Genetic identification of fish

CBER Contract Report 109

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15 November 2010

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Contents

Figures 3

Executive summary 4

Introduction 5

Case study 1: Genetic identification of a Northland cyprinid carcass 6

Case study 2: Genetic identification of a catfish carcass collected from Lake Rotoiti 6

Case study 3: Genetic identification of larval fish 8

Aims 8

Methods 8

Case study 1: Genetic identification of a Northland cyprinid carcass 9

Case study 2: Genetic identification of a catfish carcass collected from Lake Rotoiti 9

Case study 3: Genetic identification of larval fish 9

Results 10

Case study 1: Genetic identification of a Northland cyprinid carcass 10

Case study 2: Genetic identification of a catfish carcass collected from Lake Rotoiti 10

Case study 3: Genetic identification of larval fish 11

Discussion 11

Case study 1: Genetic identification of a Northland cyprinid carcass 11

Case study 2: Genetic identification of a catfish carcass collected from Lake Rotoiti 12

Case study 3: Genetic identification of larval fish 12

Acknowledgements 13

References 14

Appendices 16

Figures

Figure 1. Cyprinid carcass from DoC, Northland. Left: as captured; Right: as received for analysis.....6

Figure 2. Carcass recovered from the shore of Lake Rotoiti in January 2009; length approximately 450-500 mm. (Photo by D. Atkinson). 7

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Executive summary

Our report shows that the straightforward extraction of DNA and amplification of appropriate genes can identify fish that are difficult to identify unequivocally from morphology alone. Three examples show that these techniques can be extended to identify fish from other regions or countries and could be useful for the surveillance of illegal or unregulated transfers of fish, and for identifying species from a conservation perspective.

Firstly, a headless cyprinid carcass from a small pond connected to the Waiotira Stream, (a tributary of the Manganui River) was identified as a goldfish *Carassius auratus* from a 580-nucleotide sequence of the mitochondrial COI gene. A BLAST search showed the sequence was identical to sequences from the same species in GenBank (accession numbers AB379915 - AB379921). The next closest matches had 99% similarity with a goldfish–koi carp hybrid (AY694420), 95% similarity with a Japanese crucian carp (*Carassius cuvieri*, Cyprinidae AB045144), 89% similarity to a red-tail tinfoil (*Barbonymus altus*, Cyprinidae, EF609294), but only 88% similarity to koi carp (*Cyprinus carpio*, Cyprinidae, FJ183807).
Secondly, a large, rotted catfish carcass (total length ~ 450-500 mm) was recovered from the shore of Lake Rotoiti in January 2009 and was positively identified as a brown bullhead catfish (*Ameiurus nebulosus*) from a 417-nucleotide sequence of mitochondrial cytochrome b gene. A BLAST search showed 99.2% similarity with brown bullhead catfish Genbank sequences (AY458889 and DQ275634). The large size of the specimen raised suspicions that the carcass was a channel catfish (*Ictalurus punctatus*).

Thirdly, larval fish from Lake Rotomanu, Taranaki, was identified as a rudd (*Scardinius erythrophthalmus*) from a 160-nucleotide sequence of the mitochondrial cytochrome b gene. This sequence was 99% similar to rudd strain Se1 (Genbank accession number AY509835) from the Mediterranean. As a further test of genetic larval identification, two larval fish from Oranga Lake (University of Waikato Hamilton campus) were conclusively identified as common bully *Gobiomorphus cotidianus*, and with 100% sequence similarity to specimens collected from Martins Stream, Waikato River.

Introduction

Biosurveillance and conservation management depends crucially on the correct identification of specimens (Hebert *et al.* 2003). However, the morphological identification of decomposed and larval specimens is often difficult. An alternative is "DNA barcoding", which uses genetic sequence data to identify specimens independently of morphology (Hebert *et al.* 2003). Barcoding uses sequence data from genes, particularly mitochondrial genes, to assign unknown specimens to reference DNA sequences obtained from specimens of known identity such as adult animals or freshly-obtained cadavers.

