Development of genetic tools to detect three New Zealand indigenous freshwater mussels in environmental DNA

A thesis submitted in partial fulfilment
of the requirements for the degree
of

Master of Science in Biological Science

at

The University of Waikato

by

Xiaoxue Hu

THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

2017

Abstract

As “ecological engineers”, freshwater mussels are key components in the freshwater ecosystem. However, surveying mussels can be challenging since mussel populations may be difficult to locate. Conventional methods for identifying mussel species are also problematic and require expertise since mussels are often morphologically cryptic. The same problems apply to New Zealand indigenous freshwater mussels. New Zealand had long been considered to have two indigenous species, *Echyridella menziesii* and *Echyridella aucklandica,* until a recent revision based on molecular evidence recognised a new species, *Echyridella onekaka*, which was previously mis-identified as *Echyridella menziesii*. Results from molecular studies have placed all three freshwater mussel species under a single genus, *Echyridella,* specific to New Zealand. For conservation and ecosystem management, a fast and reliable method for surveying and identifying New Zealand mussels is required.

This research aims to develop molecular tools that utilise environmental DNA (eDNA) collected from freshwater environments to detect mussel species. These tools target a widely used DNA marker, cytochrome c oxidase subunit I (*COI*), for species identification. The *COI* targets in eDNA samples are detected by digital polymerase chain reaction (dPCR) using genus-specific primers and species-specific TaqMan probes. Three species-specific assays were developed, followed by assessment of

specificity and sensitivity. Assay for *E. aucklandica* showed high specificity and tolerance to inhibition from high levels of non-target DNA. A trial of duplexing simultaneously assays detecting *E. onekaka* and *E. aucklandica* also showed promising results. With further assessment of assays using *in vivo* and *in situ* environmental samples in the future, these techniques hold great promise for being a rapid and cost-effective method for surveying and identifying New Zealand freshwater mussels.

Additionally, to increase detection rate when target concentration in eDNA is extremely low, known mitochondrial sequences for all three species were extended through PCR and *de novo* sequencing to allow future manufacturing of a customised commercial target DNA enrichment kit (MYbaits). This work is necessary since known mitochondrial genomic sequences for these three species are too short to generate effective MYbaits oligos. Consensus primers were designed based on available complete mitochondrial genomes of closely related mussel species. Most consensus primers successfully amplified in PCR, and about 1700bp have been sequenced so far. Once the sequencing of extended region is completed, a MYbaits kit will be manufactured for future research.

Acknowledgements

First, I would like to express my gratitude to Dr Charles K. Lee for his great guidance and patience. I must be a terrible student but you never give up encouraging me. Thanks also to Professor Craig Cary for sharing your knowledge and understanding about research. The heated discussion between you and Charles gave me a lesson about what's the real passion of science.

Also, a special thank you goes out to Roanna Richards-Babbage, Lynne Parker, Judith Burrows and all the technicians for helping me along throughout my lab work. I would also like to thank John Longmore for the help of DNA sequencing. I would like to thank Georgia Wakerley for all your help with my research and in general. I would also like to thank Caitlin Lowe, Jay Wiseman, Maria Monteiro, Rachelle Anderson, Shelly Brandt and all the past and present lab mates in TRU for the encouraging words and comradery you've given me over the last year.

Thanks also to Mark Hamer from the Waikato Regional Council, Sue Clearwater from NIWA for providing pressures mussel specimens. This project cannot go on without your help.

Huge thanks go out to my father Ronggui, I'd never have made it this far without you. Thank you for all the love and support throughout my study. Thank you also goes out to my friends and family for allowing me to express my feelings and

asking about my projects even when you knew you'd never have any idea what I was saying.

Table of contents

List of figures

List of tables

Chapter I Introduction

Mussel is the common name for a range of invertebrate species enclosed by two symmetrical, elongated shells. They are found all over the world from the Arctic to Antarctica and in marine, freshwater, and brackish water. They belong to the class *Bivalvia*, together with clams, cockles, and scallops, and share the phylum *Mollusca* with oysters, snails, octopi, and squid. Among the six orders of *Bivalvia*, there is a monophyletic order *Unionoida* representing freshwater mussels exclusively (Nowak & Kozlowski, 2013). *Unionoida* is distributed worldwide and contains about 800 species, including the freshwater pearl mussel (Lopes-Lima *et al.*, 2014).

Freshwater mussel is a dominant component of the biological community in many rivers and lakes (Barnhart & Neves, 2005). They are a food source of many species such as fish, reptiles, birds, and mammals. Young mussels are also food for ducks, herons, fish as well as other invertebrates. Additionally, the shells of live and dead mussel also provide habitats for aquatic insects, algae, and plants. It is not only the physical structure of the shells but also other features related to shells such as sediment stabilisation, bioturbation, biodeposition of organic matter that provide and improve habitat for other organisms (Nowak & Kozlowski, 2013).

More importantly, freshwater mussels are critical components of freshwater ecosystems. As filter feeders, they filter suspended matter and particles from water to obtain food including detritus, bacteria, phytoplankton, and zooplankton. This

feature enables mussels to clarify water, with filtration rates up to 6L/hour per mussel, thus, they are known as “ecosystem engineers” (Nowak & Kozlowski, 2013).

Freshwater mussels are declining precipitously worldwide (Strayer *et al.*, 2004; Lopes-Lima *et al.*, 2017). As filter feeders, freshwater mussels are intimately exposed to both dissolved and suspended contaminants which make them highly sensitive to water quality degradation (Watters, 1999). Consequently, with increasing human impact, nearly one-third of freshwater mussel species are classified as Endangered or Threatened species, while 17% of them are considered data-deficient for assessment (IUCN 2016). It was suggested that the decline results from anthropogenic activities such as impoundment, toxic pollution, increased nutrient loading, species introductions, and overharvest (Strayer, 2014).

To protect and restore the freshwater ecosystem, more understanding on the relation of freshwater mussel with the environment and human disturbance is required. However, studies related to freshwater mussel distribution usually based on relatively low numbers of sampling sites, failing to directly answer many important questions in aquatic conservation and restoration (Cao *et al.*, 2015).

In New Zealand, freshwater mussels are a poorly known component of local freshwater ecosystems although they are found throughout New Zealand (Walker *et al.*, 2001). The most common species *Echyridella. menziesii* (known as “kākahi” in Māori), is widespread in New Zealand. However, shell morphology of *E.menziesii* is

particularly variable (Roper & Hickey, 1994), resulting in misconceptions by early taxonomists that there are several species and many subspecies of freshwater mussels in New Zealand (Suter, 1913; McMichael, 1958). The second mussel species *Echyridella. aucklandica* was recognised by Dell (1953). Compare with *E. menziesii*, *E. aucklandica* is less widespread. In North Island, the majority of *E. aucklandica* appear in the vicinity of Kaeo in the Far North District, and in South Island records are only found from Lake Manapouri to Lake Hauroko (Walker*, et al.*, 2001). The reason of its patchy distribution is unknown (Marshall *et al.*, 2014).

Recent molecular methodologies have enhanced taxonomic studies of mussels and resolved many taxa at the species level (Graf & Cummings, 2007; Lopes-Lima*, et al.*, 2014). New Zealand indigenous freshwater mussels (Mollusca: Bivalvia: Unionidae) were also revised recently (Marshall*, et al.*, 2014). The revision utilised DNA evidence as support and recognised a third species *Echyridella. onekaka* which only present in north-western South Island (Marshall*, et al.*, 2014). The genus *Echyridella* (McMichael & Hiscock,1958) was also identified as a monophyletic group endemic to New Zealand (Marshall*, et al.*, 2014). Therefore, more questions were raised in terms of the evolution, physiology and biogeography of New Zealand freshwater mussels. It is unclear how their most recent common ancestor arrived in New Zealand and what caused the divergence into three species, and why some *E. aucklandica* share the same habitat with *E. menziesii* without interbreeding. Of particular interest are the factors that contributed to the unique distribution of *E.*

aucklandica and *E. onekaka.* It is unclear why *E. onekaka* were restricted only in north-western South Island (Marshall*, et al.*, 2014).

In addition to the challenges of species identification, it is also difficult to assess the occurrence (presence or absence) of mussels using conventional survey method (Cao*, et al.*, 2015). Traditional visual search requires investigators to either wade, swim, or dive. It can be easily thwarted by low mussel density, poor visibility in turbid or deep water, or when the mussel population is buried out of sight (Strayer & Smith, 2003). Extensive labour and expertise for species identification is needed to up-scale environmental surveys of mussels using traditional techniques (Cao*, et al.*, 2015).

Instead of relying on manual count data, tracing genetic material recovered from (potential) habitats has quickly become a potential new avenue of detecting and numerating organisms (Lopes-Lima*, et al.*, 2017). Animals are known to continuously expel DNA to their surroundings as they interact with the environment (Thomsen & Willerslev, 2015). This DNA, known as environmental DNA (eDNA), can persists in the aquatic environment for up to a month (Dejean *et al.*, 2011). They can be sampled, extracted, and analysed typically using polymerase chain reaction (PCR) (Thomsen & Willerslev, 2015). Using species-specific primers, DNA of a species of interest can be amplification for analysis. Moreover, the region amplified by PCR is usually a standardised DNA regions used as “barcode” to identify and discriminate taxa (Deagle *et al.*, 2014). There are assays for freshwater mussels, but specific

assays for New Zealand freshwater mussel species do not currently exist (Marshall, *et al.*, 2014).

The aim of this research is to develop quantitative assays for detecting and identifying three New Zealand endemic freshwater mussel in eDNA.

In Chapter II, a review about freshwater mussels and eDNA based species detection is present. The first part introduces the general knowledge of freshwater mussels and then focused on the difficulties of species identification and survey of freshwater mussels, especially New Zealand freshwater mussels. The second part presents the use of environmental DNA to detect species, including molecular techniques used for species identification and common issues related to species detection.

Chapter III describes the design and validation of species-specific assays. These three assays use digital PCR in combination with TaqMan probes. Compared with conventional PCR, digital PCR is more sensitive and specific in reducing false positive and false negative results.

False positive and false negative are common errors in species detection. In dPCR, false positive is reduced by combination of primers and TaqMan probe which are designed specifically for the three New Zealand mussel species. Digital PCR also reduces false negative errors. Environmental DNA is prone to PCR inhibition

(Green & Field, 2012). It may contain substances that can inhibit PCR reaction, leading to an underestimated quantification or ultimately false negative (Schriewer *et al.*, 2011). Also, a high total volume of eDNA content with little target can cause interference with PCR efficiency (Green & Field, 2012). Digital PCR reduces false negative since it partitions the reaction mix into thousands of partitions that allows better target accessibility. Thus, it is less affected by inhibition. dPCR also reduces false negative by allowing more eDNA template. Subsampling of total extracted eDNA can be challenge since the higher the amount of sample to be screened for detection the better. The tolerance of inhibition enables dPCR to allow more eDNA than standard PCR and qPCR.

Although dPCR reduce false negative by allowing more eDNA template, false negative still exists when concentration of target in eDNA sample is low, leading to a low detection rate. Chapter IV describes preparations to facilitate a target enrichment approach through capturing the target molecules. This method enriches the target by positively select known target for enrichment. MYbaits was chosen as the target capture methods to increase the detection possibility and eliminate issues raised by the large non-target eDNA present in sample.

Chapter IV describes the preparations for ordering customised MYbaits, including designing consensus primers and sequencing. To manufacture a custom MYbaits library for capturing *Echyridella* mtDNA fragments, a multiple sequence

alignment of mtDNA from targeted mussel species is required to identify regions suitable for bait design. However, the available *Echyridella* sequences are too short for effective MYbaits design. To sequence the extended region of available *COI* sequence, records of *Unionoida* were obtained to identify closely related mussel species of *Echyridella.* Assuming their conserved region are similar or even identical, consensus primers were designed based on conserved region of complete mitochondrial sequences from closely related mussel species. These primers were used to amplify mtDNA of *Echyridella* mussel samples and be used for sequencing.

