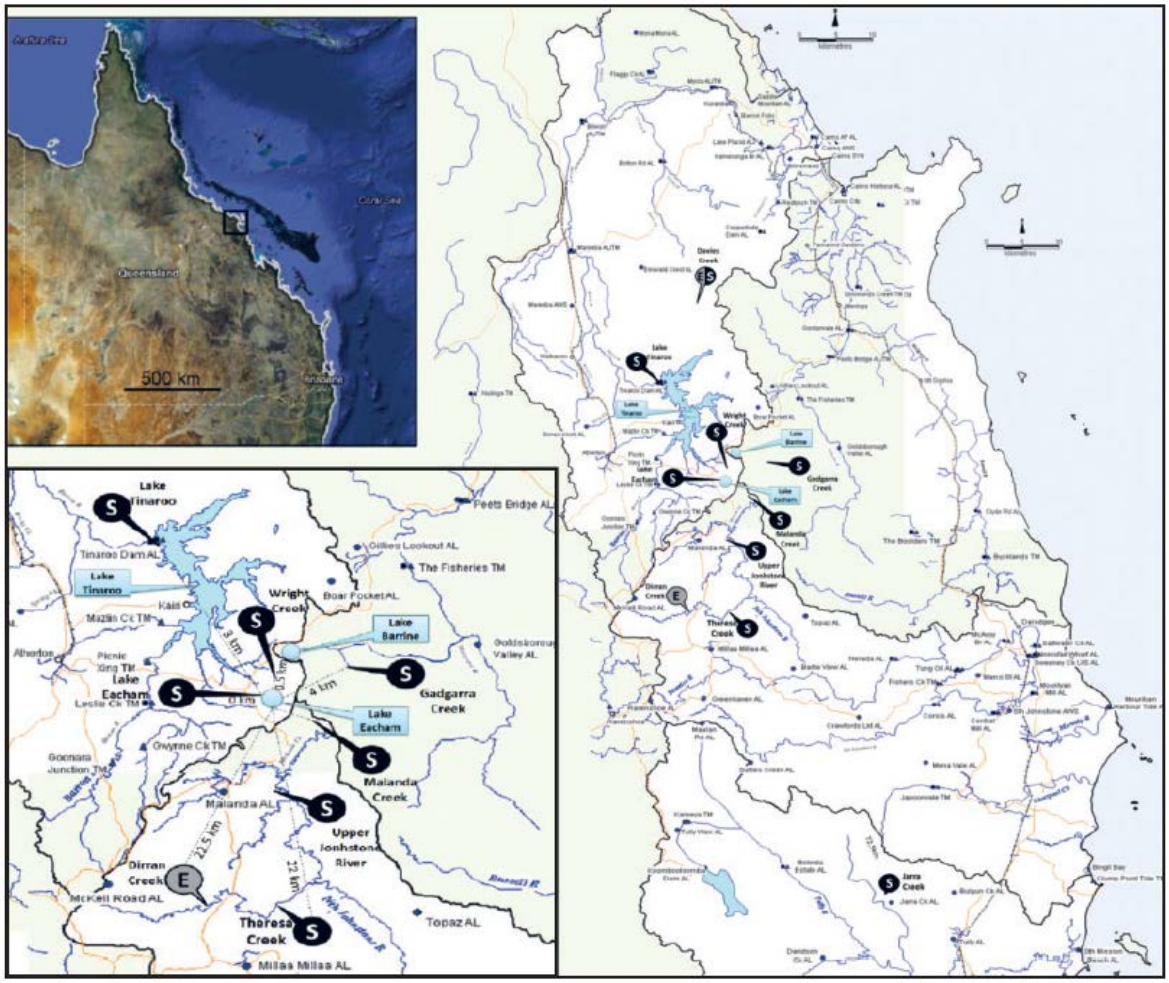
Methods

Between 2007 and 2011 we collected fish from the surrounding areas at various distances from Lake Eacham. Because we suspected the fish in the lake was *M. splendida* rather than *M. eachamensis*, most of the samples were taken from populations known to contain the former (Table 1). As a reference we included a sample from the captive-reared population of *M. eachamensis* that once inhabited Lake Eacham as well as the best known wild population of *M. eachamensis* from Dirran Creek (Fig. 1*b*). We sampled various locations including a single lowland population of *M. splendida* from the Tully River system, which is less likely to be the source population given both its geographical location in a disjunct drainage and the great linear distance to Lake Eacham. Davies Creek is also a significant distance and geographically isolated from Lake Eacham but it is part of the Barron River catchment which drains from Lake Tinaroo, offering another geographically distant population. This population has attracted the attention of aquarium enthusiasts because of its unusual appearance. Wright Creek is north of Lake Eacham and runs into Lake Tinaroo. Lake Barine is an isolated crater-lake