Mitochondria are intracellular organelles containing non-nuclear DNA and have become the preferred source of DNA for barcoding studies. Mitochondrial genes are comparatively easy to amplify because of the high number of mitochondria per cell (in contrast with the single nucleus found in most cells), the circular mitochondrial genome increases the stability of the DNA post-collection compared with nuclear DNA (Avise 1994), and the availability of universal primers will amplify genes from a wide range of taxa. A drawback of using mitochondrial genomes is that the almost universal maternal mode of mitochondrial inheritance excludes information on the paternal lineage; thus, if a specimen is a hybrid of two species, mitochondrial genes provide no information on the paternal genetic sequence.

In the last decade, genetic identifications have focused on using the mitochondrial cytochrome *c* oxidase subunit 1 (COI) gene as a global identification system for animals because several studies across a range of taxa have found that intraspecific variation in COI is very low, whereas variation among species, even closely related species, is relatively high (see (Hebert *et al.* 2003). Other genes are preferred for some animal groups (for example, the mitochondrial cytochrome oxidase b gene for fish, although the availability of fish COI sequences is increasing). Factors limiting the utility of genetic data for specimen identification include the availability of primers that will amplify a wide range of taxa, the amounts of intra and interspecific genetic variation and the availability of suitable reference material from unequivocally identified specimens. In this report we provide reaction conditions and primer sequences that amplified useful genes from fish and demonstrate the feasibility of using genetic data to identify fish specimens.

Case study 1: Genetic identification of a Northland cyprinid carcass

Goldfish, *Carassius auratus*, were first introduced to New Zealand in European colonial times and often since then (McDowall 2000). Goldfish resemble koi carp, *Cyprinus carpio*, but can be distinguished from koi carp by the absence of two pairs of small barbels near each corner of the mouth (McDowall 2000). Goldfish occur widely throughout New Zealand, although their precise distribution is poorly documented (McDowall 2000). By contrast, koi carp are found only in the North Island, especially in the lower Waikato River and intermittently in Northland, Taranaki and Hawkes Bay (McDowall 2000). Goldfish are considered to have few adverse effects on New Zealand freshwater ecosystems whereas carp are considered a noxious pest as they dig up the substrates of waterways, increasing turbidity (McDowall 2000) and increase the frequency of algal blooms through resuspension of deposited nutrients (Lougheed *et al.* 1998). Possession of koi carp is illegal without a permit (McDowall 2000).

We were asked to genetically identify a degraded fish carcass (Fig. 1) that was collected by Amy MacDonald (Department of Conservation, DoC) from a small pond connected to the Waiotira Stream, (a tributary of the Manganui River) (35.93981^{o}S, 174.18791^{o}E), Northland.

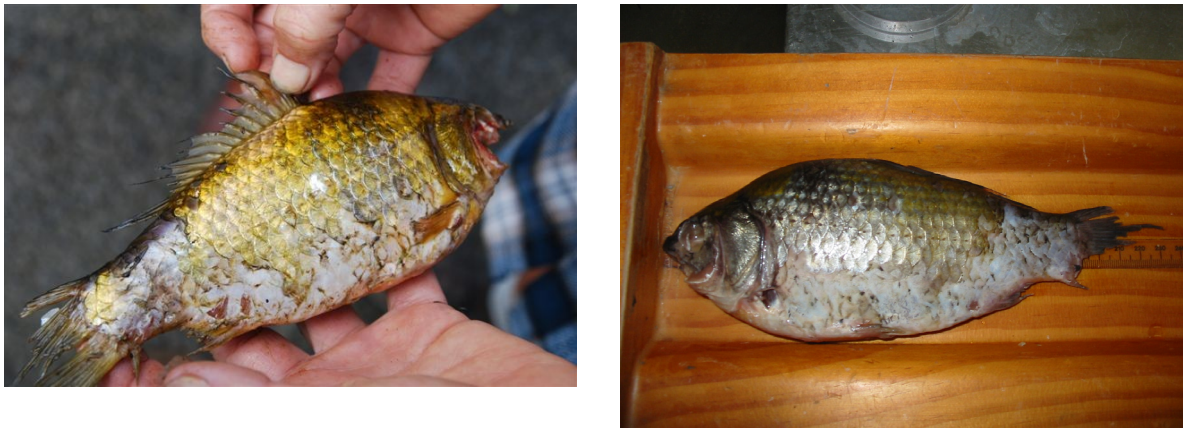

Figure 1. Cyprinid carcass from DoC, Northland. Left: as captured; Right: as received for analysis.