Chapter V concludes all the works this study, including three assays developed in this study and a trial of duplex assay to identify *E. aucklandica* and *E. onekaka* simultaneously. It also makes some discussions about possible future direction.

Chapter II Literature Review

II-1 Freshwater Mussels

II-1-1 Introduction

Mussels are invertebrate species enclosed by two symmetrical, elongated shells. They are found all over the world from the Arctic to Antarctica, and in marine, freshwater, and brackish water. Taxonomically, mussels share the phylum *Mollusca* with oysters, snails, octopi, squids, and others.(Nowak & Kozlowski, 2013).

Freshwater mussels belong to the class *Bivalvia,* which encompasses six orders. The order *Unionoida,* a monophyletic and exclusively freshwater group, has the most extensive radiation with six modern families across about 800 species (Lopes-Lima*, et al.*, 2014). Species of *Unionida* have been studied since the beginning of the nineteenth century, especially the larger and conspicuous species (Lopes-Lima*, et al.*, 2014).

The research on *Unionoida* has long been focused on taxonomy since the first publication on this topic, in which some freshwater bivalves were classified with some marine species (Linnaeus, 1758). Few anatomical and physiological studies had been carried out until freshwater mussels were listed under the U.S. Endangered Species Act, which caught the attention of research scientists, environmental managers, and policy makers (Stansbery, 1970; Bogan, 1993;

Williams *et al.*, 1993). As a result, an explosion in mussel research that focuses on conservation, ecology, physiology, and toxicology occurred and continues to the present (Lopes-Lima*, et al.*, 2014).

Freshwater mussel populations are declining precipitously worldwide (Strayer*, et al.*, 2004; Lopes-Lima*, et al.*, 2017). Of the 533 assessed freshwater mussel species in the 2016 IUCN Red List of Threatened Species (Version 2016-3. http://www.iucnredlist.org), 32 (6%) are extinct, 162 (30%) are endangered or threatened, 52 (9%) are nearly threatened, 91(17%) are data deficient, and 196 (37%) are least concern. Global awareness of the freshwater mussel crisis arises from North American *Unionida*, which constitute the continent's most endangered fauna (Williams*, et al.*, 1993; Strayer*, et al.*, 2004). In North America, over 70% of extant species are considered imperilled (Williams*, et al.*, 1993), and 37 species are extinct (Lydeard *et al.*, 2004). The decline is usually attributed to various anthropogenic activities with many of them under active research, such as dams and other habitat change (Ellis, 1936; Watters, 1999), pollution (Naimo, 1995; Cope *et al.*, 2008), species introductions (Strayer, 1999; Sousa *et al.*, 2014), and overharvest (Anthony & Downing, 2001).

II-1-2 Special physiology of freshwater mussels

Freshwater mussels have an unusual and complex life cycle highlighted by a parasitic larval stage (Nowak & Kozlowski, 2013). In early summer, male mussels

release sperms into the water. Females then capture these sperms to fertilise eggs inside them. After fertilisation, eggs breed into glochidia in the gills of female mussels. Glochidia are parasitic larvae that attach to host fish by clamping valves of their shell on fins or gill filaments. Using a variety of host-attracting strategies, they attach to the host fish and eventually become enclosed in the fish tissue through the migration of host epithelial cells (Rogers-Lowery & Dimock Jr, 2006; Barnhart *et al.*, 2008). Some glochidia have only one suitable host fish species while others may have many (Trdan & Hoeh, 1982; Gordon & Layzer, 1993). Host specificity is likely due to adaptions for surviving the innate defensive responses of the host fish (Barnhart*, et al.*, 2008). Glochidia eventually develop into juveniles and are discharged from host when the water temperature changes (Watters & O'dee, 2000). This special reproduction process implies a vulnerable lifecycle because of the species-specific parasitism, the temperature-triggered discharge of juveniles, and the susceptibility of juveniles to drift, siltation, micropredators, and sediment toxicity (Barnhart & Neves, 2005).

Species of *Unionoida* are known to exhibit doubly uniparental inheritance (DUI), an exception to strict maternal inheritance of mitochondria in the animal kingdom (Gusman *et al.*, 2016). It is characterised by the inheritance of both gender-associated mitochondrial lineages. The female-type mtDNA is transmitted through the eggs to all offspring, and the male-type mtDNA in sperm enters all eggs at the time of fertilisation but is only retained in male offspring. The female-type mtDNA

predominate all tissues of both genders in adults, except in the male gonad where the male-type mtDNA prevails. Thus, adult females are essentially homoplasmic, and adult males are heteroplasmic (Breton *et al.*, 2007; Passamonti & Ghiselli, 2009; Zouros, 2013).

First reported by Zouros *et al.* (1994), followed by a phylogenetic study from Hoeh *et al.* (1996), more DUI species have been identified (Hoeh *et al.*, 2002; Soroka, 2008; Breton *et al.*, 2009). DUI was initially found in species of seven different bivalve families (Breton*, et al.*, 2007; Passamonti *et al.*, 2011), but soon the number increased to 12 (Gusman*, et al.*, 2016). However, existing data are still insufficient to resolve the evolutionary origin of DUI, and much more complete studies of taxonomic distribution of DUI across *Bivalvia* are required (Gusman*, et al.*, 2016)

Studies of DUI have also advanced from sequencing certain mitochondrial genes to characterising the two sex-linked mitochondrial genomes to reveal the transmission route in mitochondrial genome evolution (Curole & Kocher, 2002; Passamonti*, et al.*, 2011; Śmietanka *et al.*, 2016). It has been found that there is a 200-codon extension of the Cytochrome c oxidase subunit II (*COII*) gene present in the male genome (Curole & Kocher, 2002). This was soon used as a simple and effective method of identifying UDI species in *Unionoida* (Walker *et al.*, 2006). Other male-type mtDNA markers are also used to develop sex identification method

(Mioduchowska *et al.*, 2016). This has implications for molecular analyses that target mtDNA genes, and in practice signals associated with male-type mtDNA are ignored for phylogenetic analyses.

II-1-3 Ecological importance of freshwater mussels

In a freshwater ecosystem, mussels have important functional roles (Vaughn & Hakenkamp, 2001). They increase water clarity, affect nutrient dynamics, bioturbation, biodeposition, and provide living habitat for other organisms (Levinton, 1995; Navarro & Thompson, 1997; Welker & Walz, 1998; Vaughn & Hakenkamp, 2001). Functional roles of freshwater mussels have been extensively reviewed by Vaughn and Hakenkamp (2001) (Figure II-1).

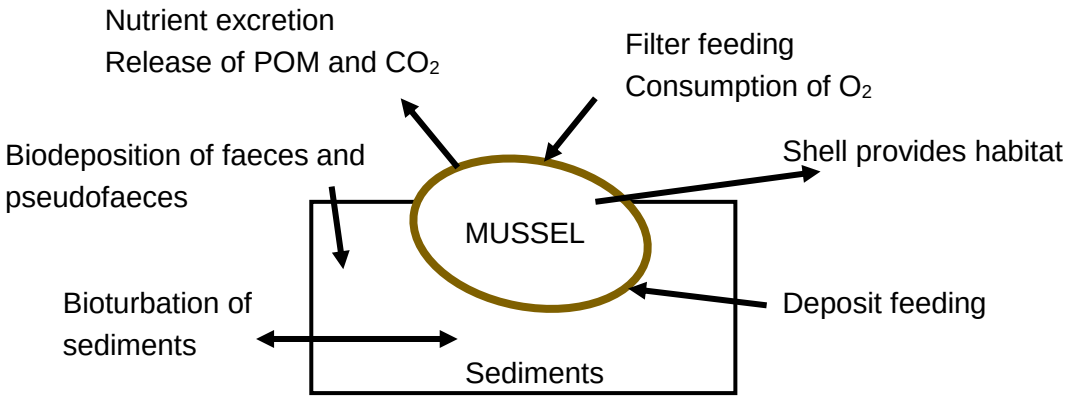


Figure II-1 Potential ecosystem functions performed by freshwater mussels.
POM: particulate organic matter. Figure reproduced from Vaughn and Hakenkamp (2001)

Freshwater mussels filter detritus, bacteria, phytoplankton, and particulate organic matter from the water column. This filtration can significantly decrease phytoplankton and other particles in the water column (Strayer *et al.*, 1999). Welker

and Walz (1998) found that mussels in the River Spree, Germany can cause "biological oligotrophication" by decreasing total phosphorus and phytoplankton and increasing water clarity. Moreover, the volume of water filtered by mussels can equal or exceed daily stream discharge within dense mussel beds (Welker & Walz, 1998).

Mussels also affect nutrient dynamics in freshwater systems. It is believed that they can feed and pump back out nutrient as well as change the chemical form of nutrients like their marine relatives (Kuenzler, 1961; Vaughn & Hakenkamp, 2001). Moreover, bioturbation of sediments through mussel movements exposes sediment to water, increases oxygen content, and releases nutrients to the water column.

Freshwater mussels are also attractive taxa for studying sediments and water pollution effects (Nowak & Kozlowski, 2013). Pollutants are present in water, sediments, as well as in the tissues of aquatic organisms including mussels. Mussels tend to accumulate toxins and pollutants including heavy metals, organic pollutants, and pathogenic bacteria (Stankovic & Jovic, 2012; Stankovic *et al.*, 2012). The vast amount of water filtered by mussels makes them susceptible to pollutants. Also, glochidia and juveniles of freshwater mussels have been found to be typically sensitive to certain contaminants in pesticides (Bringolf *et al.*, 2007). The disappearance of freshwater mussels usually indicates chronic water pollution problems (Helfrich *et al.*, 2005). Moreover, the type, extent, and the time of

contamination events occurred in rivers and lakes can be determined by pollutants in mussel tissues and shells (Nowak & Kozlowski, 2013).

Since mussels are a major component of freshwater ecosystem, it is necessary to obtain a comprehensive description of their distribution and populations for conservation assessment. This is essential for further studies regarding environmental influences and species interactions for sustaining and restoring mussel biodiversity and their ecological functions (Haag & Williams, 2014).

II-1-4 New Zealand indigenous freshwater mussels

New Zealand freshwater mussels are a poorly known component of New Zealand freshwater ecosystems, but are found throughout New Zealand (Walker*, et al.*, 2001). The most common species is *Echyridella. menziesii*, known as “kākahi” in Māori. It is widespread in North and South Islands as well as the Great Barrier Island.

Influenced by habitat variation (sediment grade, water chemistry, etc.) and infestation by the obligate inquiline commensal larvae, shell morphology of *E. menziesii* is particularly variable (Roper & Hickey, 1994). Their shell shapes vary widely, ranging from “narrowly ovate with a weakly concave or broadly rounded ventral margin”, to “broadly ovate with a rounded ventral margin” (Gray & Dieffenbach, 1843; Walker*, et al.*, 2001; Marshall*, et al.*, 2014). These morphological

variations resulted in a misconception by early taxonomists that there are several species and many subspecies of freshwater mussels in New Zealand (Suter, 1913; McMichael, 1958). *E. menziesii* is historically significant to Maori as a food source and serves as water quality indicator in many studies due to its biology and wide distribution (Hickey *et al.*, 1995; Ellis, 1997; Butterworth, 2008; Moore, 2013; Clearwater *et al.*, 2014).