catchment but overflows into Toohey Creek during very wet seasons and ultimately into the Mulgrave River. Gadgarra Creek is a small tributary of Toohey Creek. The remaining populations are part of the North Johnstone River catchment (see Fig. 2).

Table 1. Locations of fish populations sampled and the distance from Lake Eacham. Note that distances are linear and do not represent in-stream distances, which are substantially greater.

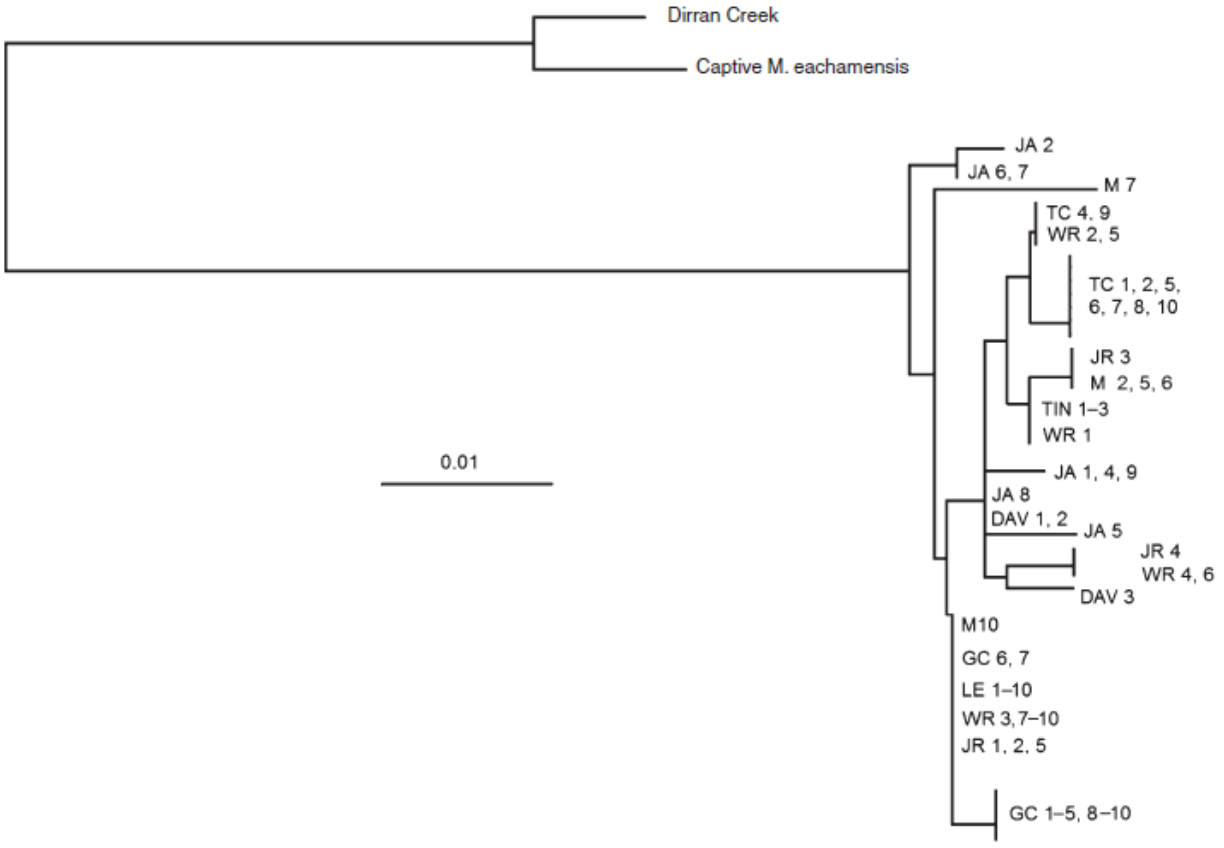
Species	Location	Key	Catchment	Latitude	Longitude	Distance (km)
M. eachamensis	Captive			17.008926	145.583499	n.a.
M. eachamensis	Dirran Creek		North Johnstone	17.470336	145.553167	22.5
M. splendida	Lake Eacham	LE	Lake Eacham	17.285142	145.629010	0
M. splendida	Wright Creek	WR	Barron	17.280111	145.632099	0.5
M. splendida	Malanda Creek	M	North Johnstone	17.299520	145.634724	2.5
M. splendida	Lake Tinaroo	TIN	Barron	17.171739	145.551381	3
M. splendida	Gadgarra Creek	GC	Mulgrave	17.273081	145.666196	4
M. splendida	Johnstone River	JR	North Johnstone	17.363136	145.642399	9.5
M. splendida	Theresa Creek	TC	North Johnstone	17.475396	145.655945	22
M. splendida	Davies Creek	DAV	Barron	17.008926	145.583499	31.5
M. splendida	Jarra Creek	JA	Tully	17.897901	145.850853	72.5

