# Fish biosurveillance by genetic methods: a feasibility study

CBER Contract Report 90

by

Matthew A. Knox

Brendan J. Hicks

Jonathan C. Banks

and

Ian D. Hogg

Centre for Biodiversity and Ecology Research
Department of Biological Sciences
School of Science and Engineering
The University of Waikato
Private Bag 3105
Hamilton, New Zealand

26 May 2009

Email: b.hicks@waikato.ac.nz

THE UNIVERSITY OF WAIKATO
*Te Whare Wānanga o Waikato*

CBER
University of Waikato
Centre for Biodiversity and Ecology Research

## Table of contents



## List of tables

## List of Figures



## List of abbreviations

| | |
|---|---|
| *CO1* | Cytochrome *c* oxidase subunit 1 |
| DNA | Deoxyribonucleic acid |
| PCR | Polymerase chain reaction |
| RNA | ribonucleic acid |
| rRNA | ribosomal ribonucleic acid |
| TRFLP | Terminal Restriction Fragment Length Polymorphism |
| WM | White muscle |

*Reviewed by*:

Ray T. Cursons
University of Waikato

*Approved for release by*:

Nick Ling
University of Waikato

# Executive summary

We evaluated the possibility of isolating DNA from environmental samples to detect and identify invasive fish species based on variation in the sequences for mitochondrial gene regions. Goldfish *CO1* DNA was successfully amplified from the water in a tank containing goldfish. Sequencing results verified that the DNA extracted from water matched goldfish sequences obtained from DNA extracted from tissue from other sources.

We also evaluated the potential of a DNA-based system to detect and identify invasive fish species based on differences in the length of several gene regions. Sequences for the 12S and 16S rRNA genes, cytochrome *c* oxidase subunit 1 (*CO1*) gene and the d-loop region downloaded from GenBank suggested that sequences from these genes would contain enough variation to distinguish six pest fish species (koi carp, rudd, brown bullhead catfish, goldfish, perch and gambusia) introduced to New Zealand freshwaters. A 250-nucleotide region of the 16S rRNA gene was successfully amplified and sequenced from fish tissue, faecal material and fish mucus. While sequences from these regions could distinguish fish species, there was insufficient variation in length to identify each fish species. Future directions for this research are suggested.

# 1. Introduction

Introducing new species into habitats can have significant adverse effects on the environment (Vitousek et al. 1997). Freshwater ecosystems are no exception; the introduction of new species into freshwater habitats has had detrimental effects on physical properties of the environment such as water quality and on other species such as native freshwater fish

(McDowall 1990), and introduced novel pathogens and parasites (McDowall 2000, Townsend 2003, McDowall 2004). There is also concern that the relatively low diversity of New Zealand freshwater ecosystems may increase the vulnerability of New Zealand habitats to the adverse effects of introduced species (Townsend and Winterbourn 1992).

Twenty one species of introduced fish are present in New Zealand freshwaters (McDowall 2000). Due to interest in the control and eradication of invasive species from freshwater habitats (for example, Anonymous 2000), we investigated the possibility of using the polymerase chain reaction to amplify fish DNA from the water column to characterise and identify the fish species present in a water body. The DNA of the American bullfrog (*Rana catesbeiana*), an invasive bullfrog native to North America, has been extracted from European ponds with surface areas ranging from 1000 - 10 000 $m^2$ and used to infer the presence or absence of the bullfrog in ponds (Ficetola et al. 2008).

Isolation of DNA from individual species in environmental samples is challenging as the DNA is likely to be somewhat sparse and degraded by exposure to the environment, or overwhelmed by non-target DNA (Matsui et al. 2001). Because of these problems, mitochondrial DNA was selected as a target as there are several thousand copies per cell (in contrast with vertebrate nuclear DNA which has two copies per cell); the circular structure of most vertebrate mitochondrial DNA makes it resistant to environmental degradation; the mitochondrial genome is well characterised for many species resulting in the availability of "universal primers" and the maternal inheritance of mitochondria generally results in a single genome per organism allowing unambiguous sequences to be obtained (Avise 1994).

A second aim of this study was to identify regions of the fish mitochondria that differ in length among species with the aim of using the length of a selected sequence to identify the fish DNA present in a water or sediment sample. Extensive use has been made of variation in the length of hypervariable regions of bacterial genomes to identify species and to characterise bacterial communities (for example, Fisher and Triplett 1999, Cardinale et al. 2004, Hewson and Fuhrman 2004). This technique is known as ARISA (Automated ribosomal intergenic spacer analysis) and uses polymerase chain reaction (PCR) primers labelled with a fluorescent dye to measure the length of the sequences generated by PCR. Biological communities from habitats such as estuaries, lakes and faecal flora (Hewson and Fuhrman 2004, Banks et al. 2009, Fisher and Triplett 1999) have been characterised using ARISA. However, ARISA has not been applied to vertebrate communities.

The overall aims of this project were to investigate the feasibility of extracting DNA shed into the water column and lake sediments from invasive fish species. The fish DNA could then be used to identify the fish present in a lake and with a view to using the ARISA technique to assess the effectiveness of pest fish control and lake management strategies.