The conservation status of *E. menziesii* in the New Zealand Freshwater Invertebrates list is “At Risk” with a large population but a predicted decline of 10–70% in next ten years (Grainger *et al.*, 2014). Due to the unique life cycle of freshwater mussels, the drop in *E. menziesii* populations is thought to be the result of the decline of its host fish. The *E. menziesii* glochidia attach themselves to small native fish such as Koaro (*Galaxias. brevipennis*), Giant Bully (*Gobiomotphus. gobioides*), and Common Bully (*Gobiomorphus. cotidianus*) (Phillips, 2007). Koaro populations have decreased significantly since European arrival, which is hypothesised to be a cause of kākahi decline (McDowall, 2002).

Compare with *E. menziesii*, *Echyridella. aucklandica* is less widespread. In North Island, the majority of *E. aucklandica* appear in the vicinity of Kaeo in the Far North District, and in South Island records are only found from Lake Manapouri to Lake Hauroko (Walker*, et al.*, 2001). The reason of its patchy distribution is unknown but possibly because of the distribution of host vector fish or disjunction by historical

consequence from volcanic activities (Marshall*, et al.*, 2014). *E. aucklandica* is sympatric with *E. menziesii* throughout its range, which means they can occupy roughly the same areas without interbreeding. In the Conservation Status of New Zealand Freshwater Invertebrates, *E. aucklandica* is listed as " Nationally Vulnerable" (Grainger*, et al.*, 2014).

II-1-5 Taxonomy of New Zealand freshwater mussels and its revision

Freshwater mussels have long been known to be plastic and variable since shell morphology can be highly variable in response to the local environment (Roper & Hickey, 1994; Hornbach *et al.*, 2010). The cryptic shell morphology posted many difficulties to taxonomists, and those of New Zealand freshwater mussels are no exception (Dell, 1953). Early workers suggested that there are several species and many subspecies of freshwater mussels in New Zealand (Suter, 1913; McMichael, 1958). However, Dell (1953) concluded that there are only two species *Hyridella menziesii* and *Hyridella aucklandica*. He examined a large collection of mussels from a wide range of localities, and found that *H. menziesii* is a species highly variable with its constituent populations had previously been recognised as species or subspecies (Dell, 1953; McMichael, 1958). However, for a long time, the actual number of New Zealand freshwater mussel taxa and their phylogenetic relationships remain unclear (Fenwick & Marshall, 2006).

Recent molecular methodologies have enhanced taxonomic studies of mussels and resolved many taxa at the species level (Graf & Cummings, 2007; Lopes-Lima*, et al.*, 2014). With the rapid development of molecular techniques, DNA sequencing became the technique adopted by most studies, mainly using mitochondrial DNA as markers (Lopes-Lima*, et al.*, 2014). Molecular techniques help aiding in the taxonomy, identification of the phylogenetic relationships as well as the phylogeographical and genetic diversity patterns (Lopes-Lima*, et al.*, 2014).

The taxonomy study of New Zealand mussels also benefited from molecular techniques. By sequencing and comparing partial Cytochrome c oxidase subunit I (*COI*) sequences, Fenwick and Marshall (2006) found a third freshwater mussel species, *Echyridella. onekaka,* which was previously classified as common kākahi. So far this mussel species was found only to the west of a line between Takaka and Cape Foulwind in north-western South Island (Fenwick & Marshall, 2006). They lives in close proximity to populations of *E. menziesii* near Cape Farewell, yet there is no record of these two species living together (Marshall*, et al.*, 2014). Current knowledge of *E. onekaka* is limited, resulting in a conservation status in New Zealand Freshwater Invertebrates as “Data Poor” (Grainger*, et al.*, 2014).

The DNA sequences provide not only species identification but also the phylogeny of mussels. It was suggested that *H. Menziesii* and *H. Aucklandica,* along with *E. onekaka,* are under the New Zealand Genus *Echyridella* (Fenwick &

Marshall, 2006; Marshall*, et al.*, 2014). This also further support the hypothesis that the New Zealand mussels have a Gondwanan origin rather than resulted from dispersal from Australia (Marshall*, et al.*, 2014).

Although their phylogeny was recently revised, the known distribution of New Zealand freshwater mussels is based only on museum records and survey data in selected locations (Marshall*, et al.*, 2014). Moreover, modern studies of New Zealand mussels have been primarily focused on potential environmental functions such as bioindicator and biomanipulation rather than their biology and ecology (Hickey*, et al.*, 1995; Clearwater*, et al.*, 2014; Hamilton *et al.*, 2016; Collier *et al.*, 2017).

II-1-6 Difficulties in studying freshwater mussels

In addition to the challenges of species identification, it is also hard to assess the occurrence (presence or absence) of mussels using conventional survey methods. Traditional visual search requiring investigators to either wade, swim, or dive. It can be easily thwarted by low mussel density, poor visibility in turbid or deep water, or when the mussel population is buried out of sight (Strayer & Smith, 2003).

It is also difficult to up-scale environmental surveys of mussels using traditional techniques. A recent study mapping freshwater mussels in wadeable streams throughout Illinois, U.S.A conducted sampling using “a four-person-hour search over

a c. 200 m subreach at each site by crews of 3–6 people" (Cao*, et al.*, 2015). They searched all available habitats and collected, identified, measured, and returned every single mussel encountered. For morphologically cryptic individuals, the mussels have to be collected and identified in the laboratory. Although described as a more cost-effective approach to determining species presence at a location than time-based sampling (Strayer & Smith, 2003), the method is still known to miss species and populations in Illinois streams (Cao*, et al.*, 2015).

The difficulties of mussel sampling and identification call for an alternative method. Recently developed molecular techniques targeting environmental DNA (eDNA) have the potential to fulfil this demand. Aquatic eDNA contains DNA from the faeces, urine, saliva, and skin cells of animals either occupying the water body or visiting the environment, such as mammals and birds (Rees *et al.*, 2014). The presence of an organism can be examined by detecting its species-specific DNA in water without direct observation or trapping. Ficetola *et al.* (2008) firstly utilised freshwater samples to detect an invasive American bullfrog. They stated that eDNA is "useful for studying secretive aquatic or semi-aquatic species, which release DNA into the environment through mucus, faeces, urine and remains." eDNA techniques therefore allow scientists to non-invasively detect target species without direct observation or trapping.

II-2 Species Detection using Environmental DNA

II-2-1 Introduction

Environmental DNA (eDNA) is DNA directly extracted from an environmental sample such as soil, sediments, snow, and water (Rees*, et al.*, 2014). Although the first reference to eDNA dated back to 1987, the term emerged at the start of the 2000s when it was mostly used in microbiological studies (Ogram *et al.*, 1987; Rondon *et al.*, 2000; Taberlet *et al.*, 2012). Environmental DNA is characterised by a mixture of genomic DNA originated from various organisms and by possible degradation. It consists of cellular DNA from living cells or organisms, and extracellular DNA from natural cell death and following the destruction of cell structure (Levy-Booth *et al.*, 2007; Pietramellara *et al.*, 2009; Taberlet*, et al.*, 2012).

For more than a decade, eDNA has been used by microbiologists in the study of microbial communities. DNA extracted from the environment enables microbiologists to access the genetics of uncultivable microorganisms and subsequently allows the identification of microbial taxa in environmental samples. Moreover, the sequencing of eDNA can help identify biochemical functions by analysing protein-coding genes complete genomes of microorganisms (Taberlet*, et al.*, 2012).

Recently, species identification by eDNA has been further extended from microorganisms to meiofauna and macroorganisms (Turner *et al.*, 2014). Higher organisms are known to continuously expel DNA from tissues or excreted cells such

as urine (e.g., Valiere and Taberlet, 2000), faeces (e.g. Poinar et al., 1998), hairs, and skin (e.g., Bunce et al., 2005; Lydolph et al., 2005) to their surroundings. DNA may also come from deceased individuals. Once DNA is exposed to the environment, it persists from hours to weeks in temperate water (Dejean et al., 2011; Thomsen et al., 2012b), enabling detection of the recent presence of living species in the water body.

Detection of freshwater species using eDNA is an emerging field that shows promise in the applications of aquatic organism survey and informing conservation schemes (Rees*, et al.*, 2014). Bhadury *et al.* (2006) first reported the identification of meiofaunal groups using eDNA. They successfully amplified the 18S rDNA of nematodes from DNA sample extracted from marine and estuarine sediments. cFicetola*, et al.* (2008) identified bullfrog in eDNA extracted from freshwater. From then on, considerable interest has been put into identifying different organisms. Many species have been studied including amphibians (Ficetola*, et al.*, 2008; Dejean *et al.*, 2012; Olson *et al.*, 2012; Thomsen *et al.*, 2012; Goldberg *et al.*, 2013; Pilliod *et al.*, 2013, 2014), reptiles (Piaggio *et al.*, 2014), fishes (Jerde *et al.*, 2011; Minamoto *et al.*, 2012; Takahara *et al.*, 2012; Collins *et al.*, 2013), and mammals (Foote *et al.*, 2012).

Although most of the freshwater eDNA studies have focused on vertebrates, especially fishes and amphibians, some studies have demonstrated the possibility of

detecting invertebrates such as arthropods, gastropods, and mussels (Thomsen*, et al.*, 2012; Goldberg*, et al.*, 2013; Deiner & Altermatt, 2014). Deiner and Altermatt (2014) reported that a freshwater sessile mussel species *Unio tumidus* was detectable up to 9.1 km downstream of the source population. In their research, PCR primers were designed with maximised mismatches against closely related co-occurring species. After using multiple sequence alignments to check for species specificity, the annealing temperature and concentrations of PCR primers were optimised using DNA extracted from target specimens. The optimised PCR assay was applied to eDNA extracted from water samples and successfully detected *Unio tumidus*. The detection rate of *Unio tumidus* significantly decreased with increased distance to source population (Deiner & Altermatt, 2014). The study demonstrated that mussel populations could be studied using eDNA techniques.

II-2-2 Advantages of eDNA in species detection

eDNA-based species detection has several advantages over traditional survey methods. Methods using eDNA is non-invasive whereas traditional survey methods usually disturb or capture the species of interest, which impacts the animal welfare. Mammals, insects, amphibians, and fish in freshwater habitats have all been detected with no disturbance to the target organisms (Ficetola*, et al.*, 2008; Thomsen*, et al.*, 2012).

Moreover, eDNA potentially has greater sensitivity than visual-based searching. Dejean*, et al.* (2012) demonstrated that eDNA method is more sensitive with less sampling effort in the detection of American bullfrog *Rana catesbeiana* at 38 sites using eDNA comparing to 7 sites using conventional methods. A case study also proved that within a given budget, eDNA sampling is more cost effective than trapping methods, and efficiency increases with the number of sites sampled (Smart *et al.*, 2016). Smart*, et al.* (2016) suggested that the cost of detection through traditional survey methods is 2–10X higher than eDNA detection.

II-2-3 Marker Genes

To identify a species in eDNA, a marker gene is required. The marker should be unique to differentiate target DNA fragments from other co-existing eDNA fragments and should be abundant for detection (Wood *et al.*, 2013). Moreover, a shorter marker is preferred since long eDNA fragment are comparatively limited due to degradation (Rees*, et al.*, 2014).

Marker genes have been identified and informally standardised for many taxa groups and serve as barcodes for species identification. For animals, the most popular marker is a 658-bp region of the mitochondrial cytochrome c oxidase I (*coxI* or *COI*) (Hebert *et al.*, 2003b; Hebert *et al.*, 2003a). And for plants, the most common markers are 500–800-bp plastid fragments of the maturase K gene (matK) and the

large subunit of ribulose 1,5-bisphosphate carboxylase gene (rbcL) (Group *et al.*, 2009).