Fig. 2. Location of the sample sites in the Atherton Tablelands. The lower left-hand box shows an enlargement of the area around Lake Eacham, showing the sites and the linear distances to Lake Eacham. S = *M. splendida*, E=*M. eachamensis*.



The fish were captured at each location either by seine net or in a standard bait trap (Queensland Fisheries permit #100562). A minimum of 10 fish were photographed, fin clipped and released. The fin clips were held in 75% ethanol in individually labelled eppendorf tubes for DNA extraction.

Fig. 3. A molecular phylogenetic tree based on the sequence of the mitochondrial D-loop. *M. eachamensis* populations are represented by Dirran Creek and the captive *eachamensis* stock. The remaining populations are all *M. splendida*. The fish currently in Lake Eacham (LE) clearly cluster within the *M. splendida* populations near the bottom of the tree. Numbers next to the location abbreviations refer to specimen numbers. Refer to Table 1 for the key to collection location abbreviations.

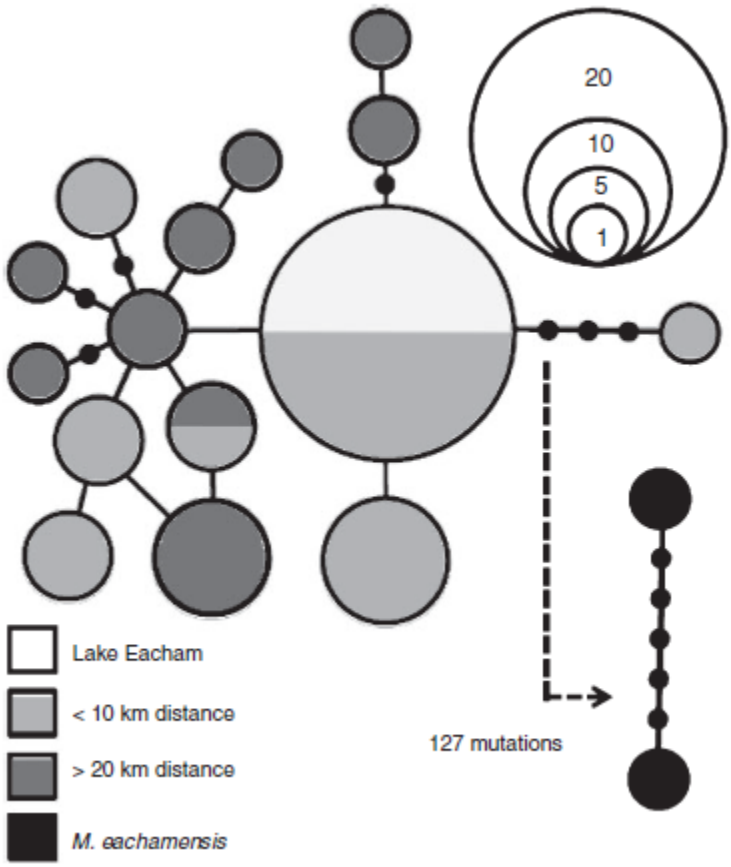


DNA was extracted from fin clips using the proteinase K/salting-out method (Sunnucks and Hales 1996), with a 3-h proteinase K digestion at 55°C. The D-loop (control region) of mitochondrial DNA was amplified in a PCR using 0.2 µM final concentration of universal primers CRAf and CREr (CCTCTA ACTCCCAAAGCTAG and CCTGAAGTAGGAACCAGATG respectively: Lee *et al*. 1995). PCRs were performed in 50-mL volumes using *GoTaq* white (Promega) in the buffer supplied with the enzyme, with the addition of RNAse A to a final concentration of 10 mg mL^{-1}. The temperature cycles for the PCR were: 94°C for 3 min; 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, 35 cycles; 72°C for 5 min. The success of amplification was assessed by electrophoresis on 2% agarose gels. PCR products were purified using ExoSap-IT (USB) digestion according to the manufacturer's instructions. Purified PCR products were sequenced using BigDye ver. 3.1 chemistry (Applied Biosystems) with the reverse primer (CREr).