# 2. Methods

## *(a) Extraction of amplifiable fish DNA from tissue and water samples*

We investigated the feasibility of extracting DNA from water samples using a simple DNA extraction protocol. Aliquots (1 mL) of fish-tank water were centrifuged at 13 200 rpm for 10 minutes. This process was repeated up to five times. Pellets were then immediately subjected

to a chloroform isoamyl extraction and DNA was resuspended in 25 – 50 μL tris EDTA buffer.

DNA was also extracted from fish white muscle, faeces or mucus from 11 species of fish (Table 2) using a phenol: chloroform: isoamyl DNA extraction (Hillis et al. 1996) to provide comparative samples. A total of 86 samples from various sources were extracted (Tables 1 and 2).

Table 1. Fish species from which DNA was extracted.

| Common name | Species | Family |
|---|---|---|
| Catfish | *Ameiurus nebulosus* | Ameiuridae |
| Shortfin eel | *Anguilla australis* | Anguillidae |
| Goldfish | *Carassius auratus* | Cyprinidae |
| Koi carp | *Cyprinus carpio* | Cyprinidae |
| Rudd | *Scardinius erythrophthalmus* | Cyprinidae |
| Tench | *Tinca tinca* | Cyprinidae |
| Common bully | *Gobiomorphus cotidianus* | Eleotridae |
| Perch | *Perca fluviatilis* | Percidae |
| Gambusia | *Gambusia affinis* | Poeciliidae |
| Common smelt | *Retropinna retropinna* | Retropinnidae |
| Rainbow trout | *Oncorhynchus mykiss* | Salmonidae |

## 16S rRNA PCR

The fish-specific primers 16S Fish F: AGACCCTATGGAGCTTTAGAC and16S2R: CGCTGTTATCCCTATGGTAACT (Deagle et al. 2005) were used to amplify a 250

nucleotide portion of the 16S rRNA gene. Thermal cycling conditions were: 94°C for 10 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 45 s with a final step at 72°C for 2 min. Products were run on a 1.5% TBE agarose gel and successful reactions were purified using exosap (0.1 μL SAP (1 U/μL), 0.2 μL EXO (10 U/ μL) and 2.7 μL $H_2O$ added to 10 μL PCR product) and sequenced in both directions using Big Dye v.3 chemistry.

## Cytochrome *c* oxidase subunit I PCR

The fish - specific primers for *CO1*, FishF1-5' TCAACCAACCACAAAGACATTGGCAC3' and FishR1-5'TAGACTTCTGGGTGGCCAAAGAATCA3' (Ward et al. 2005), were used to amplify a 700 nucleotide portion of *CO1*. Thermal cycling conditions were: 95°C for 2 min followed by 35 cycles of 94°C for 30 s, 54°C for 30 s, 72°C for 60 s, with a final step at 72°C for 10 min. Products were run on a 1.5% TBE agarose gel and successful reactions were purified and sequenced as above.

**Table 2**. Source of extracted DNA and number of extractions. The species type for DNA extractions from water refer to the species in the tank prior to sampling.

| Species | DNA source | | | |
|---|---|---|---|---|
| | White muscle | Faeces | Mucus | Water |
| Catfish | 4 | - | 3 | - |
| Shortfin Eel | 3 | 1 | 4 | - |
| Goldfish | 4 | 5 | 3 | 5 |
| Koi carp | 6 | - | 2 | - |
| Rudd | 4 | 2 | 2 | - |
| Tench | 6 | 2 | - | - |
| Common bully | 5 | - | - | - |
| Perch | 4 | 2 | 2 | - |
| Gambusia | 3 | - | - | - |
| Common smelt | 2 | - | - | 4 |
| Rainbow trout | 1 | - | 1 | 4 |

16S rRNA and *CO1* sequences were verified as being derived from fish DNA using the BLAST algorithm in GenBank. Sequences were aligned using Sequencher (Gene Codes ver. 4.1.2 for Macintosh) and a neighbour joining phylogram was constructed using PAUP* 4.0b10 (Swofford 2003).

## *(b) Identification of regions in the genome with sufficient length variation to identify New Zealand freshwater pest-fish*

Six New Zealand pest fish species (goldfish, common carp, gambusia, rudd, perch and brown bullhead catfish) that covered a wide taxonomic range or that have been declared noxious fish were selected for this evaluation. Mitochondrial sequences were downloaded from GenBank (www.ncbi.gov) to determine which genes had already been sequenced and hence if primers were available, and to investigate levels of variability among the six species to determine if enough variation existed to distinguish species. Based on experience with other taxa, 12S rRNA, 16S rRNA and cytochrome c oxidase subunit 1 (*CO1*) genes, and the d-loop region were selected for the evaluation. Sequences were aligned in Sequencher 4.8 (Gene Codes Corporation, Ann Arbor) and levels of variability analysed using Paup* 4.10b (Swofford 2003).

## *(c)* In-silico *assessment of Terminal Restriction Fragment Length Polymorphism (TRFLP)*

The Vector NTI software (Invitrogen) was used to predict fragment sizes after digestion with the restriction enzymes Alu1, BstKT1 or HpyCH4I from the *CO1* sequences generated from the 10 fish species assessed in (b).