Case study 2: Genetic identification of a catfish carcass collected from Lake Rotoiti

Brown bullhead catfish, *Ameiurus nebulosus*, were introduced to New Zealand in 1877 from the United States (McDowall 1978). Brown bullheads are from the family Ictaluridae which comprises 49 described species. Ictaluridae all have four pairs of barbels around the mouth, an adipose fin, pelvic fins on the abdomen and lack scales (Page and Burr 2006). While

brown bullhead catfish in New Zealand are unlikely to be confused with any other naturalised species (McDowall 2000), very large individuals develop a somewhat atypical appearance (Fig. 2). The large fish in Fig. 2 was found dead on the shores of Okawa Bay, Lake Rotoiti, and subsequently buried by a member of the public. When Michel Deudal of DoC Turangi saw a photograph of the carcass he commented that it was, "a very strange looking catfish". After this, Johlene Kelly of the Department of Conservation was notified of the discovery of the catfish, and the catfish was exhumed for further examination. It was then decided that, if possible, genetic methods should be used to confirm the identity of the fish as that of a brown bullhead catfish.

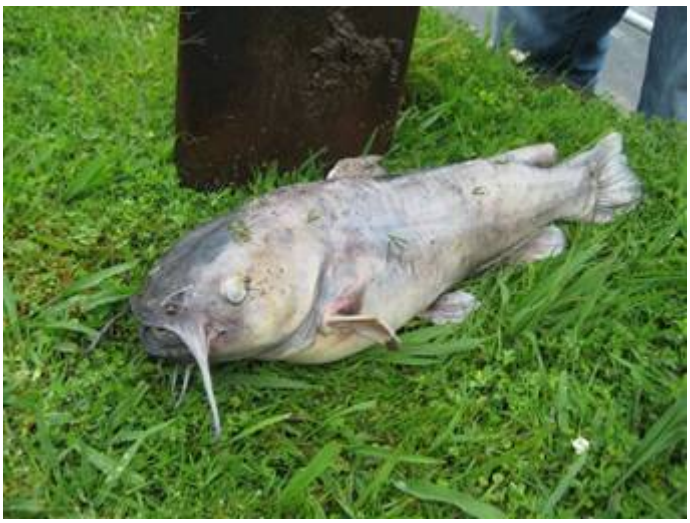

Figure 2. Carcass recovered from the shore of Lake Rotoiti in January 2009; length approximately 450-500 mm. (Photo by D. Atkinson).

The most likely alternative identity for the carcass is the channel catfish, *Ictalurus punctatus*, (Ictaluridae). Channel catfish were imported into New Zealand quarantine facilities in 1987 to assess the suitability of the species for aquaculture but the species was assessed as posing an unacceptable risk to the aquatic environment and the quarantine population was destroyed (Townsend and Winterbourn 1992). However, there have been instances in the past of individuals of aquaculture species escaping destruction orders following adverse environmental impact assessments or illegal imports to establish aquaculture populations in New Zealand (for example marron, *Cherax tenuimanus*, http://www.maf.govt.nz/mafnet/press/230305marron.htm).

Case study 3: Genetic identification of larval fish

The identification of juvenile animals, including larval fish, is sometimes difficult because diagnostic morphological characters often do not develop until an animal is sexually mature. Genetic data may help in these instances as DNA is unlikely to alter during an animal's lifetime and the identity of a juvenile specimen can be inferred if its genetic sequence matches sequences obtained from identifiable adult animals.

Larval fish (approximately 10 mm in length) were collected from Lake Rotomanu, New Plymouth, Taranaki on 27 November 2008 and Lake Oranga, University of Waikato campus, Hamilton. Based on the known distributions of fish, it was thought that the Lake Rotomanu larval fish would be perch, *Perca fluviatilis*, and we were unsure of the likely identity of the Lake Oranga larval fish as the larvae did not appear to match any fish species known to inhabit the lake.

Aims

Case study 1: Genetic identification of a Northland cyprinid carcass

Use genetic sequences to identify a partially degraded cyprinid fish carcass.

Case study 2: Genetic identification of a catfish carcass collected from Lake Rotoiti

Use genetic data to identify an ictalurid fish carcass found in Okawa Bay, Lake Rotoiti.