For identification at the species level, it is of great importance to choose a DNA marker that is capable of distinguishing species within a single genus. Recent studies favour mitochondrial DNA (mtDNA) for species detection because mtDNA has a fast mutation rate and enough sequence divergence to enable differentiation between closely related species (Hebert*, et al.*, 2003b). Moreover, mtDNA benefits eDNA-based species detection due to considerably higher copy numbers of mtDNA than the nuclear DNA per cell and consequently a greater level of mtDNA in eDNA (Rees*, et al.*, 2014).

II-2-4 Common techniques for species identification

There are many molecular techniques used for species identification and monitoring in eDNA. Wood*, et al.* (2013) made a comprehensive review of these techniques with applications used in New Zealand. The advantages and limitations are shown in (Table II-1)

Most of these techniques are Polymerase Chain Reaction (PCR) based techniques. PCR is a widely-used technique in molecular biology for amplifying a specific segment of DNA sequence to exponentially more copies. This sequence-specific amplification is guaranteed by oligonucleotides called primers that

specifically binds to targeted DNA fragment. After primer binding to single strand DNA, elongation of complimentary DNA is initiated with polymerase enzyme and deoxyribonucleotides (dNTP). This process is referred as one cycle. By altering the temperature, PCR cycles can be carried for multiple times to exponentially increase the number of amplicons. After completion of PCR, amplicons can be validated by gel electrophoresis which enables the visualisation of DNA amplicon size.

PCR-based techniques employ PCR not only to make sufficient targets but also utilise this specific amplification to identify species. This identification is ensured by primers that target a specific marker gene. According to the scope of research, the identification can be carried out by PCR coupled with sequencing or by PCR platforms alone (PCR, quantitative PCR or digital PCR). For identification at single species level, quantitative PCR (qPCR) is commonly used due to its high sensitivity and specificity (Wood*, et al.*, 2013; Rees*, et al.*, 2014). PCR coupled with sanger sequencing or next generation sequencing (NGS) allows identification at various levels (Rees*, et al.*, 2014). There are also specific applications such as Automated Ribosomal Intergenic Spacer Analysis (ARISA) and Terminal Restriction Fragment Length Polymorphism (T-RFLP), which is developed to characterise microbial communities from environmental samples (Liu *et al.*, 1997; Ranjard *et al.*, 2001).

Table II-1 Molecular methods used for aquatic species detection in environmental DNA.

Adapted from (Wood*, et al.*, 2013)

Technique/platform		**Advantages**	**limitations**
PCR-based techniques	PCR-Sanger sequencing	Inexpensive Potential for high specificity Able to amplify small amount of DNA	Post-PCR handling time-consuming Potential exposure to toxic reagents
	PCR- next generation sequencing (NGS)	Multiplexing available Massive sequence high-throughput compared to other NGS platforms Good capacity for multiplexing	Library prep/run very time-consuming and expensive Sequence reads (<150 base pair) Substantial demands on bioinformatics
	Quantitative PCR (qPCR/RT-PCR)	Highly specific low detection level Allows quantification Allows rapid diagnostics Potential for high throughput analyses	Expertise- reliant assay development Relatively expensive.
	Digital PCR (dPCR)	Highly specific low detection level Absolute quantification Rapid diagnostics	Expertise- reliant assay development Relatively expensive Consumable required

	Automated ribosomal intergenic spacer analysis (ARISA)	Relatively rapid and inexpensive Effective for monitoring microbial community changes	Cannot provide absolute abundance Unrelated organisms can have identical spacers length Can under or over-estimate diversity
	Terminal restriction fragment length polymorphism (T-RFLP)	Rapid Inexpensive Effective for high-throughput Simplifies comparisons among many samples	Cannot provide absolute abundance. Can under- or over-estimate diversity.
Non-PCR based techniques	Fluorescence in situ Hybridization (FISH)	High specificity Low detection level Allows visual verification Detection/enumeration capability	Expensive Expertise-reliant Long processing time Limited scope for multiplexing
	Sandwich hybridization arrays (SHA)	Relatively cheap per sample High specificity allows visual verification Rapid and portable	Limited by amount of material Limited scope of multiplexing

Non-PCR based techniques have their own advantage that suits specific applications. For rapid visualisation of species identification, fluorescent in situ hybridization (FISH) allows visual verification of target using fluorescent microscopy to visualise the probe binding site such as tissue section or location on chromosomes (Mountfort *et al.*, 2007). Sandwich hybridization arrays (SHA) also allows visual identification with rapid diagnostics and enumeration of organisms (Ayers *et al.*, 2005). These techniques are ideal for direct species identification in which target are abundant for visualisation such as marine algae and marine bioinvasives (Wood*, et al.*, 2013).

II-2-5 Difficulties of PCR-based species detection

Assuming that the null hypothesis in species detection is the absence of target species, PCR-based techniques have two types of error: false positive and false negative. Various possible sources of errors have been reviewed by Darling and Mahon (2011). They distinguished the errors into two categories: method errors that are attributable to the methods being employed, and process errors that derived from the sampling process. Since the focus of this thesis is method development, this literature review focuses on the false positive and false negative caused by method errors (Table II-2).

Table II-2 Possible result of species detection with method error.

	Positive detection	Negative detection
Target DNA present	True positive	False negative
Target DNA absent	False positive	True negative

False positive results caused by method error typically result from unintended amplification by target DNA contamination (e.g., mishandling of positive control) or “look-like” non-target(s). PCR-based species detection methods are usually optimised to detect rare targets. However, it also makes them prone to false positive since even a small amount of contaminating DNA will be amplified (Darling & Mahon, 2011). In addition, PCR may amplify “look-like” non-targets due to insufficient primer specificity. Although PCR primers are designed to recognise target DNA exclusively, cross-reactivity with non-target may occur when the similarity between target and the non-target sequence is sufficiently high, leading to a false positive (Raut *et al.*, 2007). Darling and Mahon (2011) pointed out that the contamination can be prevented by utmost caution with quality assurance and control protocols, and for the non-target amplification, the specificity of the method should be improved.

A false negative, on the other hand, refers to the absence of target signal for a sample in which target DNA fragments are present. A false negative

result is usually caused by a method not sensitive enough to detect the target, or the method simply failed to work as expected (Darling & Mahon, 2011). For normal PCR assays, false negative may occur when there are PCR inhibitors or target concentration is too low to be amplified sufficiently. To increase the detection rate of the rare target, screening more of the extracted eDNA samples is necessary (Machler *et al.*, 2016). However, using more eDNA template in single PCR reaction can be problematic since co-extracted substance from eDNA usually inhibits PCR and high concentration of double stranded DNA also cause inhibition of PCR (SantaLucia, 2007; McKee *et al.*, 2015). As a result, increase the ratio of target vs. non-target becomes the key to reduce false negative.

II-2-6 qPCR in species detection

To quantify the precise concentration of DNA template, quantitative PCR (qPCR) was developed. The advances of qPCR over PCR is that the progression of amplification after each cycle could be monitored in real-time through detection of a fluorescent signal (Hindson *et al.*, 2011) (Table II-3). This improvement can rapidly (less than 2h) determine whether target DNA fragments are present in a sample while also calculating the number of copies of the target.

Table II-3 Comparison of PCR, qPCR and dPCR for species detection

	PCR	qPCR	dPCR
Quantification	Not possible	Relative quantification with internal reference or standard	Absolute quantification
Measurement	End-point Gel electrophoresis	Real-time fluorescence detection	End-point positive/negative fluorescence detection
Reproducibility	Reproducible	Reliant on standard	Reproducible
Inhibition	Sensitive to inhibitors	Sensitive to inhibitors	Tolerant to inhibitors by partitions
Specificity	Primers	Primers+ probe	Primers+ probe

To measure the fluorescent signal in real-time, two strategies are used. One is intercalating dyes which are chemical compounds intercalating between the planar base pairs of the DNA helix with a high affinity for DNA. When the dye binds to double-stranded DNA, it releases fluorescent signals. SYBR Green (Becker et al. 1996), LCGreen (Wittwer et al. 2003), and SYTO9 (Monis et al. 2005) are the common intercalating dyes. Intercalating dyes are generally defined as non-specific to target since they bind non-specifically to any double-stranded DNA.

The other detection method is probe-based detection systems. It utilises a fluorescent-labelled probe, which is a specific sequence of the desired PCR product. Upon amplification of a target sequence, the fluorescent reporter molecule attached to probe becomes cleaved and fluoresces. The fluorescent

signal is detected immediately. Common probes include TaqMan (Heid et al. 1996), minor groove binder eclipse probe (Afonina et al. 2002), and fluorescence resonance energy transfer (Chen & Kwok 1999). This probe-based detection is template-specific since the amplification of target is ensured by three sequences. The accuracy of qPCR is therefore higher than standard PCR.

By default, quantitative PCR allows relative quantification (Dhanasekaran *et al.*, 2010). Absolute quantification, which gives the exact number of target DNA molecules, can be obtained by comparing the result of amplification with DNA standards of known quantities. It is not necessarily reliable since the quantification requires the same amplification efficiency for samples and standards, which is not always the case (Bar *et al.*, 2012).

II-2-7 Advantages of dPCR in eDNA study

More recently, digital PCR (dPCR) which inherently enables absolute quantitation of nucleic acids in a sample was developed (Hindson*, et al.*, 2011). This application employs a classic PCR together with fluorescence-based detection but partitions a single PCR reaction into hundreds or thousands of subreactions. When targets are present in a sample at an optimal concentration, some of the subreactions carry target sequence for

amplification, while others do not. The subreactions are analysed individually at end point for the amplification of interest, generating a ratio of positive signal to negative signal. Since the target molecules are distributed randomly into each subreaction, which follows a Poisson distribution, the starting amount of the target template in the original sample can be calculated by fitting the positive reactions into a Poisson distribution. According to the Poisson's law of small numbers, when there is a random distribution of quantifiable, independent events, predictions can be made about the likelihood with which these events occur. In the model $\lambda = -\ln(1 - \mathrm{p})$, λ refers to the average number of target DNA molecules per replicate reaction and p is the fraction of positive end-point reactions. Thus, given the fluorescence data, we can use the Poisson distribution to determine the number of template molecules in a reaction (Hindson*, et al.*, 2011).

Partitioning of templates makes the dPCR more tolerant to inhibition. In PCR, the binding of primer, probe, polymerase to target sequence take place stochastically, but the inhibitors may binds DNA or polymerase can prevent amplification. Thus, a higher target/non-target ratio, in which the target is more and inhibitor containing non-target content is less, becomes the key to high PCR efficiency. If a DNA sample contains 200 targeted DNA fragments and 20,000 non-target DNA fragments. The ratio in PCR or qCPR is always 1:100.

However, in dPCR where the sample is partitioned into 20,000 subreactions (ideally, each subreaction contains one non-target, and 200 of them contains one target each), the ratio is only counted in target-containing subreaction, which will be about 1:1. This is a significantly higher ratio than bulk reaction of standard PCR or qPCR. And untimely presenting a higher tolerance to inhibition.

Analysis at end-point in dPCR also provides advantage over qPCR which monitors reaction in real time. PCR amplification efficiency varies from sample to sample due to minor inhibitors or delayed amplification start caused by target accessibility. In qPCR, these factors influence the accuracy of quantification. However, the accessibility issue can be ameliorated by partitioning the sample into smaller subreactions. In dPCR, if the amplification takes an event and moves forward, any delay on the subreaction will not influence the end-point positive result, which ultimately enables quantification. Thus, the quantification is more reliable than qPCR.

Due to the higher tolerance to inhibitors, dPCR clearly outperforms qPCR in eDNA-based studies. qPCR is prone to inhibition in eDNA studies since the co-extracted substance in eDNA usually inhibits qPCR, leading to an incorrect result inferring a species is absent or in low abundance (McKee*, et al.*, 2015).