# 3. Results

## *(a) Extraction of amplifiable fish DNA from tissue and water samples*

The phenol: chloroform: isoamyl extraction generated high quality DNA from white muscle tissue, faeces and mucus for all species tested. The yield of DNA (approximately 5 ng/μL) from faeces and mucus was lower than that from white muscle tissue, however the amount was sufficient for subsequent PCR.

### 16S rRNA PCR

Twenty five sequences for the 16S rRNA gene were obtained from eight species from the DNA extracted from faeces and mucus. A phylogenetic analysis using the neighbour joining algorithm found that DNA extracted from different types of sample types from the same species produced sequences that tended to group together (Fig. 1). For example, 16S sequences obtained from eel genomic DNA extracted from white muscle, faeces and mucus grouped together. The exceptions are a 16S sequence amplified from DNA extracted from eel faeces that grouped with the mosquito fish and DNA extracted from goldfish faeces that grouped with the eels.

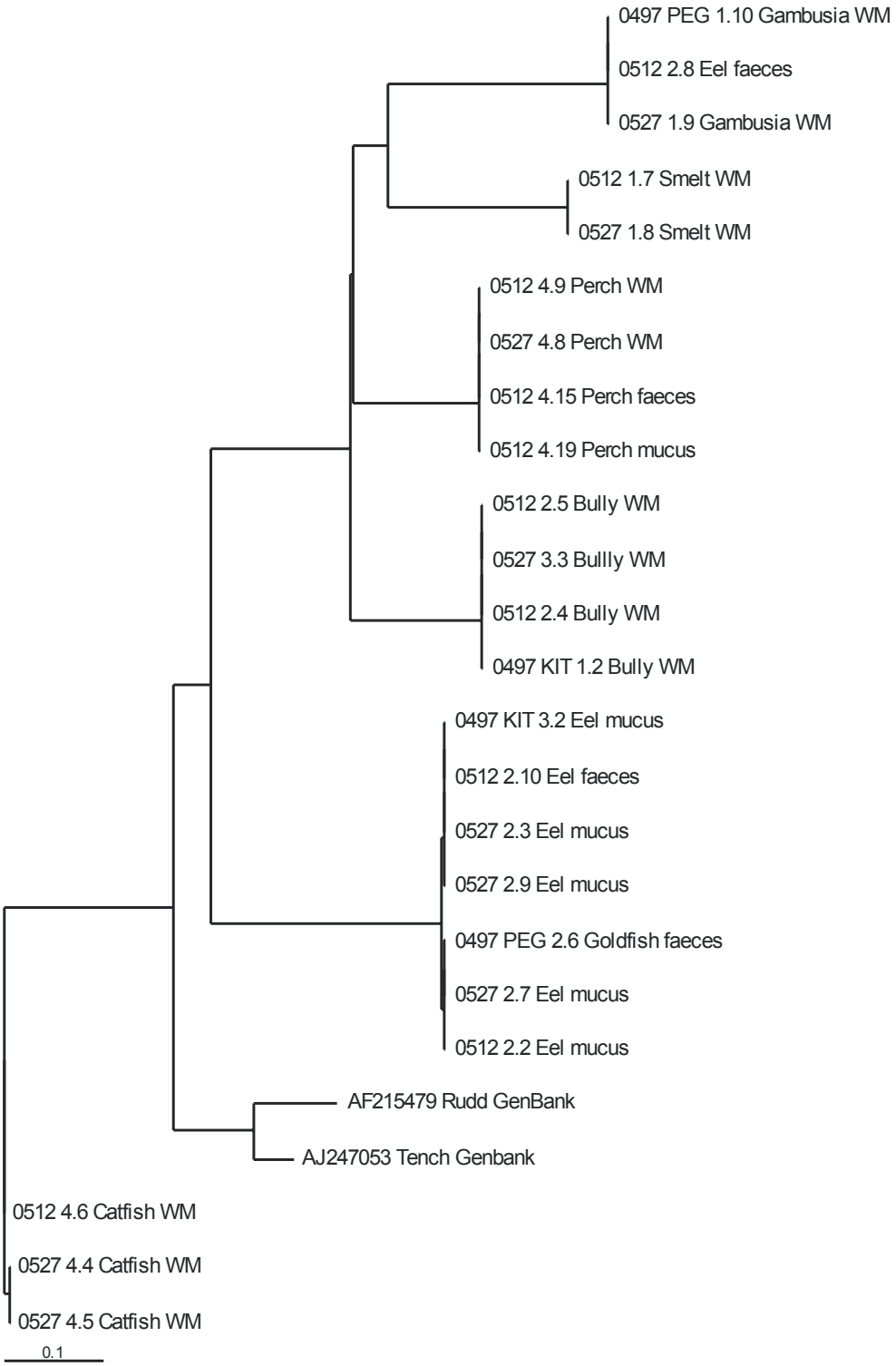


**Figure 1**. Phylogeny estimated by the neighbour joining algorithm for eight species of fish from a portion of the 16S rRNA gene from DNA extracted a variety of sources. WM = white muscle.

