Who's your mama? Riverine hybridisation of threatened freshwater Trout Cod and Murray Cod.

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1.1 Abstract

Rates of hybridization and introgression are increasing dramatically worldwide because of translocations, restocking of organisms and habitat modifications (Allendorf et al., 2001) thus determining whether hybridization is beneficial or detrimental for the species involved is commensurately important for conservation. Restocking programs are sometimes criticized because of the genetic consequences of hatchery-bred fish breeding with wild populations. These concerns are important to conservation restocking programs, including Percichthyidae.

Two of the better known Australian Percichthyidae are the Murray Cod, *Maccullochella peelii* (Mitchell, 1938) and Trout Cod, *Maccullochella macquariensis* (Cuvier, 1829) which were formerly widespread over the Murray Darling Basin. In much of the Murrumbidgee River Trout Cod and Murray Cod were sympatric until the late 1970s when Trout Cod were extirpated. Here we use genetic single nucleotide polymorphism (SNP) data to examine hybridization and introgression between Murray Cod and Trout Cod in the upper Murrumbidgee River and consider implications for restocking programs.

For the first time, we have confirmed restocked riverine Trout Cod as reproducing in the wild. We detected hybrid Trout Cod-Murray Cod in the Upper Murrumbidgee, recording the first hybrid larvae in the wild. Although hybrid larvae, juveniles and adults have been recorded in hatcheries and impoundments, and hybrid adults have been recorded in rivers previously (Douglas, Gooley & Ingram, 1994; Douglas et al., 1995), this is the first time fertile F1 have been recorded in the wild. The F1 backcrosses with Murray cod have also been found to be fertile. All backcrosses noted were with pure Murray Cod. Such introgression has not been

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recorded previously in these two species, and the imbalance in hybridization direction may have important implications for restocking programs.

1.2 Introduction

Hybridization and introgression play important roles in speciation and evolution (Dowling & Secor, 1997). Within breeding programs hybridization is sometimes encouraged to produce 'hybrid vigour'. Hybridization and introgression in the wild occurs in more than 10% of animal species, and is most common in more recently diverged species (Mallet, 2005). Rates of hybridization and introgression are increasing dramatically worldwide because of translocations, restocking of organisms and habitat modifications (Allendorf et al., 2001) thus determining whether hybridization is beneficial or detrimental for the species involved is commensurately important for conservation.

Introgression is now seen as an important phenomenon in many taxa, contributing to adaptation and speciation in plants, fish, and insects (Baack & Rieseberg, 2007). While introgression initially increases genetic diversity it can eventually reduce genetic diversity of one or both of the parent species, particularly if they are not naturally sympatric and one is introduced. Furthermore, the synergistic effects of multiple extinction drivers, including genetic consequences, are only starting to be understood (Brook, Sodhi & Bradshaw, 2008). The genetic consequences of introgression are of increasing interest to conservation biologists as many species are on an irreversible path to extinction (the extinction vortex) which can be initiated without any obvious signs (Blomqvist et al., 2010; Fagan et al., 2005).

Restocking programs are questioned and often criticized because of potential genetic consequences of hatchery-bred fish breeding with wild populations, and such concerns are of critical importance to conservation restocking programs and in particular the potential changes to genetic diversity. For instance Allendorf et al., (2001) discuss introductions as a particular genetic problem for native trout species in the USA and Nock et al., (2011) describe adverse effects of stocking on an endangered Australian percichthyid, including loss of heterozygosity and allelic richness.

Two of the better known Australian Percichthyidae are Murray Cod *(Maccullochella peelii)* and Trout Cod *(Maccullochella macquariensis)*. These iconic Australian species (only formally recognized as separate species in 1972) are highly sought after by anglers and previously overfished by commercial fishing until populations became so low the industry collapsed. The Murray Cod is Australia's largest freshwater fish and can grow to as large as 180cm in length. Both Trout Cod and Murray Cod are large bodied species reaching maximum recorded weights of 16 and 113.6kg respectively, with fecundity ranging from 1 200-11 000 and 9 000-120 000 annual eggs in Trout Cod and Murray Cod respectively. Both are limited to parts of the Murray Darling Basin (MDB) where the Trout Cod is endangered, and the Murray Cod is listed as vulnerable under the Australian Environment Protection and Biodiversity Conservation Act (1999) (Department of Environment, 2016).