Common treatments in qPCR to reduce inhibition such as 10-fold dilution or a column purification of the eDNA template also co-dilute target DNA and potentially result in non-detections or underestimation (McKee*, et al.*, 2015; Goldberg *et al.*, 2016). These treatments will be less necessary since dPCR is tolerant to inhibition.

The subreactions also benefits dPCR with a higher detection rate by accepting more eDNA template per reaction. In many DNA-based detections of macroinvertebrates, the maximum volume of template per qPCR reaction ranges from 1µl to 5 µl (Machler*, et al.*, 2016). The subsampling of total extracted eDNA into qPCR reaction leads to an uncertainty of whether the targeted DNA is sampled into reactions. The uncertainty can be even higher when detecting a rare target or inhibition is present qPCR. To generate reliable detection result, screening more eDNA is desired. dPCR accepts more template eDNA than qPCR per reaction since the reaction mix is divided into thousands of subreactions. In dPCR, the maximum volume of eDNA template causing inhibition in qPCR will be partitioned. Targets will be amplified with less inhibition per subreactions, which means higher template volume is acceptable by dPCR. Since dPCR allows more template eDNA per reaction, the chance of the presence of a target in the reaction is much higher than qPCR, leading to a higher detection rate.

Experimentally, dPCR has been shown to be more accurate and reliable than qPCR for species detection. A study evaluated the accuracy in quantifying the species abundance of common carp *Cyprinus carpio L* in mesocosm experiments. They compared the quantification accuracy of droplet digital PCR and qPCR and suggested that dPCR is more accurate than qPCR in the quantification of carp eDNA concentration (Doi *et al.*, 2015). Hunter *et al.* (2017) developed a new Grass Carp (*Ctenopharyngodon idella*) TaqMan assay and tested it on both quantitative and droplet digital PCR using eDNA in water samples. The results suggested that digital PCR had higher occurrence estimates compared to quantitative PCR due to increased sensitivity and dilution of inhibitors at low concentrations.

dPCR outcompetes qPCR not only in quantification but also in multiplexing. Multiplexed reactions utilise multiple template-specific primers and fluorescent probes to enumerate more than one target in a single reaction (Handy *et al.*, 2006). Multiplexing in qPCR usually requires additional optimisation since a varied level of targets can be present. The varied target levels may cause monopolisation and depletion of reaction components (e.g., polymerase and dNTPs) where the lower abundance target may fail to amplify (Bizouarn, 2014). However, the partitioning of dPCR provides a dilution effect on the more abundant target. The ratio of abundant target to less abundant

target in single subreaction will reduce significantly, give a much better chance of amplification and subsequent detection and quantification (Bizouarn, 2014).

Reference

Anthony, J. L., & Downing, J. A. (2001). Exploitation trajectory of a declining fauna: a century of freshwater mussel fisheries in North America. *Canadian Journal of Fisheries and Aquatic Sciences, 58*(10), 2071-2090.

Bar, T., Kubista, M., & Tichopad, A. (2012). Validation of kinetics similarity in qPCR. *Nucleic Acids Research, 40*(4), 1395-1406.

Barnhart, C., & Neves, D. (2005). Overview of North American freshwater mussels. Mussels Toxicity Testing Workshop Hosted by U.S. EPA, Chicago, Illinois,2005.

Barnhart, M. C., Haag, W. R., & Roston, W. N. (2008). Adaptations to host infection and larval parasitism in Unionoida. *Journal of the North American Benthological Society, 27*(2), 370-394.

Bhadury, P., Austen, M. C., Bilton, D. T., Lambshead, P. D., Rogers, A. D., & Smerdon, G. R. (2006). Molecular detection of marine nematodes from environmental samples: overcoming eukaryotic interference. *Aquatic Microbial Ecology, 44*(1), 97-103.

Bizouarn, F. (2014). Introduction to digital PCR. *Methods Mol Biol, 1160*, 27-41.

Bogan, A. E. (1993). Freshwater bivalve extinctions (Mollusca: Unionoida): a search for causes. *American Zoologist, 33*(6), 599-609.

Breton, S., Beaupré, H. D., Stewart, D. T., Hoeh, W. R., & Blier, P. U. (2007). The unusual system of doubly uniparental inheritance of mtDNA: isn't one enough? *Trends in Genetics, 23*(9), 465-474.

Breton, S., Beaupré, H. D., Stewart, D. T., Piontkivska, H., Karmakar, M., Bogan, A. E., Blier, P. U., & Hoeh, W. R. (2009). Comparative Mitochondrial Genomics of Freshwater Mussels (Bivalvia: Unionoida) With Doubly Uniparental Inheritance of mtDNA: Gender-Specific Open Reading Frames and Putative Origins of Replication. *Genetics, 183*(4), 1575.

Bringolf, R. B., Cope, W. G., Eads, C. B., Lazaro, P. R., Barnhart, M. C., & Shea, D. (2007). Acute and chronic toxicity of technical‐grade pesticides to glochidia and juveniles of freshwater mussels (unionidae). *Environmental Toxicology and Chemistry, 26*(10), 2086-2093.

Butterworth, J. (2008). Lake Rotokakahi: the kakahi (Hyridella menziesi) in a general framework of lake health (

Cao, Y., Stodola, A., Douglass, S., Shasteen, D., Cummings, K., & Holtrop, A. (2015). Modelling and mapping the distribution, diversity and abundance of freshwater mussels (Family Unionidae) in wadeable streams of Illinois, U.S.A. *Freshwater Biology, 60*(7), 1379-1397.

Clearwater, S. J., Thompson, K. J., & Hickey, C. W. (2014). Acute Toxicity of Copper, Zinc, and Ammonia to Larvae (Glochidia) of a Native Freshwater Mussel *Echyridella. menziesii* in New Zealand. *Archives of Environmental Contamination and Toxicology, 66*(2), 213-226.

Collier, K. J., Clearwater, S. J., Neijenhuis, P. H. M. W., & Wood, S. A. (2017). Factors influencing biodeposit production by the New Zealand freshwater mussel *Echyridella. menziesii*. *New Zealand Journal of Marine and Freshwater Research*, 1-15.

Collins, R. A., Armstrong, K. F., Holyoake, A. J., & Keeling, S. (2013). Something in the water: biosecurity monitoring of ornamental fish imports using environmental DNA. *Biological Invasions, 15*(6), 1209-1215.

Cope, W. G., Bringolf, R. B., Buchwalter, D. B., Newton, T. J., Ingersoll, C. G., Wang, N., Augspurger, T., Dwyer, F. J., Barnhart, M. C., & Neves, R. J. (2008). Differential exposure, duration, and sensitivity of unionoidean bivalve life stages to environmental contaminants. *Journal of the North American Benthological Society, 27*(2), 451-462.

Curole, J. P., & Kocher, T. D. (2002). Ancient Sex-Specific Extension of the Cytochrome c Oxidase II Gene in Bivalves and the Fidelity of Doubly-Uniparental Inheritance. *Molecular Biology and Evolution, 19*(8), 1323-1328.

Darling, J. A., & Mahon, A. R. (2011). From molecules to management: Adopting DNA-based methods for monitoring biological invasions in aquatic environments. *Environmental Research, 111*(7), 978-988.

Deiner, K., & Altermatt, F. (2014). Transport distance of invertebrate environmental DNA in a natural river. *PloS one, 9*(2), e88786.

Dejean, T., Valentini, A., Miquel, C., Taberlet, P., Bellemain, E., & Miaud, C. (2012). Improved detection of an alien invasive species through environmental DNA barcoding: the example of the American bullfrog Lithobates catesbeianus. *Journal of Applied Ecology, 49*(4), 953-959.

Dell, R. (1953). The Fresh-water Mollusca of New Zealand Part 1—The Genus Hyridella. *Transactions and Proceedings of the Royal Society of New Zealand, 81*.

Dhanasekaran, S., Doherty, T. M., & Kenneth, J. (2010). Comparison of different standards for real-time PCR-based absolute quantification. *Journal of Immunological Methods, 354*(1–2), 34-39.

Doi, H., Uchii, K., Takahara, T., Matsuhashi, S., Yamanaka, H., & Minamoto, T. (2015). Use of Droplet Digital PCR for Estimation of Fish Abundance and Biomass in Environmental DNA Surveys. *PLoS ONE, 10*(3), e0122763.

Ellis, M. (1936). Erosion silt as a factor in aquatic environments. *Ecology, 17*(1), 29-42.

Ellis, R. J. (1997). Biomonitoring of Waikato river water quality using the freshwater mussel Hyridella menziesi. thesis.

Fenwick, M. C., & Marshall, B. A. (2006). A new species of Echyridella from New Zealand, and recognition of Echyridella lucasi (Suter, 1905)(Mollusca: Bivalvia: Hyriidae). *Molluscan Research, 26*(2), 69-76.

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

Foote, A. D., Thomsen, P. F., Sveegaard, S., Wahlberg, M., Kielgast, J., Kyhn, L. A., Salling, A. B., Galatius, A., Orlando, L., & Gilbert, M. T. P. (2012). Investigating the Potential Use of Environmental DNA (eDNA) for Genetic Monitoring of Marine Mammals. *Plos One, 7*(8).

Goldberg, C. S., Sepulveda, A., Ray, A., Baumgardt, J., & Waits, L. P. (2013). Environmental DNA as a new method for early detection of New Zealand mudsnails (Potamopyrgus antipodarum). *Freshwater Science, 32*(3), 792-800.

Goldberg, C. S., Turner, C. R., Deiner, K., Klymus, K. E., Thomsen, P. F., Murphy, M. A., Spear, S. F., McKee, A., Oyler‐McCance, S. J., Cornman, R. S., Laramie, M. B., Mahon, A. R., Lance, R. F., Pilliod, D. S., Strickler, K. M., Waits, L. P., Fremier, A. K., Takahara, T., Herder, J. E., & Taberlet, P. (2016). Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods in Ecology and Evolution, 7*(11), 1299-1307.

Gordon, M. E., & Layzer, J. B. (1993). Glochidial host of Alasmidonta atropurpurea (Bivalvia: Unionoidea, Unionidae). *Transactions of the American Microscopical Society*, 145-150.

Graf, D. L., & Cummings, K. S. (2007). Review of the systematics and global diversity of freshwater mussel species (Bivalvia: Unionoida). *Journal of Molluscan Studies, 73*(4), 291-314.

Grainger, N., Collier, K. J., Hitchmough, R., Harding, J. S., Smith, B., Sutherland, D., & New Zealand. Department of, C. (2014). *Conservation status of New Zealand freshwater invertebrates, 2013* (Vol. 8.; 8). Wellington, New Zealand: Publishing Team, Department of Conversation.

Gray, J., & Dieffenbach, E. (1843). Catalogue of the species of Mollusca and their shells, which have hitherto been recorded as found at New Zealand, with the description of some lately discovered species. In *Travels in New Zealand; with contributions to the geography, geology, botany and natural history of that country* (Vol 2, pp. 228-256): J. Murray London.

Group, C. P. W., Hollingsworth, P. M., Forrest, L. L., Spouge, J. L., Hajibabaei, M., Ratnasingham, S., van der Bank, M., Chase, M. W., Cowan, R. S., Erickson, D. L., Fazekas, A. J., Graham, S. W., James, K. E., Kim, K.-J., Kress, W. J., Schneider, H., van AlphenStahl, J., Barrett, S. C. H., van den Berg, C., Bogarin, D., Burgess, K. S., Cameron, K. M., Carine, M., Chacón, J., Clark, A., Clarkson, J. J., Conrad, F., Devey, D. S., Ford, C. S., Hedderson, T. A. J., Hollingsworth, M. L., Husband, B. C., Kelly, L. J., Kesanakurti, P. R., Kim, J. S., Kim, Y.-D., Lahaye, R., Lee, H.-L., Long, D. G., Madriñán, S., Maurin, O., Meusnier, I., Newmaster, S. G., Park, C.-W., Percy, D. M., Petersen, G., Richardson, J. E., Salazar, G. A., Savolainen, V., Seberg, O., Wilkinson, M. J., Yi, D.-K., & Little, D. P. (2009). A DNA barcode for land plants. *Proceedings of the National Academy of Sciences, 106*(31), 12794-12797.