## Cytochrome *c* oxidase subunit 1 PCR

Twenty eight sequences for a 617 nucleotide portion of the *CO1* gene were obtained from the ten fish species from DNA extracted from white muscle, faeces and/or mucus. One sequence was obtained from DNA extracted from a water sample. There were no insertions or deletions in the sequences. A phylogenetic analysis using the neighbour joining algorithm found that DNA extracted from different types of sample types from the same species produced sequences that grouped together. For example, *CO1* sequences obtained from rudd genomic DNA extracted from white muscle or mucus grouped together (Fig. 2). Amplification of the *CO1* gene from DNA extracted from tank water in which goldfish had been kept grouped with *CO1* amplified from goldfish DNA extracted from white muscle.

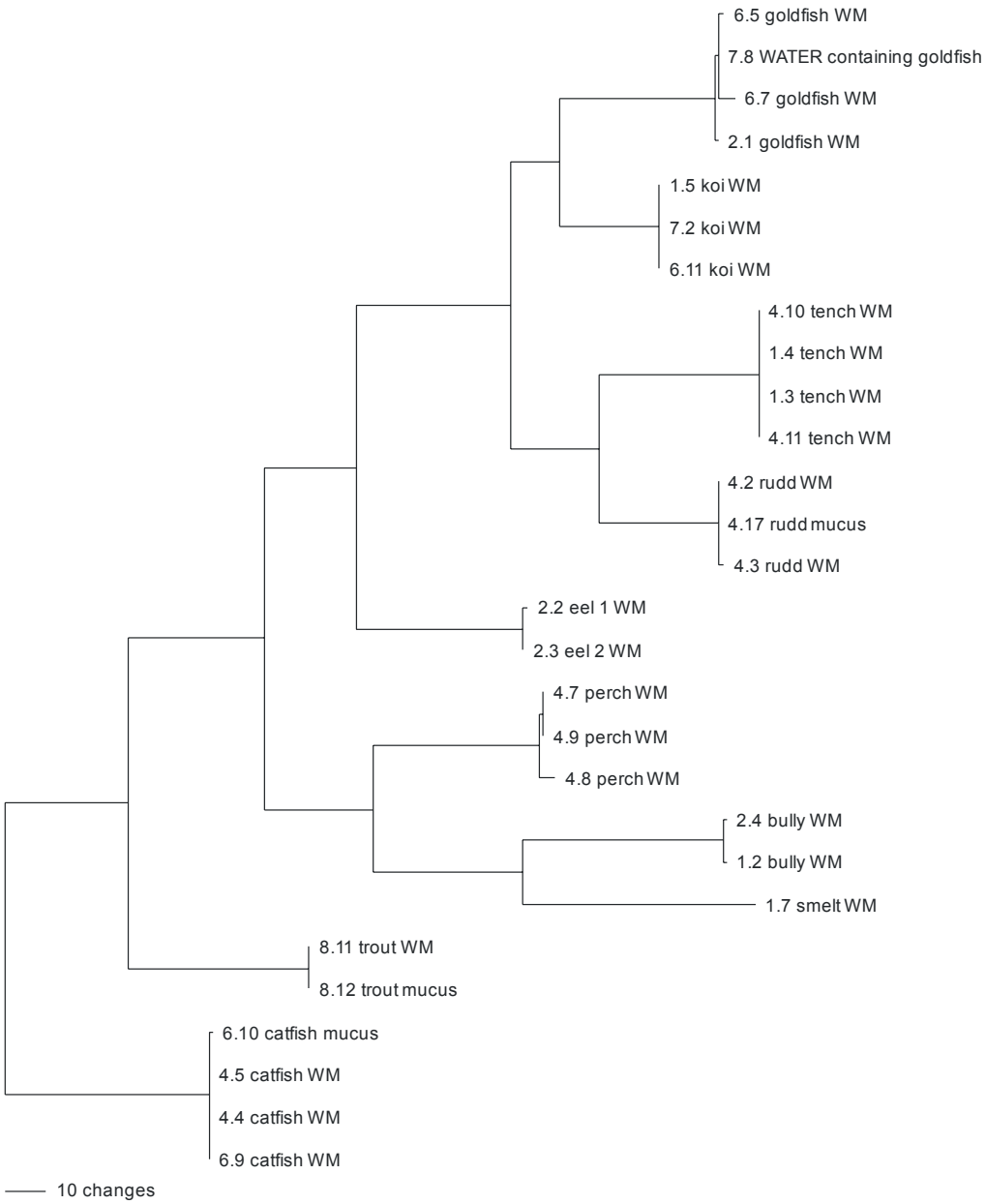


**Figure 2**. Phylogenetic tree estimated from *CO1* sequences for 10 species of fish amplified from a variety of sources (WM is white muscle).

## *(b) Identification of regions in the genome with sufficient length variation to identify New Zealand freshwater pest-fish*

Sequences were available for all six taxa for the four regions selected for the evaluation. The nucleotide sequence varied between species for all regions evaluated (Table 3). Genetic distances were obtained from the six taxa for all four regions (Tables 4 - 7).