Trout Cod was originally widely distributed in the Murray-Darling Basin (Lintermans 2007), but by the 1980s had been reduced to just a single natural wild population and two translocated populations (Koehn et al., 2013). Trout Cod was one of the first Australian freshwater species identified and listed as threatened, with recovery actions now spanning more than 25 years

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(Koehn et al., 2013). In much of the Murrumbidgee River Trout Cod and Murray Cod were sympatric until the late 1970s when Trout Cod were extirpated (Lintermans, Kukolic & Rutzou, 1988).

A Trout Cod conservation restocking program in the Upper Murrumbidgee released 326 200 Trout Cod fingerlings between 1988 and 2009 on 35 occasions across 8 sites (Koehn et al., 2013). However, low numbers were stocked prior to 1992 (a total of 11 000 individuals across 2 releases) with the majority of fish (a total of 205 000 across 16 releases) stocked between 2004 and 2007, with stocking ceasing in 2008. This means 24 years have elapsed since the first stocking, but only 8-10 years since the majority of the stocking occurred. Trout Cod become sexually mature after 3-5 years (Lintermans, 2007), producing noticeably more recruits after 6 years of age (Lyon, Todd & Nicol, 2012). This means that there has been opportunity for at least 4 generations to occur by 2011 – the beginning of this study.

First generation interspecific hybridization between Murray Cod and Trout Cod has been recorded in impoundments (Harris & Dixon, 1986), hatcheries (Ho et al., 2008) and wild sympatric populations (Douglas et al., 1995). Fisherman too have recorded catching hybrid fish (based on phenotypic characteristics) although not all of these anecdotal records are reliable (Cleaver, 2015) as misidentification of these two cod species was only resolved in 1972 with the formal re-description of Trout Cod (Berra & Weatherley, 1972). In any case morphological distinction between species remains difficult.

A recent review of the national Trout Cod recovery program found that there were encouraging signs of recovery for this species, but genetic considerations were not widely canvased in the review (Koehn et al. 2013). Hybridization was identified as a major concern for one population of Trout Cod translocated to Cataract Dam, a water reservoir (Harris & Dixon, 1986; Douglas, Gooley & Ingram, 1994). This then resulted in initial stocking site selection criteria in the program excluding sites where Murray Cod were known to be present (Douglas, Gooley & Ingram, 1994, p.37). However, minimal detection of hybrids between Murray Cod and Trout Cod in riverine environments (*e.g.*, Douglas et al., 1995) has meant that site selection criteria has been relaxed in recent years and stocking now regularly occurs where Murray Cod are present. Here we use genetic single nucleotide polymorphism (SNP) data to examine hybridization and introgression between Murray Cod and Trout Cod in the upper Murrumbidgee River and consider the implications for restocking programs.

1.3 Methods

1.3.1 Animal Material

We examined 251 *Maccullochella* larvae which were collected in 2011, 2012, and 2013 from six sites in Murrumbidgee River in the Australian Capital Territory (ACT) (Figure 1, Table 1) using standard larval driftnets with 500um mesh. For reference purposes two Trout Cod controls were included, one hatchery sourced larvae obtained from NSW DPI Narrandera, the other an adult fish, from a stocked impoundment (Bendora Reservoir) in the ACT. Murray Cod control samples were obtained from the upper Murrumbidgee River. Fish were collected under ACT Government licences LT2011516, LT2012590 and LT20133653. Research was conducted under approvals

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CEAE 11-15 and CEAE 13-17 from the University of Canberra Committee for Ethics in Animal Experimentation.