Case study 3: Genetic identification of larval fish

Identify larval fish collected from Lake Rotomanu, New Plymouth, Taranaki on 27 Nov 2008 and Lake Oranga, University of Waikato campus, Hamilton, using genetic sequences.

Methods

Genomic DNA was extracted from a small piece of muscle tissue using a DNeasy blood and tissue kit, (Qiagen, Mississauga, Canada) following the manufacturer's protocol for all three case studies. Individual polymerase chain reaction (PCR) reagents and conditions are listed under the heading for each case study. PCR products were purified using a Strataprep PCR purification kit (Stratagene, La Jolla, USA) and automated sequencing was carried out on an ABI 3130XL DNA sequencer. Sequences from all samples were compared to sequences from identified individuals using a Basic Local Alignment Search Tool (BLAST) available from

the Genbank website (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All sequences in this report are listed in Appendices 1 – 5 and archived in the BOLD database (www.barcodinglife.org, (Ratnasingham and Hebert 2007) under the accession numbers NZEF001 to NZEF005.

Case study 1: Genetic identification of a Northland cyprinid carcass

Cytochrome c oxidase subunit 1 gene (*COI*) amplification was carried out using 2.5 µL of 10X buffer with $MgCl_2$, 2.5 µL of dNTPs (2 mmol/L each), 1 µL of 10 µmol/L primers COIFWD (CTW TAT CTW GWA TTT GGT GC, R. Lobb unpub.) and COIREV (GTG TTG RTA DAG RAT YGG, R. Lobb unpub.), 1 µL of *Taq* polymerase (1 unit/µL), 15 µL of water and 2 µL of extracted DNA. Reaction conditions were 94°C for 2 minutes, followed by 40 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 30 s and a final extension at 72°C for 5 minutes.

Case study 2: Genetic identification of a catfish carcass collected from Lake Rotoiti

Seven months after burial, a tissue sample was taken from the decomposed carcass of the fish found dead at Okawa Bay. The tissue sample was preserved in 100% ethanol before being transferred to the University of Waikato. A polymerase chain reaction was carried out on 2 µL of extracted DNA to amplify a portion of the cytochrome oxidase b gene (*cytb*) using 2.5 µL of 10X buffer, 1uL of $MgCl_2$ (25 mmol/L), 2.5 µL of dNTPs (2 mmol/L each), 1 µL of 10 µmol/L primers cyb2 (CCC TCA GAA TGA TAT TTG TCC TCA, Kocher et al. 1989) and tgludg (TGA CTT GAA RAA CCA YCG TTG, Palumbi et al. 1991), 1 µL of *Taq* polymerase (1 unit/µL) and 15 µL of water. Reaction conditions were 94°C for 2 minutes, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 30 s and a final extension at 72°C for 5 minutes.

Case study 3: Genetic identification of larval fish

A portion of *cytb* from the two Lake Oranga larval fish was amplified using 2.5 µL of 10X buffer, 1 µL of $MgCl_2$ (25 mmol/L), 2.5 µL of dNTPs (2 mmol/L each), 1 nmol of primers cyb2 (CCC TCA GAA TGA TAT TTG TCC TCA, (Kocher *et al.* 1989) and tgludg (TGA CTT GAA RAA CCA YCG TTG, (Palumbi *et al.* 1991), 1 µL of *Taq* polymerase (1 unit/µL), 15 µL of water and 2 µL of extracted DNA. The solution was incubated at 94°C for 2

minutes, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 30 s and a final extension at 72°C for 5 minutes.

A portion of the cytochrome oxidase b gene (*cytb*) from the Lake Rotomanu larval fish specimens was amplified using 2.5 µL of 10X buffer, 1.5 µL of $MgCl_2$ (25mmol/L), 2.5 µL of dNTPs (2 mmol/L each), 1 nmol of primers cyb224f (CCA YAT CTG CCG AGA YGT, R. Lobb unpub.) and cyb351r (ACA TCT CGG CAG ATR TGG, R. Lobb unpub.), 1 µL of *Taq* polymerase (1 unit/µL), 5 µL of bovine serum albumin (0.2 mg/µL), 8.5 µL of water and 2 µL of extracted DNA. Reaction conditions were 94°C for 2 minutes, followed by 35 cycles of 94°C for 10 s, 55°C for 10 s, 72°C for 35 s and a final extension at 72°C for 5 minutes.