**Table 3**. Potential mitochondrial regions to distinguish fish species based on length variation.

| Gene | Universal primer sites | Length | Distinguish taxa from length | Distinguish taxa from sequence |
|---|---|---|---|---|
| 12S rRNA | Yes | ~360 | No | Yes |
| 16S rRNA | Yes | ~500 | No | Yes |
| *CO1* | Yes | ~650 | No | Yes |
| D-loop | Unlikely | ~800 | Yes | Yes |

**Table 4**. Pairwise genetic distances calculated from GenBank sequences for six fish species for the 12S rRNA gene.

| | Koi carp | Rudd | Catfish | Goldfish | Perch | Gambusia |
|---|---|---|---|---|---|---|
| Koi carp | 0.0000 | | | | | |
| Rudd | 0.0713 | 0.0000 | | | | |
| Catfish | 0.1310 | 0.1422 | 0.0000 | | | |
| Goldfish | 0.0230 | 0.0856 | 0.1347 | 0.0000 | | |
| Perch | 0.1987 | 0.2125 | 0.1812 | 0.1907 | 0.0000 | |
| Gambusia | 0.2328 | 0.2193 | 0.1947 | 0.2234 | 0.1384 | 0.0000 |

**Table 5**. Pairwise genetic distances calculated from GenBank sequences for six fish species for the 16S rRNA gene.

| | Koi carp | Rudd | Catfish | Goldfish | Perch | Gambusia |
|---|---|---|---|---|---|---|
| Koi carp | 0.0000 | | | | | |
| Rudd | 0.1118 | 0.0000 | | | | |
| Catfish | 0.1668 | 0.2000 | 0.0000 | | | |
| Goldfish | 0.0376 | 0.1086 | 0.1806 | 0.0000 | | |
| Perch | 0.2480 | 0.2801 | 0.2419 | 0.2675 | 0.0000 | |
| Gambusia | 0.3163 | 0.3260 | 0.3253 | 0.3285 | 0.2272 | 0.0000 |

**Table 6**. Pairwise genetic distances calculated from GenBank sequences for six fish species for the *CO1* gene.

| | Koi carp | Rudd | Catfish | Goldfish | Perch | Gambusia |
|---|---|---|---|---|---|---|
| Koi carp | 0.0000 | | | | | |
| Rudd | 0.1562 | 0.0000 | | | | |
| Catfish | 0.2597 | 0.2515 | 0.0000 | | | |
| Goldfish | 0.1184 | 0.1754 | 0.2613 | 0.0000 | | |
| Perch | 0.2294 | 0.2635 | 0.2380 | 0.2452 | 0.0000 | |
| Gambusia | 0.2682 | 0.2762 | 0.2528 | 0.2646 | 0.2563 | 0.0000 |

**Table 7**. Pairwise genetic distances calculated from GenBank sequences for six fish species for the d-loop region.

| | Koi carp | Rudd | Catfish | Goldfish | Perch | Gambusia |
|---|---|---|---|---|---|---|
| Koi carp | 0 | | | | | |
| Rudd | 0.28739 | 0 | | | | |
| Catfish | 0.343195 | 0.410029 | 0 | | | |
| Goldfish | 0.143275 | 0.271386 | 0.327381 | 0 | | |
| Perch | 0.464286 | 0.504348 | 0.44152 | 0.458084 | 0 | |
| Gambusia | 0.456522 | 0.516616 | 0.496951 | 0.471875 | 0.318713 | 0 |

## *(c)* In-silico *assessment of Terminal Restriction Fragment Length Polymorphism (TRFLP)*

The *in-silico* assessment of the fragment sizes produced by three of the restriction enzymes in the Vector NTI database found that the restriction enzymes Alu1, BstKT1 and HpyCH4I would produce a mix of fragment lengths from *CO1* which would uniquely identify each of the 10 fish species (Table 8 and Figs 3-5).

**Table 8**. Fragment sizes predicted by Vector NTI to be produced by the restriction enzymes Alu1, BstKT1 and HpyCH4I from the portion of *CO1* that we sequenced.

| Taxon | Size of fragments produced by Alu1 (nucleotides) | Size of fragments produced by BstKT1 (nucleotides) | Size of fragments produced by HpyCH4I (nucleotides) |
|---|---|---|---|
| Bully | 366, 208 and 43 | 309, 167, 99 and 42 | 213, 156, 148, 78 and 22 |
| Catfish | 257, 182, 109 and 69 | 381, 204 and 32 | 254, 224 and 139 |
| Eel | 259, 251, 56, 39 and 21 | 619 | 485 and 132 |
| Goldfish | 243, 187, 64, 61, 38, 24 | 423, 85, 77 and 32 | 393 and 224 |
| Koi carp | 304, 251, 38, 18 and 6 | 508 and 109 | 461 and 156 |
| Perch | 226, 201, 109, 56 and 25 | 408, 173 and 36 | 254, 132, 116 and 115 |
| Rudd | 289, 216, 61, 33 and 18 | 515 and 102 | 617 |
| Smelt | 326, 187 and 10 | 196, 172, 119 and 36 | 324, 101, 78, 15 and 5 |
| Tench | 556 and 61 | 481, 85 and 51 | 329, 156 and 132 |
| Trout | 251, 211 and 155 | 294, 204 and 119 | 456 and 161 |