Adult *Maccullochella* were identified based on morphological features after (Lintermans, 2007). Larvae and tissue samples were preserved in 95% ethanol at room temperature until 2014 after which samples were stored at -20° C. Larval fish were aged using otolith daily increments (Humphries, 2005).

1.3.2 Genomic DNA Extraction and Sequencing

Total DNA of different genotypes was isolated from whole larval heads, or for adults, from approximately 0.25 cm^3 of caudal fin or muscle tissue. The DNA extraction protocol is detailed in Couch and Young, (2016), and is based on a turtle DNA extraction protocol (FitzSimmons, Moritz & Moore, 1995). Each tissue sample was placed into a 1.5 mL ep tube with 300 µL extraction buffer, 30 µL SDS (20%) and 15 µL Prot K (20 mg/mL). It was incubated at 55 °C overnight, while rotating at 14rpm.

1.5 μL RNase A (4 mg/mL) was added and the mix was incubated for 30 minutes at 37°C. The protein was precipitated by spinning at 13 000 rpm for one minute at room temperature. After aspirating the lysate while avoiding the cell debris pellet, the lysate was transferred to new tube for the second stage of protein precipitation. 150 μL of ammonium acetate was added and mixed. It was spun at 13 000 rpm for 30 minutes at room temperature and the lysate was transferred to a 1.5 mL tube, again avoiding any remaining cell debris/SDS pellet.

To precipitate the DNA 600 μL of isopropanol was added and mixed. The mixture was cooled at -80 °C for 5 minutes then at 4 °C for 30 minutes. It was spun for 20 minutes at 13 000 rpm at 4°C. The isopropanol was decanted, leaving the DNA pellet behind. 600 μL of cold 70% ethanol was added to each tube, and gently mixed. It was spun at 13 000 rpm 4 °C for 10 minutes. The ethanol was aspirated and the remainder air dried while covered for 15 minutes. The DNA pellet was re-suspended in 40µL filtered water.

A sub-sample of the extract was run on a 0.8% Agarose gel electrophoresis for one hour at 90v with Hyperladder 1 kb+ size standard to check the extraction quality.

Sequencing was done using DArT PL DArTseq™ which represents a combination of DArT complexity reduction methods and next generation sequencing platforms (Kilian et al., 2012; Courtois et al., 2013; Raman et al., 2014; Cruz, Kilian & Dierig, 2013). DArTseq™ represents a new implementation of sequencing of complexity reduced representations (Altshuler et al., 2000) and more recent applications of this concept use next generation sequencing platforms (Baird et al., 2008; Elshire et al., 2011). The technology is optimized for each organism and application by selecting the most appropriate complexity reduction method (both the size of the representation and the fraction of a genome selected for assays). Four methods of complexity reduction were tested in *Maccullochella* (data not presented) and the PstI-SphI method was selected.

DNA samples were processed in digestion/ligation reactions principally as per (Kilian et al., 2012) but replacing a single PstI-compatible adaptor with two different adaptors corresponding to two different restriction enzyme overhangs. The PstI-compatible adapter was designed to include Illumina flowcell attachment sequence, sequencing primer sequence and “staggered”,

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varying length barcode region, similar to the sequence reported by Elshire et al., (2011). The reverse adapter contained flowcell attachment region and SphI-compatible overhang sequence.

Only "mixed fragments" (PstI-SphI) were effectively amplified in 30 rounds of polymerase chain reaction (PCR). Amplifications consisted of an initial denaturation step of 94 °C for one minute, followed by 30 cycles of PCR with the following temperature profile: denaturation at 94 °C for 20 seconds, annealing at 58 °C for 30 seconds, and extension at 72 °C for 45 seconds, with an additional final extension at 72 °C for seven minutes.

After PCR equimolar amounts of amplification products from each sample of the 96-well microtiter plate were bulked and applied to c-Bot (Illumina) bridge PCR followed by sequencing on Illumina Hiseq2500. The sequencing (single read) was run for 77 cycles.