Gusman, A., Lecomte, S., Stewart, D. T., Passamonti, M., & Breton, S. (2016). Pursuing the quest for better understanding the taxonomic distribution of the system of doubly uniparental inheritance of mtDNA. *PeerJ, 4*, e2760.

Hamilton, D. P., Collier, K. J., & Howard-Williams, C. (2016). Lake Restoration in New Zealand. *Ecological Management & Restoration, 17*(3), 191-199.

Handy, S. M., Hutchins, D. A., Cary, S. C., & Coyne, K. J. (2006). Simultaneous enumeration of multiple raphidophyte species by quantitative real-time PCR: capabilities and limitations. *Limnology and Oceanography: Methods, 4*(6), 193-204.

Hebert, P. D. N., Ratnasingham, S., & de Waard, J. R. (2003a). Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London B: Biological Sciences, 270*(Suppl 1), S96-S99.

Hebert, P. D. N., Cywinska, A., Ball, S. L., & deWaard, J. R. (2003b). Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences, 270*(1512), 313-321.

Helfrich, L. A., Neves, R. J., & Chapman, H. (2005). Sustaining America's Aquatic Biodiversity. Freshwater Mussel Biodiversity and Conservation.

Hickey, C. W., Roper, D. S., & Buckland, S. J. (1995). Metal concentrations of resident and transplanted freshwater mussels Hyridella menziesi (Unionacea: Hyriidae) and sediments in the Waikato River, New Zealand. *Science of The Total Environment, 175*(3), 163-177.

Hindson, B. J., Ness, K. D., Masquelier, D. A., Belgrader, P., Heredia, N. J., Makarewicz, A. J., Bright, I. J., Lucero, M. Y., Hiddessen, A. L., Legler, T. C., Kitano, T. K., Hodel, M. R., Petersen, J. F., Wyatt, P. W., Steenblock, E. R., Shah, P. H., Bousse, L. J., Troup, C. B., Mellen, J. C., Wittmann, D. K., Erndt, N. G., Cauley, T. H., Koehler, R. T., So, A. P., Dube, S., Rose, K. A., Montesclaros, L., Wang, S., Stumbo, D. P., Hodges, S. P., Romine, S., Milanovich, F. P., White, H. E., Regan, J. F., Karlin-Neumann, G. A., Hindson, C. M., Saxonov, S., & Colston, B. W. (2011). High-Throughput Droplet Digital PCR System for Absolute Quantitation of DNA Copy Number. *Analytical Chemistry, 83*(22), 8604-8610.

Hoeh, W. R., Stewart, D. T., & Guttman, S. I. (2002). High Fidelity of Mitochondrial Genome Transmission under the Doubly Uniparental Mode of Inheritance in Freshwater Mussels (Bivalvia: Unionoidea). *Evolution, 56*(11), 2252-2261.

Hoeh, W. R., Stewart, D. T., Sutherland, B. W., & Zouros, E. (1996). Multiple Origins of Gender-Associated Mitochondrial DNA Lineages in Bivalves (Mollusca: Bivalvia). *Evolution, 50*(6), 2276-2286.

Hornbach, D. J., Kurth, V. J., & Hove, M. C. (2010). Variation in Freshwater Mussel Shell Sculpture and Shape Along a River Gradient. *American Midland Naturalist, 164*(1), 22-36.

Hunter, M. E., Dorazio, R. M., Butterfield, J. S. S., Meigs-Friend, G., Nico, L. G., & Ferrante, J. A. (2017). Detection limits of quantitative and digital PCR assays and their influence in presence–absence surveys of environmental DNA. *Molecular Ecology Resources, 17*(2), 221-229.

Jerde, C. L., Mahon, A. R., Chadderton, W. L., & Lodge, D. M. (2011). "Sight-unseen" detection of rare aquatic species using environmental DNA. *Conservation Letters, 4*(2), 150-157.

Kuenzler, E. J. (1961). Structure and energy flow of a mussel population in a Georgia salt marsh. *Limnology and Oceanography, 6*(2), 191-204.

Levinton, J. (1995). Bioturbators as ecosystem engineers: control of the sediment fabric, inter-individual interactions, and material fluxes. In *Linking Species & Ecosystems* (pp. 29-36): Springer.

Levy-Booth, D. J., Campbell, R. G., Gulden, R. H., Hart, M. M., Powell, J. R., Klironomos, J. N., Pauls, K. P., Swanton, C. J., Trevors, J. T., & Dunfield, K. E. (2007). Cycling of extracellular DNA in the soil environment. *Soil Biology and Biochemistry, 39*(12), 2977-2991.

Linnaeus, C. v. (1758). Systema naturae, 10th edn, vol. 1. *Stockholm: L. Salvii*.

Liu, W. T., Marsh, T. L., Cheng, H., & Forney, L. J. (1997). Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Applied and Environmental Microbiology, 63*(11), 4516-4522.

Lopes-Lima, M., Teixeira, A., Froufe, E., Lopes, A., Varandas, S., & Sousa, R. (2014). Biology and conservation of freshwater bivalves: past, present and future perspectives. *Hydrobiologia, 735*(1), 1-13.

Lopes-Lima, M., Sousa, R., Geist, J., Aldridge, D. C., Araujo, R., Bergengren, J., Bespalaya, Y., Bódis, E., Burlakova, L., Van Damme, D., Douda, K., Froufe, E., Georgiev, D., Gumpinger, C., Karatayev, A., Kebapçi, Ü., Killeen, I., Lajtner, J., Larsen, B. M., Lauceri, R., Legakis, A., Lois, S., Lundberg, S., Moorkens, E., Motte, G., Nagel, K.-O., Ondina, P., Outeiro, A., Paunovic, M., Prié, V., von Proschwitz, T., Riccardi, N., Rudzīte, M., Rudzītis, M., Scheder, C., Seddon, M., Şereflişan, H., Simić, V., Sokolova, S., Stoeckl, K., Taskinen, J., Teixeira, A., Thielen, F., Trichkova, T., Varandas, S., Vicentini, H., Zajac, K., Zajac, T., & Zogaris, S. (2017). Conservation status of freshwater mussels in Europe: state of the art and future challenges. *Biological Reviews, 92*(1), 572-607.

Lydeard, C., Cowie, R. H., Ponder, W. F., Bogan, A. E., Bouchet, P., Clark, S. A., Cummings, K. S., Frest, T. J., Gargominy, O., & Herbert, D. G. (2004). The global decline of nonmarine mollusks. *BioScience, 54*(4), 321-330.

Marshall, B. A., Fenwick, M. C., & Ritchie, P. A. (2014). New Zealand Recent Hyriidae (Mollusca: Bivalvia: Unionida). *Molluscan Research, 34*(3), 181-200.

McDowall, B. (2002). Decline of the kakahi–identifying cause and effect. *Water and Atmosphere 10 (4)*, 8-9.

McKee, A. M., Spear, S. F., & Pierson, T. W. (2015). The effect of dilution and the use of a post-extraction nucleic acid purification column on the accuracy, precision, and inhibition of environmental DNA samples. *Biological Conservation, 183*, 70-76.

McMichael, D. F. (Compiler) (1958). *The nature and origin of the New Zealand freshwater mussel fauna*. Transactions of the Royal Society of New Zealand.

Minamoto, T., Yamanaka, H., Takahara, T., Honjo, M. N., & Kawabata, Z. i. (2012). Surveillance of fish species composition using environmental DNA. *Limnology, 13*(2), 193-197.

Mioduchowska, M., Kaczmarczyk, A., Zając, K., Zając, T., & Sell, J. (2016). Gender-Associated Mitochondrial DNA Heteroplasmy in Somatic Tissues of the Endangered Freshwater Mussel Unio crassus (Bivalvia: Unionidae): Implications for Sex Identification and Phylogeographical Studies. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, 325*(9), 610-625.

Moore, S. (2013). *Echyridella. menziesii*. The IUCN Red List of Threatened Species. Version 2015.2. .

Naimo, T. J. (1995). A review of the effects of heavy metals on freshwater mussels. *Ecotoxicology, 4*(6), 341-362.

Navarro, J., & Thompson, R. (1997). Biodeposition by the horse mussel Modiolus modiolus (Dillwyn) during the spring diatom bloom. *Journal of Experimental Marine Biology and Ecology, 209*(1-2), 1-13.

Nowak, J., & Kozlowski, M. (2013). *Mussels: ecology, life habits and control*. Hauppauge, New York: Nova Science Publishers, Inc.

Ogram, A., Sayler, G. S., & Barkay, T. (1987). The extraction and purification of microbial DNA from sediments. *Journal of microbiological methods, 7*(2), 57-66.

Olson, Z. H., Briggler, J. T., & Williams, R. N. (2012). An eDNA approach to detect eastern hellbenders (Cryptobranchus a. alleganiensis) using samples of water. *Wildlife Research, 39*(7), 629-636.

Passamonti, M., & Ghiselli, F. (2009). Doubly Uniparental Inheritance: Two Mitochondrial Genomes, One Precious Model for Organelle DNA Inheritance and Evolution. *DNA Cell Biol, 28*.

Passamonti, M., Ricci, A., Milani, L., & Ghiselli, F. (2011). Mitochondrial genomes and Doubly Uniparental Inheritance: new insights from Musculista senhousia sex-linked mitochondrial DNAs (Bivalvia Mytilidae). *BMC Genomics, 12*(1), 442.

Phillips, N. (2007). Review of the potential for biomanipulation of phytoplankton abundance by freshwater mussels (kakahi) in the Te Arawa lakes (Environment Bay of Plenty.

Piaggio, A. J., Engeman, R. M., Hopken, M. W., Humphrey, J. S., Keacher, K. L., Bruce, W. E., & Avery, M. L. (2014). Detecting an elusive invasive species: a diagnostic PCR to detect Burmese python in Florida waters and an assessment of persistence of environmental DNA. *Molecular Ecology Resources, 14*(2), 374-380.

Pietramellara, G., Ascher, J., Borgogni, F., Ceccherini, M., Guerri, G., & Nannipieri, P. (2009). Extracellular DNA in soil and sediment: fate and ecological relevance. *Biology and Fertility of Soils, 45*(3), 219-235.

Pilliod, D. S., Goldberg, C. S., Arkle, R. S., & Waits, L. P. (2013). Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. *Canadian Journal of Fisheries and Aquatic Sciences, 70*(8), 1123-1130.

Pilliod, D. S., Goldberg, C. S., Arkle, R. S., & Waits, L. P. (2014). Factors influencing detection of eDNA from a stream-dwelling amphibian. *Molecular Ecology Resources, 14*(1), 109-116.

Ranjard, L., Poly, F., Lata, J.-C., Mougel, C., Thioulouse, J., & Nazaret, S. (2001). Characterization of bacterial and fungal soil communities by automated ribosomal intergenic spacer analysis fingerprints: biological and methodological variability. *Applied and environmental microbiology, 67*(10), 4479-4487.

Raut, A. D., Kapadnis, B. P., Shashidhar, R., Bandekar, J. R., Vaishampayan, P., & Shouche, Y. S. (2007). Nonspecific PCR amplification of the 16S rRNA gene segment in different bacteria by use of primers specific for Campylobacter, Arcobacter, and Helicobacter spp. *Journal of clinical microbiology, 45*(4), 1376-1377.