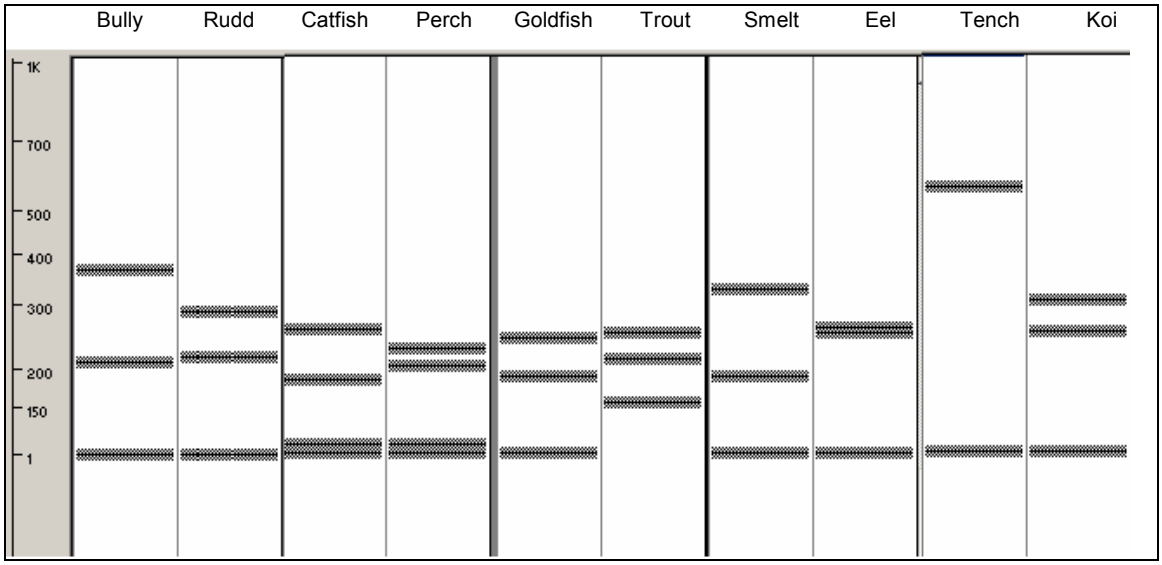


**Figure 3**. Map of the fragments predicted from digesting the portion of *CO1* that we sequenced with the restriction enzyme AluI.

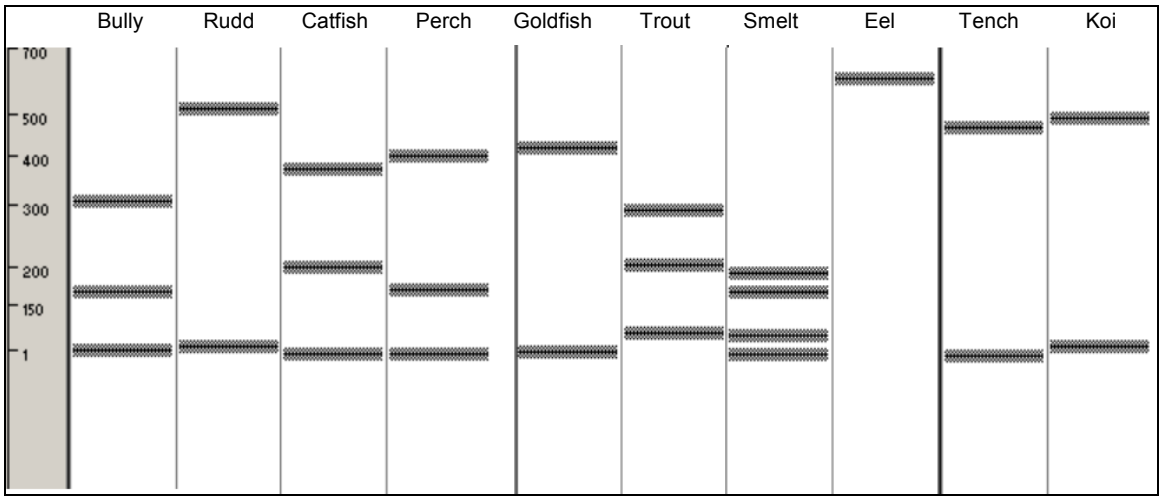


**Figure 4**. Map of the fragments predicted from digesting the portion of *CO1* that we sequenced with the restriction enzyme BstKT1.