Sequences generated from each lane were processed using proprietary DArT analytical pipelines. In the primary pipeline the fastq files were first processed to filter poor quality sequences, applying more stringent selection criteria to the barcode region compared to the rest of the sequence. In that way, the assignments of the sequences to specific samples carried in the "barcode split" step were very reliable. Approximately 2 000 000 sequences per barcode/sample were identified and used in marker calling. Finally, identical sequences were collapsed into "fastqcoll files". The fastqcoll files were "groomed" using DArT PL's proprietary algorithm which corrects a low quality base from a singleton tag into a correct base using collapsed tags with multiple members as a template. The "groomed" fastqcoll files were used in the secondary pipeline for DArT PL's proprietary SNP and SilicoDArT (presence/absence of restriction fragments in representation) calling algorithms (DArTsoft14). For SNP calling, all tags from all libraries included in theDArTsoft14 analysis are clustered using DArT PL's C++ algorithm at the threshold distance of 3, followed by parsing of the clusters into separate SNP loci using a range of technical parameters, especially the balance of read counts for the allelic pairs. Additional selection criteria were added to the algorithm based on analysis of approximately 1000 controlled cross populations. Testing a range of tag count parameters facilitated selection of true allelic variants from paralogous sequences, In addition multiple samples were processed from DNA to allelic calls as technical replicates and scoring consistency was used as the main selection criteria for high quality/low error rate markers. Calling quality was assured by high average read depth per locus (>60).

1.3.3 Marker Scoring and Statistical Analysis

DArTsoft (Diversity Arrays Technology, Building 3, University of Canberra, Australia), a software package developed by DArT PL (http://www.diversityarrays.com/software.html), was used to both identify and score the markers that were polymorphic. The results of polymorphic scoring are presented in Microsoft™ Excel in binary format where "1" denotes the presence and "0" the absence of a marker in genomic representation of a sample.

1.3.4 Mitochondrial DNA sequencing

We amplified a portion of the cytochrome *b* (cyt*b*) gene *via* PCR. Samples were amplified *via* nested PCR due to the low quantity of DNA present. In the first reaction we used the primers Glu18 TAACCAGGACTAATGRCTTGAA and hd.macc.632

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GATTTTATCTGAATCTGAGTTTA followed by Glu31 TGRCTTGAAAAACCACCGTTGT and hd.Mac.538 GGGAAGAGGAAGTGGAAGGC in the second reaction.

The first reaction used 1 μL of template DNA, 0.5 μL of each primer, 5 μL of Bioline MyTaq Red Mix and 3 μL of water 9.5 μL in total. Amplification parameters were as follows: 94°C for 2 min followed by 35 cycles of 94°C for 20 s, 48°C for 20 s, and 72°C for 60 s, and 72°C for 7 min. This first PCR reaction was then diluted to 1:49 and 1 μL from that was used in the second 25 μL reaction with the same PCR conditions listed above. We examined PCR products on a 2% agarose gel using SYBR safe DNA gel stain (Invitrogen, Eugene, OR, USA). Sequences were obtained *via* cycle sequencing with Big Dye 3.0 dye terminator ready reaction kits using 1/16th reaction size (Applied Biosystems, Foster City, CA, USA).

Sequencing reactions were run with an annealing temperature of 52°C and following the manufacturer's protocol. Sequenced products were purified using sephadex columns. Sequences were obtained using an Applied Biosystems 3730 XL automated sequencer at the Brigham Young University DNA Sequencing Center. All sequences obtained in this study were deposited in GenBank, accession numbers KX355263 - KX355274.

1.3.5 Analysis of Mitochondrial DNA sequence data

Sequences were edited using Chromas Lite 2.0 (Technelysium) and imported into BioEdit 7.0.5.2 (Hall, 1999). Sequences coding for amino acids were aligned by eye and checked *via* amino acid coding in MEGA 6.06 (Tamura et al., 2013) to test for unexpected frame shift errors or stop codons.