Rees, H. C., Maddison, B. C., Middleditch, D. J., Patmore, J. R. M., & Gough, K. C. (2014). REVIEW: The detection of aquatic animal species using environmental DNA – a review of eDNA as a survey tool in ecology. *Journal of Applied Ecology, 51*(5), 1450-1459.

Rogers-Lowery, C. L., & Dimock Jr, R. V. (2006). Encapsulation of attached ectoparasitic glochidia larvae of freshwater mussels by epithelial tissue on fins of naive and resistant host fish. *The Biological Bulletin, 210*(1), 51-63.

Rondon, M. R., August, P. R., Bettermann, A. D., Brady, S. F., Grossman, T. H., Liles, M. R., Loiacono, K. A., Lynch, B. A., MacNeil, I. A., & Minor, C. (2000). Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. *Applied and environmental microbiology, 66*(6), 2541-2547.

Roper, D. S., & Hickey, C. W. (1994). Population structure, shell morphology, age and condition of the freshwater mussel Hyridella menziesi (Unionacea: Hyriidae) from seven lake and river sites in the Waikato River system. *Hydrobiologia, 284*(3), 205-217.

Smart, A. S., Weeks, A. R., Rooyen, A. R. v., Moore, A., McCarthy, M. A., & Tingley, R. (2016). Assessing the cost‐efficiency of environmental DNA sampling. *Methods in Ecology and Evolution, 7*(11), 1291-1298.

Śmietanka, B., Wenne, R., & Burzyński, A. (2016). Complete male mitochondrial genomes of European Mytilus edulis mussels. *Mitochondrial DNA Part A, 27*(3), 1634-1635.

Soroka, M. (2008). Doubly uniparental inheritance of mitochondrial DNA in the freshwater bivalve Anodonta woodiana (Bivalvia: Unionidae). *Folia Biol (Krakow), 56*(1-2), 91-5.

Sousa, R., Novais, A., Costa, R., & Strayer, D. L. (2014). Invasive bivalves in fresh waters: impacts from individuals to ecosystems and possible control strategies. *Hydrobiologia, 735*(1), 233-251.

Stankovic, S., & Jovic, M. (2012). Health risks of heavy metals in the mediterranean mussels as seafood. *Environmental chemistry letters, 10*(2), 119-130.

Stankovic, S., Jovic, M., Stankovic, A. R., & Katsikas, L. (2012). Heavy metals in seafood mussels. Risks for human health. In *Environmental chemistry for a sustainable world* (pp. 311-373): Springer.

Stansbery, D. H. (1970). Eastern Freshwater Mollusks: The Mississippi and St. Lawrence River Systems. I (Institute of Malacology.

Strayer, D. L. (1999). Effects of alien species on freshwater mollusks in North America. *Journal of the North American Benthological Society, 18*(1), 74-98.

Strayer, D. L., & Smith, D. R. (2003). *A guide to sampling freshwater mussel populations* (Vol. no. 8; 8). Bethesda, Md: American Fisheries Society.

Strayer, D. L., Caraco, N. F., Cole, J. J., Findlay, S., & Pace, M. L. (1999). Transformation of freshwater ecosystems by bivalves. *BioScience, 49*(1), 19-27.

Strayer, D. L., Downing, J. A., Haag, W. R., King, T. L., Layzer, J. B., Newton, T. J., & Nichols, S. J. (2004). Changing perspectives on pearly mussels, North America's most imperiled animals. *BioScience, 54*(5), 429-439.

Suter, H. (1913). Manual of the New Zealand mollusca: With an atlas of quarto plates (Vol. 1: J. Mackay, Government Printer.

Taberlet, P., Coissac, E., Hajibabaei, M., & Rieseberg, L. H. (2012). Environmental DNA. *Molecular ecology, 21*(8), 1789-1793.

Takahara, T., Minamoto, T., Yamanaka, H., Doi, H., & Kawabata, Z. i. (2012). Estimation of Fish Biomass Using Environmental DNA. *Plos One, 7*(4).

Thomsen, P. F., Kielgast, J. O. S., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., Orlando, L., & Willerslev, E. (2012). Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology, 21*(11), 2565-2573.

Trdan, R. J., & Hoeh, W. R. (1982). Eurytopic host use by two congeneric species of freshwater mussel (Pelecypoda: Unionidae: Anodonta). *American Midland Naturalist*, 381-388.

Turner, C. R., Barnes, M. A., Xu, C. C. Y., Jones, S. E., Jerde, C. L., & Lodge, D. M. (2014). Particle size distribution and optimal capture of aqueous macrobial eDNA. *Methods in Ecology and Evolution, 5*(7), 676-684.

Vaughn, C. C., & Hakenkamp, C. C. (2001). The functional role of burrowing bivalves in freshwater ecosystems. *Freshwater Biology, 46*(11), 1431-1446.

Walker, J. M., Curole, J. P., Wade, D. E., Chapman, E. G., Bogan, A. E., Watters, G. T., & Hoeh, W. R. (2006). Taxonomic distribution and phylogenetic utility of gender-associated mitochondrial genomes in the Unionoida (Bivalvia). *MALACOLOGIA-PHILADELPHIA-, 48*(1/2), 265.

Walker, K. F., Byrne, M., Hickey, C. W., & Roper, D. S. (2001). Freshwater mussels (Hyriidae) of Australasia. In *Ecology and evolution of the freshwater mussels Unionoida* (pp. 5-31): Springer.

Watters, G. T. (Compiler) (1999). *Freshwater mussels and water quality: a review of the effects of hydrologic and instream habitat alterations*. Proceedings of the First Freshwater Mollusk Conservation Society Symposium.

Watters, G. T., & O'dee, S. (Compiler) (2000). *Glochidial release as a function of water temperature: beyond bradyticty and tachyticty*. Proceedings of the conservation, captive care, and propagation of freshwater mussels symposium. Ohio Biological Survey, Ohio.

Welker, M., & Walz, N. (1998). Can mussels control the plankton in rivers?—a planktological approach applying a Lagrangian sampling strategy. *Limnology and Oceanography, 43*(5), 753-762.

Williams, J. D., Warren Jr, M. L., Cummings, K. S., Harris, J. L., & Neves, R. J. (1993). Conservation status of freshwater mussels of the United States and Canada. *Fisheries, 18*(9), 6-22.

Wood, S. A., Smith, K. F., Banks, J. C., Tremblay, L. A., Rhodes, L., Mountfort, D., Cary, S. C., & Pochon, X. (2013). Molecular genetic tools for environmental monitoring of New Zealand's aquatic habitats, past, present and the future. *New Zealand Journal of Marine and Freshwater Research, 47*(1), 90-119.

Zouros, E. (2013). Biparental Inheritance Through Uniparental Transmission: The Doubly Uniparental Inheritance (DUI) of Mitochondrial DNA. *Evolutionary Biology, 40*(1), 1-31.

Zouros, E., Ball, A. O., Saavedra, C., & Freeman, K. R. (1994). Mitochondrial DNA inheritance. *Nature, 368*.

Chapter III Design and validation of species-specific dPCR assay

III-1 Introduction

The aim of this project is to develop assays to detect and quantify three New Zealand indigenous freshwater mussel species in environmental DNA (eDNA). Among current techniques for species detection in eDNA, we chose digital PCR (dPCR) because of its advantages in reducing false positive and false negative errors. This chapter briefly explains the advantages of dPCR, followed by descriptions of the development of dPCR assays, including primer design, probe design, and assay validations.

False positive and false negative PCR results usually arise from misinterpretations of PCR results in which method errors are present (Table III-1). More specifically, false positive results are caused by insufficient specificity of the method or target DNA contamination (e.g., mishandling of positive control). When the true target is absent, amplification of non-targets or target contaminants can easily lead to a false positive result. False negative occurs when a method has insufficient sensitivity or efficiency to detect and amplify its target, demanding a better method with higher target/non-target

ratio. A successful species detection method should minimise false positive and false negative errors.

Table III-1 Possible positive and negative result of PCR amplification

	Target present	Target absent
Positive outcome (Amplification present)	True positive	False positive (Insufficient specificity or target DNA contamination)
Negative outcome (Amplification absent)	False negative (Insufficient sensitivity or efficiency and low detection rate)	True negative

We choose dPCR because of its advantages in reducing false positive and false negative errors, relative to quantitative PCR (qPCR) and conventional PCR. dPCR utilises TaqMan probe and primer. The TaqMan probe is known to be highly sensitive among known commercially available probes. The combination of primer and TaqMan probe will increase the specificity of the assay. Altogether, these attributes reduces false positive errors.

In reducing the false negative, dPCR divides reaction mix into thousands subreactions. The partitioning of samples in dPCR can reduce the inhibition significantly by increase the target/non-target ratio. In this thesis, I used the QuantStudio™ 3D Digital PCR platform (Thermo Fisher Scientific) available in the lab. The partitioning of reaction mix for this dPCR platform is implemented by a chip containing 20,000 small wells. Each target will have significantly

higher target/non-target ratio in a well of 755pL instead of in total eDNA template. Moreover, small wells make each target more accessible to primers, probes, and polymerases.

In this study, the *COI* gene is chosen as targeted gene not only because it is a widely accepted marker but also the *COI* sequences of three NZ mussels are available in GenBank. In the development of dPCR assays, multiple candidates of dPCR primers are designed to amplify a conserved region of the *COI* gene for all three New Zealand freshwater mussel species using Geneious R9 (http://www.geneious.com, Kearse et al., 2012) (Figure III-1). Then, the feature of each primers such as product length, primer-dimer, degeneracy will be assessed, and primer pairs will be prioritised. Lastly, specificity of the best candidate primer pair will be checked by searching for any possible unintended target using Primer-BLAST (https://www.ncbi.nlm.nih.gov /tools/primer-blast/). Primers hitting non-target species that may be present in New Zealand will be excluded.

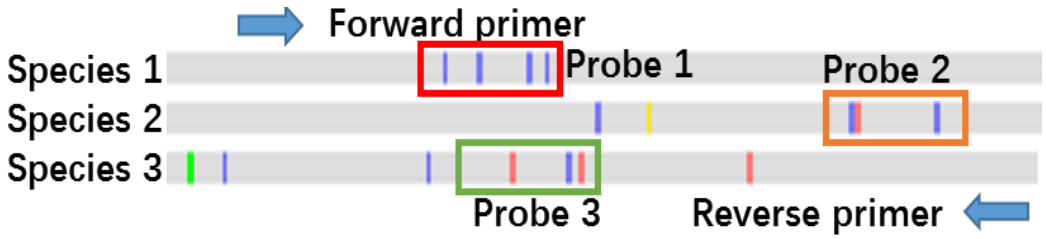


Figure III-1 Schematic diagram of multiple sequence alignment of three sequences and probe design. Each sequence represents each species. Grey shaded area indicates identical sequence. Coloured bars indicates interspecies heterogenetic bases. Targeted area of each probe shown in coloured boxes. Note each box has unique interspecies heterogenic bases.

In probe design, Geneious will be used to generate probes for each species. The probes will be checked against the alignment of *Echyridella* sequences to see if they are unique enough to distinguish individual species by looking at how many interspecies mismatches they have (Figure III-1). Probes with the most different bases compare to the other two species is selected.

To facilitate detection by fluorescence, each probe is synthesised with a designated fluorescent dye. The QuantStudio™ 3D Digital PCR platform officially supports two fluorescent molecules: FAM and VIC. Therefore, fluorescent dye assignment during probe design determines which two of the three species can be detected simultaneously in a duplex assay.