Sequences were determined on an ABI 3130*xl* Genetic Analyzer. Sequencing reactions, unincorporated dye-terminator removal and capillary electrophoresis were carried out at the Macquarie University DNA Analysis Facility. Sequence size was 384 base pairs in length. The DNA sequences were manually edited

and were entered into the Biomanager database (http://biomanager.info). Sequences were aligned using the ClustalW algorithm (Thompson *et al.* 1994). Phylogenetic relationships were assessed with a maximum-likelihood phylogram using the Tamura–Nei model of evolution and phylogenetic trees generated using MEGA ver. 5 (Tamura *et al.* 2011). All sequences were submitted to GenBank (accession nos: KC201362–426).

Fig. 4. Haplotype network for the fish sampled. The size of the circles refers to the number of sequences that belong to that haplotype and the small black dots refer to missing haplotypes. Haplotypes are shaded relative to the distance of the collection location from Lake Eacham. Samples taken from Lake Eacham are shaded in light grey. Note that only two samples of *M. eachamensis* are shown to illustrate the great differentiation between the two species.



Results

Our genetic data show that the current rainbowfish occupying Lake Eacham is clearly *M. splendida* as it clusters with several other populations belonging to this lineage (Fig. 3). Examination of the most closely related populations of *M. splendida* in the phylogenetic tree revealed that they all occurred close to Lake Eacham. Wright Creek is 0.5 km to the north while Malanda Creek is 2.5 km to the south. The latter flows into the North Johnstone River, where another two close matches were found in the main stream. Interestingly, a few fish from Gadgarra Creek also clustered with this group. Davies Creek fish all cluster with the rest of the *M. splendida* clade.

As further validation of these results, we noted that the wild population of *M. eachamensis* in Dirran Creek was closely related to the captive population originally collected from Lake Eacham during the early 1980s.