1.3.6 SNP Analyses

Of the 21_076 alleles, the number of unique *Maccullochella* SNPs analysed in the DaRT sequences was 12_299. The polymorphisms selected for genotyping were all SNPs. The length of the DNA fragments for each SNP in this sequencing was 69 base pairs.

Variation in the genome wide SNP data of the studied *Maccullochella* genotypes was analysed using Discriminant Analysis of Principal Components (DAPC) using sequential K-means and model selection to infer genetic clusters (Jombart, Devillard & Balloux, 2010) using R package 'adegenet' (Jombart, 2008). Summary and comparative statistics were created in R (R Development Core Team & R Core Team, 2013) and Tableau (Tableau, 2013). Maps were created using ARCGIS (ESRI, 2013) and Tableau.

The hybrid status of larvae was assessed, initially by K-means clustering, and then with NewHybrids 1.1 (Anderson & Thompson, 2002). NewHybrids computes, by Markov chain Monte Carlo, the posterior probability to which of each of the distinct parental or hybrid classes, i.e., F1, F2, or backcrosses, an individual belongs. The entire SNP matrix was too large to analyse in NewHybrids, so we selected 200 SNPs with the highest polymorphic information content (PIC) from the 6 364 loci with call rate above 0.98 (3_061 loci) and with a reproducibility score of 1 (2_722 loci). Independent runs were initiated from different starting points and the MCMC chain was allowed to run until the log likelihood values reached stationarity and posterior probabilities of assignment to a class did not vary. Known Trout Cod and Murray Cod

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samples were nominated *a priori* as parental taxa as they are the only two *Maccullochella* species found in the upper Murrumbidgee River within the Murray Darling Basin.

1.4 Results

1.4.1 *Maccullochella* Relationships and Hybridization

The majority of larvae are Murray Cod, with two known Trout Cod controls clearly separated in the PCA plot, with F1 hybrids and backcross hybrids being placed intermediate between the two species *(Figure 2)*. Two Trout Cod controls were included, one hatchery sourced larvae from the NSW Department of Primary Industries Hatchery at Narrandera, NSW, the other an adult fish, from a stocked impoundment, Bendora Reservoir, in the ACT. The percentage of hybrid larvae varied from 2.1-6.1 % in each year.

1.4.2 Hybrid and Control Larvae Species Assignments

To confirm hybridization and identify the directionality of that hybridization, fragments of mitochondrial DNA were sequenced from two Trout Cod controls, two Murray Cod controls and each of the F1 and F1 backcross hybrids detected using DArT sequencing. Mitochondrial sequencing indicates that six of the eight hybrid larvae had a Trout Cod as a female parent and female grandparent, while one F1 larva (fish #262) and one backcross hybrid (fish #102) had a female Murray Cod parent (Table 2).

The hybrids in Table 2 represent the product of 13 known matings; eight first generation crosses, four second generation crosses and one third generation cross (fish #106). Of these 13, 11 involved a female Trout Cod. This is a statistically significant departure from the 50% expected if matings were random, with a chi squared value of $\chi^2 = 6.23$ (df=1, p= 0.013)

1.4.3 Location and Temporal Aspects of Hybrid Larvae

Hybrid larvae were detected at four of six sites sampled; Tharwa, Lanyon, Murramore and Nerreman (Figure 1). There were three hybrid larvae detected in the 138 larvae caught and sequenced in 2013 (2.17%). This included two F1 hybrids and one backcross hybrid. There were three hybrids sampled of 49 larvae sampled in the previous year 2012 (6.12%) This included one F1 hybrid and two backcross hybrids. Two backcross hybrids were detected in the 64 larvae sampled in 2011 (3.13%).

There was no significant difference between the day of the year on which hybrid and non-hybrid larvae were sampled (t = -0.162, df = 10.415, p-value = 0.874). There was no significant difference between the age of hybrid and non-hybrid larvae sampled (t = -0.053, df = 7.12, p-value = 0.959).