Results

Case study 1: Genetic identification of a Northland cyprinid carcass

The genetic sequence for a 580 nucleotide portion of *COI* was obtained from the tissue sample (Appendix 1). A BLAST search showed the sequence was identical to sequences for goldfish, *Carassius auratus* (GenBank accession numbers AB379915 - AB379921). The next closest matches were 99% similarity with a goldfish – koi carp hybrid (AY694420), 95% similarity with a Japanese crucian carp (*Carassius cuvieri*, Cyprinidae AB045144) and 89% similarity to a red-tail tinfoil (*Barbonymus altus*, Cyprinidae, EF609294). The sequence we obtained differed by 12% from koi carp (*Cyprinus carpio*, Cyprinidae, FJ183807).

Case study 2: Genetic identification of a catfish carcass collected from Lake Rotoiti

The genetic sequence for a 417 nucleotide portion of cytochrome b (Appendix 2)was obtained for the Lake Rotoiti fish carcass. A BLAST search found that the Lake Rotoiti fish carcass differed by 0.8% from brown bullhead catfish (Genbank sequences AY458889 and DQ275634). All three nucleotide differences were synonymous (i.e., the differences did not change the amino acid sequences translated from the nucleotide sequences). The next closest matches found from the BLAST search was to a black bullhead, *Ameiurus melas*, (DQ421893, 98% similarity, seven nucleotide differences) from Montana, USA and to a snail bullhead *Ameiurus brunneus* from Alabama, USA (AY184272, 94% similarity, 23 nucleotide differences).

By contrast, the sequences we obtained from the carcass differed from channel catfish by 17.8% (Genbank accession numbers AB045119 and EU490914) and the sequence differences resulted in nine differences in the amino acid sequence. On this basis, the carcass is almost certainly that of a brown bullhead catfish.

Case study 3: Genetic identification of larval fish

A 160 nucleotide portion of *cytb* (Appendix 3) was sequenced for the larval fish from Lake Rotomanu. A blast search of this sequence found 99% similarity (one nucleotide different) to rudd *Scardinius erythrophthalmus* strain Se1 (Genbank accession number AY509835) collected from the Mediterranean area (Ketmaier *et al.* 2004). By contrast, the sequences we obtained were only 75% similar to sequences for perch, *Perca fluviatilis*. These specimens are almost certainly juvenile rudd.

A 423 nucleotide portion of *cytb* was sequenced for the two larval fish from Lake Oranga (appendices 4 and 5). A BLAST search of the sequence found 100% similarity to a common bully *Gobiomorphus cotidianus* collected from Martins Stream, Waikato (Genbank accession number EF455574). The next closest matches were also to common bullies, at 99% similarity (differing by three of 377 nucleotides), (Genbank accession numbers EF455570, EF455571, FJ389460 – FJ839465, FJ389485, FJ389486) and 98% (EF455572, EF455573, EF455575). There were also close matches (97 – 98%) between the sequences we obtained and Cran's bully, *G. basalis* (EF455572, EF455573, EF455575 - EF455584) with differences of four to eight nucleotides in 377 nucleotides. Our sequences also matched closely with Tarndale bully, *G. alpinus* (EF455576) at 98% similarity (differing by four of 377 nucleotides).

Discussion

Case study 1: Genetic identification of a Northland cyprinid carcass

The 99% sequence match of our cyprinid carcass *COI* sequence with goldfish sequences in Genbank very strongly suggests that the specimen was a goldfish. The most likely alternative identity for the specimen based on the damaged morphology was that of a koi carp; the large differences between our specimen and koi carp sequences in Genbank do not support this alternative identity. Koi carp can hybridise with goldfish (Smith and McVeagh 2005) and as mitochondrial DNA is inherited maternally, our results cannot exclude that the specimen was

a goldfish – koi carp hybrid with a goldfish mother. However, from the size of the specimen and the coloration, it is most unlikely to be a hybrid. Additionally, hybrids are less than 2% of the population where goldfish and koi ranges overlap (Tempero 2004)

Case study 2: Genetic identification of a catfish carcass collected from Lake Rotoiti