The haplotype network for *M. splendida* clearly illustrates a star cluster pattern typical of invasion and recent expansion (Fig. 4). Fifteen haplotypes were revealed from 63 sequences and a large number of fish sampled from close by shared the same haplotype as the fish presently in Lake Eacham.

Discussion

Our results clearly demonstrate that the colonisation of Lake Eacham by novelfish species is on-going despite the fact that it is a World Heritage National Park. The latest arrival in the lake has been positively identified as *M. splendida* and is most likely derived from source populations in the immediate vicinity. Wright Creek, the closest match, with several individuals sharing the same haplotype, is just 500m north of Lake Eacham. It is difficult to ascertain with any degree of certainty how these novel fish species colonised the lake but it does highlight the continuing problem of translocation in the region (Pusey *et al*. 2004).

Lintermans (2004) identified 12 possible means by which freshwater fish are dispersed by human activities in Australia, of which only two are relevant to the present context: discarding of aquarium fish and deliberate illegal stocking. Most researchers assumed that the previous simultaneous introduction of four species into the lake in the late 1980s pointed to a deliberate unauthorised translocation or a release from an aquarium (Barlow *et al*. 1987; Brown and Warburton 1997). None of the introduced species are especially targeted by anglers, so it unlikely that the release event was related to angling. A deliberate release is also possible for *M. splendida*, but this species is not commonly maintained in the aquarium hobby. Unlike the other species, however, *M. splendida* is capable of colonising new locations across land barriers without human assistance. Rainbowfish produce eggs with sticky, web-like strands that enable them to adhere to aquatic vegetation in fast-flowing water (Allen and Cross 1982). These strands could be caught on the feet or feathers of aquatic birds, or transported aloft with aquatic weeds via the same vector (Unmack 2001). Similarly, they could be accidently transferred by human bathers or on aquatic leisure craft such as kayaks. The latter is particularly likely given that Lake Eacham is a popular tourist destination. While the eggs may not be able to stay out of water for long, it would take only a few minutes for a bird to move from the suspected source streams to Lake Eacham. It is highly likely that the eggs would still be viable after such a brief trip. Further research needs to examine the viability of eggs following exposure to air.

The molecular methods utilised here allowed us to unequivocally identify the current species of rainbowfish in Lake Eacham as *M. splendida.* Confirmation that the molecular methodology is sound is shown by the close alignment between the captive population of *M. eachamensis*, which has now been in captivity for over 30 years, and the well known wild population in Dirran Creek. These results are also validated by those of earlier studies using a combination of molecular markers, including the control region sequence and multivariate morphometrics (Zhu *et al*. 1998; McGuigan *et al*. 2000).

It is interesting to note that the Lake Barine system has also been invaded by *M. splendida*, as indicated by its presence in Gadgarra Creek. Lake Barine, like Lake Eacham, is an isolated crater-lake system with only a single stream (Toohey Creek) running out of it during extremely wet seasons. Whilewe have not sampled fish in the lake itself, our sequence analysis showed that the fish in the Gadgarra Creek, a small tributary of Toohey Creek, are all relatively closely clustered together, suggesting that they may be genetically isolated from the surrounding populations. Nevertheless, they are clearly closely related to the fish currently in Lake Eacham and likely share a recent common ancestor despite the fact that they are in

the headwaters of the Mulgrave River system. We also note that the unusual morphotype in Davies Creek is another variant of *M. splendida.*

To conclude, the freshwater ichthyofauna of the Atherton Tablelands region is highly dynamic and this is facilitated by human activities such as translocation, angling and stocking practices. The new occurrences of a range of Australian native fishes presents a significant threat to several unique, threatened rainbowfish species in the region, including *M. eachamensis* and *M. utcheensis*. Further surveys of the area coupled with molecular analyses are required to characterise diversity and identify populations for specific conservation management action.

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