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1.5 Discussion

1.5.1 Hybridization and Genetic Effects

This is the first study to confidently detect hybrid Trout Cod-Murray Cod in the Upper Murrumbidgee River, and the first record of hybrid larvae in the wild. Although hybrid larvae, juveniles and adults have been recorded in hatcheries and impoundments, and hybrid adults have been recorded in rivers previously (Douglas, Gooley & Ingram, 1994; Douglas et al., 1995), this is the first time fertile first generation (F1) hybrids have been recorded in the wild as evidenced by the finding of F1 x Murray Cod backcrosses (F1xMC). These F1xMC backcrosses have also been shown to be fertile as there is one example of an F1MC backcross again backcrossing with a Murray Cod (fish #106). All backcrosses were with pure Murray Cod. Such introgression has not been recorded previously in these two species.

This is also the first time restocked riverine Trout Cod have been confirmed as reproducing in the wild, but no pure trout cod larvae were detected. Nor were any of the backcrosses with a pure Trout Cod. While successful breeding of the first born generation is used by some as a measure of success (Sarrazin & Barbault, 1996) such breeding, unless genetically sound, and sustained, is a measure of re-introduction success rather than a more important indicator of recovery success.

There are reports that Trout Cod spawn earlier than Murray Cod (Cadwallader, 1977) and reports that there is contemporaneous, or at least an overlap in spawning period (Koehn & Harrington, 2006) between these two species. In this study sampling commenced 51 days, 42 days and 24 days (2011, 2012, 2013 respectively) before the first *Maccullochella* larvae were detected dispersing. This, and the finding that hybrids did not differ significantly from Murray Cod hatching suggests that it was not a temporal sampling issue that resulted in no pure Trout Cod larvae being detected.

Potential outcomes of hybridization between two species include:

- blurring of species boundaries and the emergence of new cryptic species
- emergence of a hybrid swarm through ongoing reproduction
- collapse into one species through interspecific gene flow (introgression)
- enhanced reproductive isolation
- increased genetic diversity with potentially adaptive benefit

Most of the literature points to reduced fitness in hybrid fish (see for example (Houde, Fraser & Hutchings, 2010). However, there are some important examples of hybrid vigour (heterosis) in a number of fish species. Salmonid heterosis for resistance to amoebic gill disease is one Australian commercial fish breeding example (Maynard et al., 2016). At the present time it is unclear what evolutionary outcome is most likely from hybridization observed in this study. The relative fitness of hybrid *Maccullochella* is unknown. It is possible that reduced fitness of larval hybrids means they rarely survive to adulthood, and so the implications of hybridization are minimal for the conservation of riverine populations. Alternatively, hybrid vigour may be evident, with hybrids demonstrating enhanced fitness. Longitudinal observations will be required to determine the outcome.

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(Seehausen, 2006) provides forewarning that homogenizing environments may cause the rapid loss of such species through a reversal of the speciation process. One clear example of two species becoming one in freshwater fishes is the lacustrine stickleback study undertaken by Taylor et al., (2006). Such an outcome in an endangered and threatened species such as Trout Cod and Murray Cod is highly undesirable.

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1.5.2 Dispersal and the Limited Male Hypothesis

The absence of pure Trout Cod larvae and the relatively high levels of hybrid larvae detected, given the limited number of Trout Cod expected to have matured following restocking, raises an important question as to why this hybridization is occurring. Given that male Trout Cod are more limited in abundance than male Murray Cod, one hypothesis is that this could result in a limited number of mature stocked female trout cod succumbing to a disproportionately high mating pressure from more numerous Murray Cod males, rather than locating scarcer Trout Cod males. Both cod species have a similar reproductive strategy and spawning season (Koehn & Harrington, 2006) (Lintermans, 2007) as demonstrated by hybridization in both lentic and lotic environments (Douglas, Gooley & Ingram, 1994; Douglas et al., 1995). Consequently reproductively ripe individuals of both species are likely to be present in the river at the same time. Mitochondrial sequencing of the hybrid larvae in this study supports this 'limited male' Trout Cod hypothesis, but not exclusively as the female parent of two hybrid larvae (fish #102 and #262) were found to be a Murray Cod. Collection and testing of a larger number of larvae will provide a better estimate of the bias towards Trout Cod as the female parent of hybrids.