Identifying the carcass as a brown bullhead catfish has shown that we can obtain enough DNA of suitable quality from even extremely degraded tissues to identify specimens. In this instance the carcass was identified as a brown bullhead catfish which have been recorded from Lake Rotoiti in the past so no further action was required. This is not the first alert concerning catfish in Lake Rotoiti (Blair and Hicks 2009). In 1995, moribund juvenile catfish were observed to fall out of a hollow-framed boat trailer after a boat launching. This boat had been parked on its trailer overnight in Lake Taupo at Motuapa immediately before the boat was launched in Lake Rotoiti. Despite extensive searching around the boat ramp in Lake Rotoiti within 7 days of the launching, no catfish were observed (Rowan Strickland, Cawthron Institute, pers. comm.). In 2003, a shallow excavation reminiscent of a catfish nest in a sandy beach was seen in Lake Rotoiti (John Clayton, NIWA, pers. comm.). Subsequent boat electrofishing in Jan 2004 found no catfish (Hicks and Ring 2004).

Had the carcass been of a new, illegally introduced species, unequivocal identification of the fish may have allowed eradication measures to be started earlier than had waterways managers had to wait to collect more specimens for a definitive identification. Several studies have shown a strong positive correlation between the number of animals introduced to a new habitat and the successful establishment of new populations (Cassey et al. 2004, Lockwood et al. 2005). Thus earlier identification of specimens increases the probability of successful eradication.

Case study 3: Genetic identification of larval fish

The identification of the larval fish from Lake Rotomanu as rudd has shown that we can extract sufficient DNA from very small, juvenile animals to identify a specimen independently of morphological characters. Early identification of larval fish may allow conservation managers to take action earlier to mitigate the adverse effects of illegal transfers of pest organisms. In some instances, early action may reduce the cost and severity of control measures required to control pest organisms.

The usefulness of promptly distinguishing between pest and lower risk species is exemplified by the difference between perch and rudd. Based on morphology and the known distribution of fish in the Taranaki region, the larvae collected from Lake Rotomanu were initially thought to be perch, *Perca fluviatilis*, Percidae. Perch were introduced to New Zealand probably as a game fish although as individual perch in New Zealand tend to be small, the fish have not become sought after by fishers (McDowall 2000). Perch are distributed patchily around New Zealand but they are relatively widespread in the North Island around Northland, Auckland, Waikato, Hawkes Bay, Manawatu and Taranaki (McDowall 2000). Perch are not regarded as a serious threat to indigenous fish but more study is needed (McDowall 2000). Rudd were introduced into New Zealand illegally from the 1960s onwards probably as a sports fish and are widely distributed from the Waikato River northwards, and are occasionally recorded from elsewhere, e.g., Taranaki (McDowall 2000). Rudd are classified as noxious fish because of their adverse effects on native freshwater invertebrates and macrophytes which ultimately affect fish such as trout that are valued for recreation (McDowall 2000). Despite this noxious classification, the distribution of rudd appears to be increasing probably due to illegal releases (McDowall 2000).

The larval fish from Lake Oranga were identified as common bully. The taxonomic status and the species boundaries of common, Cran's and Tarndale bullies have been vigorously debated (McDowall 1975, Smith et al. 2003, McDowall and Stevens 2007) in part because of limited genetic differences among the three species. Given the ranges of the three species (McDowall 2000) we can be confident that the larval fish we sequenced were either common or Cran's bullies, and were almost certainly common bullies. To identify larval fish from genetic sequences, we are dependent on freely accessible databases of genetic data such as Genbank. There are still some New Zealand species of fish for which there are no genetic sequences; filling these gaps would be a useful project.

Acknowledgements

We thank Amy Macdonald, The Department of Conservation, Northland for providing the sample and the research question for case study 1. Thanks to Ian Hogg and Nick Ling for thorough reviews of earlier drafts of this report. The work was funded by contract UOWX0505 from the New Zealand Foundation for Research, Science and Technology.

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Appendices

Appendix 1. Sequence (*COI*) obtained for the Northland cyprinid (goldfish *Carassius auratus*).