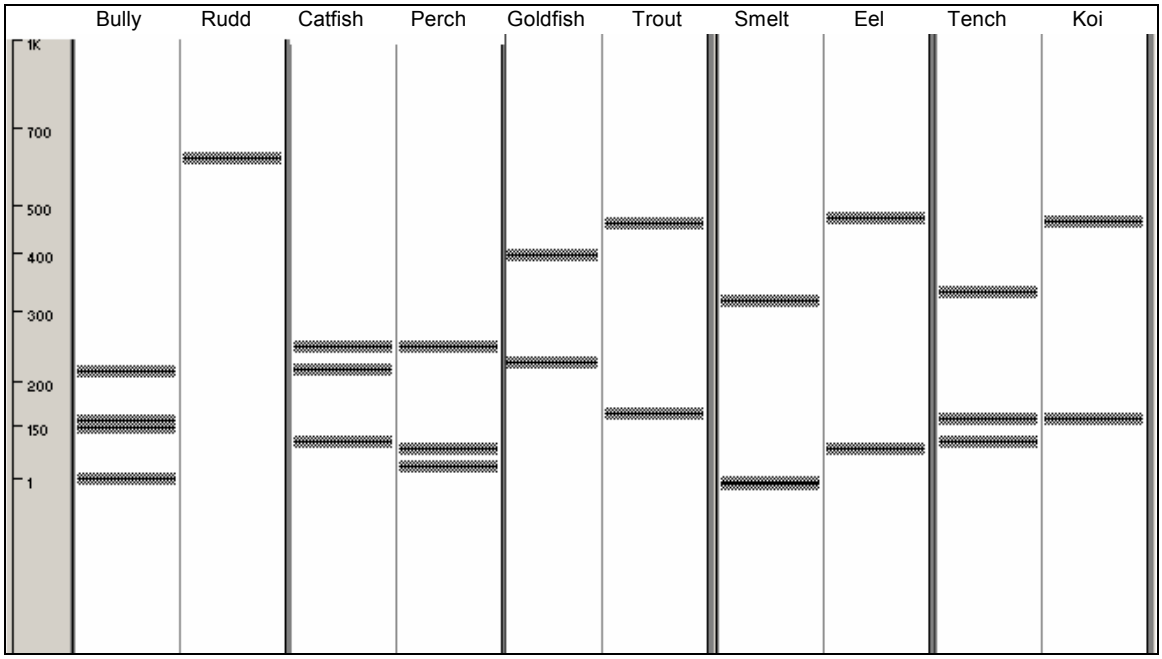


**Figure 5**. Map of the fragments predicted from digesting the portion of *CO1* that we sequenced with the restriction enzyme HpyCH4I.

# 4. Discussion

## *(a) Extraction of amplifiable fish DNA from tissue and water samples*

### 16S rRNA gene

A notable result from the 16S sequences was the grouping of a DNA sequence extracted from goldfish faeces with eels which suggested that eel DNA had passed into the faeces of goldfish. Goldfish are omnivores and eat detritus (McDowall 2000) so we may have sequenced some undigested eel DNA that the goldfish excreted in its faeces. Likewise a 16S sequence obtained from DNA extracted from an eel faecal sample grouped with 16S sequences obtained from gambusia DNA extracted from white muscle suggesting that the eel may have recently eaten gambusia. The eel was collected from Oranga Lake at the University

of Waikato and Gambusia are present in this lake. Other studies have used DNA extracted from faeces to identify the prey of animals. For example, the prey of Steller sea lions was identified from DNA extracted from the sea lions' faeces (Deagle et al. 2005).

### Cytochrome *c* oxidase subunit 1 gene

Cytochrome *c* oxidase subunit 1 readily amplified from muscle tissue, mucus and faeces for the 10 species examined. However, while PCR product was readily obtained from the DNA extracted from the water samples, with one exception the sequences obtained were of poor quality. The poor quality sequences could be due to degradation of the DNA while it is in the water. Refining the extraction method and amplifying a shorter fragment of *CO1* will likely improve the quality of the sequence obtained. Ficetola et al. (2008) successfully amplified a 79 nucleotide portion of the mitochondrial gene cytochrome b (*cytb*) DNA from water samples after adding sodium acetate to the water samples at the time of collection to reduce the adverse effects of any degrading enzymes in the water. Hajibabaei et al. (2006) have shown that fragments as small as 100 nucleotides of *CO1* can be used to identify species of Australian fish. In the future we intend to test both of these options to improve the yield of DNA from the water samples.

## *(b) Identification of regions in the genome with sufficient length variation to identify New Zealand freshwater pest-fish*

The GenBank sequences indicate that there is sufficient sequence variation to distinguish pest fish species in any of the four mitochondrial regions evaluated. However, none of the regions had enough variation in length to unequivocally distinguish the six species. A solution to this

problem would be to generate length variation using restriction enzymes (enzymes that cut the sequences obtained by PCR at specific cutting sites).

### *(c)* In-silico *assessment of Terminal Restriction Fragment Length Polymorphism (TRFLP)*

Restriction enzymes such as Alu1 cut DNA at specific sites (for example Alu1 recognises the nucleotide sequence AGCT and cuts between the G and C, Invitrogen product information) and thus introduce length variation in the fragments produced from different species if sequences differ. Our results showed that digesting the *CO1* PCR product with restriction enzymes will produce a mix of fragments specific to each fish species. One problem that may occur would be if there is intraspecific variation in the *CO1* region. However, many of the pest fish (i.e., those other than the salmonids) were established from a limited number of introductions (McDowall 1990) and populations that are recently established from a small number of individuals generally have low levels of intraspecific variation (Barton and Charlesworth 1984). Given the potential for intraspecific variation to produce conflicting results, we recommend that more study is done to assess the extent of variation within species, especially variation at potential restriction sites. We also recommend that the number of fish species in the database is expanded to obtain a more comprehensive map of the predicted fragment sizes.

## 5. Future directions

This review of progress with the genetic identification of fish within Intermediate Outcome Two of the University of Waikato Outcome Based Investment on pest fish suggests that the following sequence of actions should be taken.

1. Stop searching for length variable regions of the mitochondria to identify fish species. The GenBank data suggest that we are unlikely to find a hypervariable region in fish with enough length variation and which is flanked by conserved regions suitable for universal primers.