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Dispersal of post-juvenile Trout Cod away from stocking sites has been previously postulated as one explanation for the low detectability of Trout Cod in subsequent monitoring programs (Ebner, Thiem & Lintermans, 2007) (Ebner et al., 2006; Ebner & Thiem, 2009). Such dispersal may also contribute to low density of adult fish, and subsequent increased pressure to mate with more abundant congeners. The 'limited male' hypothesis might also be exacerbated by skewed sex ratios resulting from restocking programs. At least one study has found deviation from the expected sex ratio where females dominated by 2.5 to 1. The same authors report previous unpublished findings of highly skewed sex ratios of up to nine males to each female. (Lyon, Todd & Nicol, 2012).

In the upper Murrumbidgee River Murray Cod had a limited distribution, with the species not recorded in reaches upstream of a barrier formed by Gigerline Gorge (Figure 1) when the Trout Cod stocking program commenced in 1988 (Lintermans, 2002). Murray Cod are known to undertake upstream spawning migrations (Koehn et al., 2009) but adult Trout Cod are less mobile, at least in lowland rivers, than Murray Cod (Koehn & Nicol, 2016). The major stocking site for Trout Cod (99,500 fish from 1996-2005) was immediately upstream of Gigerline Gorge and so the presence of a migration barrier may result in aggregations of reproductively ripe Murray Cod mixing with downstream displaced trout cod below the barrier, further enhancing the chance of hybridization. Although 99,500 Trout Cod fingerlings was a substantial stocking effort over a 10 year period, the relatively high fecundity of the species means that this stocking effort only represents what would be the naturally expected reproductive output of less than 20 individuals per year based on the egg and larvae mortality estimates of Todd, Nicol & Koehn, (2004). The majority of the hybrids were detected less than 10 km downstream of Gigerline Gorge (Figure 1), with this location having one of the last naturally occurring remnant

populations of Trout Cod prior to their extirpation (Berra, 1974; Lintermans, Kukolic & Rutzou, 1988).

1.5.3 Implications for Restocking

Potential implications of genetic effects resulting from restocking have been highlighted for some time, even when there was a greater paucity of data about the genetic structure of fish in the MDB (Phillips, 2003; Gillanders, Elsdon & Munro, 2006). The findings in this study are a specific case of genetic effects resulting from stocking programs. Rourke et al., (2010) have previously noted a range of genetic effects from stocking. The introgression observed in this study, although clearly resulting from a restocking program, cannot be meaningfully compared to the expected genetic effects of the two species coexisting naturally because, although they were sympatric before extirpation of Trout Cod in the upper Murrumbidgee in the 1970s, the relevant data does not exist. However, if the limited numbers of mature female Trout Cod resulting from stocking are under a disproportionately high mating pressure from Murray Cod males compared to when high number of both species naturally coexisted, then there is likely to be proportionately more hybridization than may have occurred previously. If so this is a genetic effect that should be given more attention and then considered when making conservation restocking decisions in these and other species.

Although hybridization is a natural process and is relatively more common in fishes than other vertebrates, the occurrence of hybridization and introgression poses some real challenges for threatened species recovery programs (Gese et al., 2015). Reintroductions of threatened fish are usually resource limited, and so the number of individuals available from captive breeding programs is often only equivalent to the reproductive output of a handful of wild spawnings. Consequently, when trying to establish wild populations in the presence of an abundant congener, mis-mating is highly possible. This is in contrast to genetic swamping when a large number of hatchery-bred fish are stocked over the top of a small remnant population, as has occurred with Eastern freshwater cod (Nock et al., 2011).