CCTCCTCATCCGAGCTGAaCTTAGTCAACCCGGATCACTTCTAGGTGATGACCAAATTTa
cAAtGTAATTGTTACCGCCCACGCCTTCGTAATAATTTTcTTTataGTAATGCCTATCCT
CATTGGAGGATTCGGAAACTGACTTGTACCccTGATAATCGGAGCCCCAGACATGGCATT
CCCACGAATAAACAATATAAGCTTCTGACTTCTTCCCCCATCATTCCTGTTACTACTAGC
TTCCTCTGGTGTTGAAGCCGGAGCTGGCACCGGATGGACAGTATACCCCCCTCTTGCAGG
AAACCTGGCCCACGCAGGAGCATCAGTAGACCTAACAATTTTCTCACTACATTTAGCAGG
TGTTTCATCAATCCTGGGGGCAATCAACTTCATTACTACAACCATTAACATAAAACCTCC
AGCCATTTCCCAATACCAAACACCCCTATTTGTTTGATCCGTACTTGTAACCGCCGTCCT
CCTTCTCCTATCACTACCTGTTCTAGCTGCCGGTATTACAATGCTTTTAACAGATCGAAA
TCTTAACACCACATTCTTTGATCCCGCAGGCGGGGGAGAC

Appendix 2. Sequence (*cytb*) obtained for the Lake Rotoiti carcass (brown bullhead catfish *Ameiurus nebulosus*).

TATTCAACTATGAkAACTATGGTCACCCGAAAAACCCACCCCCTCTTCAAAATTGCCAAC
AACGCACTAATCGACCTCCCCGCCCCATCTAACATCTCTGAATGATGAAACTTTGGTTCC
CTATTATTATTATGTCTTATGATACAAATCCTAACAGGACTATTTCTAGCCATACACTAC
ACATCAGACATCTCAACTGCTTTTTCATCCGTAGTCCACATCTGCCGGGACGTAAATTAT
GGATGACTCATCCGCAATCTACACGCCAACGGAGCCTCCTTCTTCTTCATTTGCATTTAC
CTCCACATTGGACGAGGCCTTTATTACGGCTCCTATCTTTATAAGGAAACCTGAAACATT
GGGGTGGTCCTTCTTCTATTAGTAATAATAACCGCATTTGTAGGATATGTCCTACCA

Appendix 3. Sequence (*cytb*) obtained for the Lake Rotomanu larval fish (rudd *Scardinius erythrophthalmus*).

CGCTTCCATATCTGCCGAGACGTTAACTACGGCTGACTTATCCGAAGCCTACATGCCAAC
GGAGCATCCTTCTTCTTCATCTGTCTTTATATACATATCGCACGGGGACTATATTATGGG
TCATACCTTTACAAAGAGACCTGAACCATCGGAAAAGAA

Appendix 4. Sequence (*cytb*) obtained for the Lake Oranga larval fish (common bully *Gobiomorphus cotidianus*).

TATTCAACTACAAAAACCTTAATGGCCCACCTACGAAAAACGCACCCCCTCCTCAAAATC
GCAAACGACGCCCTAGTAGACCTCCCCGCCCCATCCAATATCTCCGTGTGATGAAACTTC
GGATCCCTTCTAGGCCTTTGCCTAGGCGCTCAGCTTGTTACAGGAATTTTTCTTGCAATA
CACTATACAGCCGACATCGCAACAGCATTCTCCTCCGTCGCCCACATCTGCCGAGACGTC
AACTTTGGCTGACTCATCCGAAACATACACGCCAACGGCGCCTCTTTCTTTTTTATTTGC
CTTTATTCCCACATCGGGCGGGGCCTATACTATGGCTCCTATCTTTATAAAGAGACCTGA
ATAATCGGAGTAGTTCTTTTACTTCTAGTAATAATGACCGCGTTCGTTGGCTaCGTACTG
CCT

Appendix 5. Sequence (*cytb*) obtained for the Lake Oranga larval fish(common bully *Gobiomorphus cotidianus*).

TATTCAACTACAAAAACCTTAATGGCCCACCTACGAAAAACGCACCCCCTCCTCAAAATC
GCAAACGACGCCCTAGTAGACCTCCCCGCCCCATCCAATATCTCCGTGTGATGAAACTTC
GGATCCCTTCTAGGCCTTTGCCTAGGCGCTCAGCTTGTTACAGGAATTTTTCTTGCAATA
CACTATACAGCCGACATCGCAACAGCATTCTCCTCCGTCGCCCACATCTGCCGAGACGTC
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CCT