2. Improve the yield and quality of the DNA extracted from the water samples.
This could be achieved by adding 1.5 mL of sodium acetate (3 M) and 33 mL of ethanol to 15 mL water samples at the time of collection and then using the extraction technique of Ficetola et al. (2008).

3. Improve the reliability of the PCR and the quality of the sequence obtained by amplifying a shorter fragment of *CO1*. Ficetola et al. (2008) successfully sequenced a 79-nucleotide portion of cytochrome b from DNA extracted from water samples.

4. Expand the RFLP *in-silico* database to ensure that all fish species will be distinguishable using restriction enzymes.

4. Develop methods to carry out real-time quantitative PCR (qPCR) which will quantify the DNA present in the water sample. This method offers a second benefit in that high resolution melting analysis can be incorporated into the protocol and likely used for species identification (for example see Cheng et al. 2006).

## 6. Acknowledgements

This project was funded by FRST Contract UOWX0505. Thanks to Cindy Satake-Cadman for her work on this project.

## 7. References

Anonymous. 2000. Canterbury Conservation Management Strategy Planning Series No. 10. Department of Conservation, Christchurch.

Avise, J. C. 1994. Molecular Markers, Natural History and Evolution. Chapman and Hall, New York.

Banks, J. C., I. D. Hogg, and S. C. Cary. 2009. The phylogeography of Adelie penguin faecal bacteria. Environmental Microbiology **11**:577-588.

Barton, N. H. and B. Charlesworth. 1984. Genetic revolutions, founder effects, and speciation. Annual Review of Ecology and Systematics **15**:133-164.

Cardinale, M., L. Brusetti, P. Quatrini, S. Borin, A. M. Puglia, A. Rizzi, E. Zanardini, C. Sorlini, C. Corselli, and D. Daffonchio. 2004. Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities. Applied and Environmental Microbiology **70**:6147-6156.

Cheng, J. C., C. L. Huang, C. C. Lin, C. C. Chen, Y. C. Chang, S. S. Chang, and C. P. Tseng. 2006. Rapid detection and identification of clinically important bacteria by high-resolution melting analysis after broad-range ribosomal RNA real-time PCR. Clinical Chemistry **52**:1997-2004.

Deagle, B. E., D. J. Tollit, S. N. Jarman, M. A. Hindell, A. W. Trites, and N. J. Gales. 2005. Molecular scatology as a tool to study diet: analysis of prey DNA in scats from captive Steller sea lions. Molecular Ecology **14**:1831-1842.

Ficetola, G. F., C. Miaud, F. Pompanon, and P. Taberlet. 2008. Species detection using environmental DNA from water samples. Biology Letters **4**:423-425.

Fisher, M. M. and E. W. Triplett. 1999. Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. Applied and Environmental Microbiology **65**:4630-4636.

Hajibabaei, M., M. A. Smith, D. H. Janzen, J. J. Rodriguez, J. B. Whitfield, and P. D. N. Hebert. 2006. A minimalist barcode can identify a specimen whose DNA is degraded. Molecular Ecology Notes **6**:959-964.

Hewson, I. and J. A. Fuhrman. 2004. Richness and diversity of bacterioplankton species along an estuarine gradient in Moreton Bay, Australia. Applied and Environmental Microbiology **70**:3425-3433.

Hillis, D., B. Mable, A. Larson, S. Davis, and E. Zimmer. 1996. Nucleic acids IV: sequencing and cloning. Pages 321-381 *in* D. Hillis, C. Moritz, and B. Mable, editors. Molecular Systematics. Sinauer Associates, Inc., Sunderland.

Matsui, K., M. Honjo, and Z. Kawabata. 2001. Estimation of the fate of dissolved DNA in thermally stratified lake water from the stability of exogenous plasmid DNA. Aquatic Microbial Ecology **26**:95-102.

McDowall, R. M. 1990. New Zealand Freshwater Fishes: A Natural history and guide. Heinemann Reed, Auckland.

McDowall, R. M. 2000. The Reed Guide to New Zealand Freshwater Fishes. Reed Books, Auckland.

McDowall, R. M. 2004. Shoot first, and then ask questions: a look at aquarium fish imports and invasiveness in New Zealand. New Zealand Journal of Marine and Freshwater Research **38**:503-510.

Swofford, D. L. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Townsend, C. R. 2003. Individual, population, community, and ecosystem consequences of a fish invader in New Zealand streams. Conservation Biology **17**:38-47.

Townsend, C. R. and M. J. Winterbourn. 1992. Assessment of the environmental risk posed by an exotic fish: the proposed introduction of channel catfish (*Ictalurus punctatus*) to New Zealand. Conservation Biology **6**:273-282.

Vitousek, P. M., C. M. D'Antonio, L. L. Loope, M. Rejmanek, and R. Westbrooks. 1997. Introduced species: a significant component of human-caused global change. New Zealand Journal of Ecology **21**:1-16.

Ward, R. D., T. S. Zemlak, B. H. Innes, P. R. Last, and P. D. N. Hebert. 2005. DNA barcoding Australia's fish species. Philosophical Transactions of the Royal Society of London B Biological Sciences **360**:1847-1857.