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The national reintroduction program for Trout Cod originally used several criteria for selecting stocking sites, including one criterion that stocking should not occur where Murray Cod was present (Douglas, Gooley & Ingram, 1994). This was in recognition of the possibility of hybridization (as previously demonstrated in Cataract Reservoir (Wajon, 1983) (Harris & Dixon, 1986) and was an important consideration when selecting Trout Cod stocking locations in the upper Murrumbidgee River, with all mainstem stockings prior to 2005 occurring upstream of Gigerline Gorge where Murray Cod were considered absent (Lintermans, 2002). In subsequent iterations of the stocking program, this criterion was discarded, and most stocking locations now have wild populations of Murray Cod present. However, from 2008- 2011 fishing clubs, with the assistance of NSW Fisheries instituted a stocking program in a number of tributaries in the reach upstream of Gigerline Gorge of more than 4000 Murray Cod for recreational purposes (Cooma-Monaro Express, 2015). So this reach now contains low abundances of both species, possibly leading to limited mating opportunities, and raising the potential for mis-mating.

The earliest introgression detected in this study is an F1 backcross x backcross larva (fish #106) which indicates at least three hybrid generations by 2011. This suggests the first F1 hybrid mating in this lineage took place between 1998 and 2002. Given restocking commenced in 1988

and increased after 1992, introgression in even deeper backcrosses is possible but more sampling would be required to identify evidence of this.

The national Trout Cod restocking program has been through a number of iterations and changes in approach, with stocking moving from releases of small numbers of fish (<1000) for one or two years to releases of tens of thousands of fish for 5-10 years (Lyon, Todd & Nicol, 2012; Koehn et al., 2013). The upper Murrumbidgee stocking program sits midway in this stocking approach and likely still suffered from insufficient fish being stocked over a concentrated temporal and spatial scale. Upstream of the Gigerline Gorge, stocking low numbers was probably not a major issue as Murray Cod were not present, and so hybridization and introgression could not occur there. However this is no longer the case, mature individuals of both species are now obviously present. If the upper Murrumbidgee Trout Cod restocking program is to be successful and minimize the chances of hybridization and introgression with Murray Cod, then stocking even greater numbers of Trout Cod maybe required.

1.5.4 Comment on Method

The benefit of using both nuclear genome SNPs with mitochondrial methods to detect and assign hybrid types is highlighted in this study. Hybrid larvae were detected with both methods, most by both techniques, but considered together they identified additional detail about hybrids, and increasing certainty compared with using one technique alone.

This work also highlighted the utility of DArTseqs, a technique which uses large numbers of short fragments, compared to traditional sequencing for phylogenetic work – particularly where the DNA samples are partially degraded.

1.6 Conclusion

Given the single annual spawning reproductive strategy of the Trout Cod, each hybridization event is a precious but wasted reproduction opportunity for this species, which is listed as endangered under the Australian Environmental Protection and Biodiversity Conservation Act (Department of Environment, 2016). The National Recovery Plan for the Trout Cod, *Maccullochella macquariensis* (Trout Cod Recovery Team, 2008) noted potential risks of hybridization but limited recommendation on the matter to '…caution should be exercised in stocking Murray Cod in the same waters.' The present study clearly demonstrates hybridization and introgression between these species. Surely even greater caution should be exercised when stocking Murray Cod into waters where a Trout Cod recovery program is extant but Murray Cod are not.

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Comment [Office25]: You might somewhere discuss about the case of individual "106" whom mitochondrial and NEWHYBRIDS (from SNP0 results diverged, please give any suggestion to explain such a discrepancy. Now it,'sbit hard to discuss about the result since you did not provide any information about the length of the MCMC and burnin used in NEWHYBRIDS.

Comment [Office26]: Not really, actually. I think the opposite: for me the use of DArTseq here is not highlighted since the main analysis that is conducted here is NEWHYBRIDS analysis using only a subset of 200 SNPs (because of the NEWHYBRIDS assumptions…). From my point of view a really good of the many markers provided by DArtseq would have been to conduct outliers test between the two species, see how are distributed the corresponding alleles in hybrids and use these results to discuss about hybrids fitness.

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