Among the three indigenous freshwater mussel species in New Zealand, *E. menziesii* is known to be widespread while *E. aucklandica* and *E. onekaka* are restricted to certain areas (Walker *et al.*, 2014). Moreover, *E. onekaka* was only recently recognised as a distinct species from *E. menziesii* based on DNA evidence. These observation suggest stronger needs for detecting and quantifying *E. aucklandica* and *E. onekaka* rather than the well-known *E. menziesii.* Therefore, the FAM dye was assigned to the *E. onekaka* probe,

and VIC was assigned to the *E. aucklandica* probe. FAM is also assigned to the third probe targeting *E. menziesii.*

After primer and probe design, each probe is validated empirically by examining their sensitivity and specificity against various samples (Figure III-2).

The process of assay validation includes three stages:

1. Validate assays against target mussel species

Digital PCR will be run against target sample using single-probe assay to validate each probe. Two types of sample are used. One is PCR amplicon, it is a PCR product long enough to cover the targeted region of the newly designed primer set. If PCR amplicon can be amplified in dPCR, it demonstrates that the assay works with the targeted sequence.

The other target sample is genomic DNA (gDNA). The gDNA is DNA extracted from target mussel species. If the newly designed primer set can amplfy gDNA in dPCR after validation with PCR amplicons, it proves that the assay can amplify the target with the interference of mussel genomic content.

More importantly, dPCR will be conducted against a serial dilution of the target PCR amplicons allowing examination of the limit of detection of each

probe. The number of copies per microliter of known PCR product can be calculated by the formula:

$$\text{Number of copies/}\mu\text{L} = \frac{\text{ng/}\mu\text{L} \times 6.022\times10^{23}}{\text{Length of PCR amplicon} \times 1\times10^{9}\times650}$$

Since the concentration of PCR amplicons can also be detected by dPCR. By comparing the concentrations from calculation and dPCR, the sensitivity of each assay is examined.

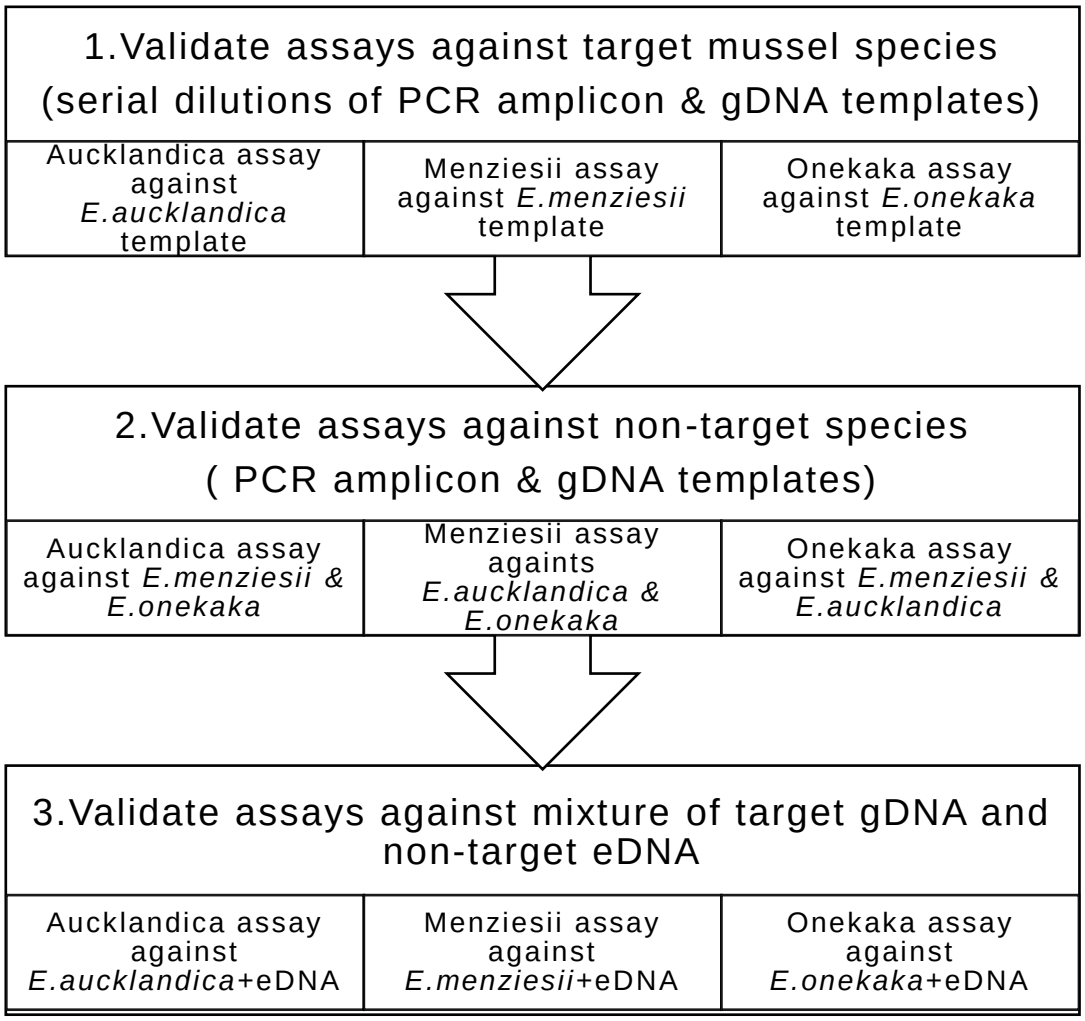


Figure III-2 Process of assay validation

The target concentration should not exceed 32,000 copies per chip since there are 20,000 wells in each dPCR chip and the recommended copies per well is 0.6~1.6 by the user guide of QuantStudio ™ 3D Digital PCR System. The PCR amplicons will be diluted 1 in 10 from 32,000 copies/µL to 3.2 copies/µL. Each chip will be loaded with 14.5µL of total dPCR reaction containing 1µL PCR amplicons as template.

2. Validate assays against non-target species

Although each probe is designed to be specific to its targeted species, the lack of cross-reactivity with the two closely related mussel species needs to be experimentally verified. To do so, dPCR will be carried using each probe against the two non-targeted species. Single-probe assays are run using long PCR amplicons and gDNA from two non-target species as template.

3. Validate assay against mixture of target DNA and non-target eDNA

To confirm that target can be detected by dPCR in the presence of large amounts of non-target eDNA, PCR amplicons will be spiked with various amounts of soil eDNA.

III-2 Material and methods

III-2-1 Mussel samples

Ten frozen mussel specimens and tissues of twenty-two mussels were obtained (Appendix I). Mussel specimens No. 1 to 5 were kindly provided by Kevin Collier (University of Waikato), and specimens No. 6 to 10 were kindly provided by Mark Hamer (Waikato Regional Council). The species identity of specimens had been identified morphologically. Frozen mussel tissue samples were kindly provided by Sue Clearwater (NIWA). Tissues were dissected from foot and mantle of mussels specimens which are suspected as *E. onekaka*. For consistency, all gDNA samples were extracted from foot tissue.

III-2-2 DNA extraction

Mussel genomic DNA was extracted from mussel tissues using E.N.Z.A Mollusc KIT (Omega Bio-tek Inc., Norcross, GA, USA). For each mussel specimen, foot tissue was cut and pulverised in liquid nitrogen with a mortar and pestle. After solubilisation with buffer and proteinase K solution provided, chloroform:isoamyl alcohol (24:1) were added into samples. The samples were centrifuged for collecting upper aqueous layer which was then added with 100% ethanol for washing. The DNA was finally extracted by centrifuge

samples in spin columns and collected by elution buffer. following the protocol provided by the kit manual. All extracted genomic DNA samples were quantified using a Qubit Fluorometer following recommended protocols for the dsDNA HS Assay, which has a high accuracy for double-stranded DNA between 1 ng/mL to 500 ng/mL (Life Technologies, Carlsbad, CA, USA). Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) was also used for assessing DNA quality and quantity. All DNA samples were stored in -20℃ freezer.

Soil DNA for spiking the target PCR amplicons was an environmental sample collected from the garden area outside the door of Thermopile Research Unit, by the carpark. The soil was scooped into a 50mL falcon tube using a sterile spatula. The soil was homogenised by shaking up and down a few times and then 0.3g was weighed out. The sample was extracted using the PowerSoil DNA isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) and stored in -20℃ freezer.

III-2-3 PCR

Standard PCR was run using a DNA Engine® Thermal Cycler (Bio-Rad Laboratories Ltd, CA, USA). Reaction volumes for the 57F - 322R pair were as follows for a 25µl reaction: 2.5 µl 10 X buffer, 2.5 µl dNTP mix (2 mM), 1.25

µl MgCl2 (50mM), 0.2 µl Taq Platinum DNA Polymerase (5U/µl), 1 µl of each primer (10 µM), 2 µl DNA (1ng/µl), 14.55 µl MiliQ water.

Samples were placed on the thermocycler with the following program:

Incubation at 95ºC for 3 min
Denaturation at 95ºC for 30 seconds
Annealing at 60ºC for 30 seconds
Elongation at 72ºC for 30 seconds
Steps (i)-(iii) repeated 34 times
A final extension at 72ºC for 5 min.

For PCR with other primer sets, elongation time varies according to the predicted length of the product. The elongation time was calculated accordingly at a rate of 500bp per 30 seconds.

III-2-4 Gel electrophoresis

PCR products were run in 1% TAE Gels consisting of 0.3g agarose, 30mL 1x TAE buffer, and 0.4µL SyberSafe (10,000X concentrate) under 70V for 25mins. The gels were viewed using the AlphaImager (ProteinSimple, CA, USA).

III-2-5 Primer design

From GenBank (https://www.ncbi.nlm.nih.gov/genbank/), partial *COI* gene sequences for *Echyridella. menziesii* (includes sequence from its synonym *Hyridella menziesii*), *Echyridella. aucklandica*, and *Echyridella. onekaka* were

downloaded. Geneious was used to make a multiple sequence alignment of these sequences.

Primers were designed using the “Design New Primers” in Geneious which integrates Primer3 (http://primer3.sourceforge.net/) for primer design. In the designing setting screen, “Included Region” that sequence within which primers can fall is set to be conserved region located by alignment. T_m is set to 60 degrees since the primer is designated to use in dPCR. Characteristics of each primer returned are recorded. Possible nonspecific amplifications of primer pairs were searched by an online tool Primer-BLAST (https://www.ncbi.nlm.nih.gov /tools/primer-blast/) to prioritise candidate primers.

III-2-6 Probe design

In Geneious, multiple sequences alignment was trimmed to the amplified region of primer pairs designed. Candidate probes for each species were designed by Geneious. The binding site of each probe was compared to the other two species on the multiple sequence alignment manually. Probes that had the highest number of bases different from the non-target species were chosen. Each probe was put into OligoAnalyzer 3.1

(https://sg.idtdna.com/calc/analyzer) to adjust its annealing temperature to 60 degrees by deleting or adding bases.

III-2-7 Digital PCR

All dPCR reactions were carried out using the QuantStudio™ 3D Digital PCR platform (Thermo Fisher Scientific). Unless specified, dPCR reaction mix is prepared as recommended by user guide and reactions were run using default thermal cycling programme. Protocol for single-probe dPCR was as follows for one chip: 7.25 µl Master Mix, 0.363 µl TaqMan probe (10 µM), 0.625 µl of each primer (10 µM), 2 µl DNA (1ng/µl), and MiliQ water to make a final volume of 14.5µl. The reaction mix was loaded onto a QuantStudio™ 3D Digital PCR Chip v2 using the included Chip Loader. dPCR was performed using the ProFlex™ 2x Flat PCR System. dPCR chips were read using the QuantStudio™ 3D Digital PCR Chip Reader. Results were viewed on the instrument touchscreen and further analysed using the QuantStudio™ 3D Analysis Suite™ (https://apps.thermofisher.com/quantstudio3d/).

During analysis, many settings were adjusted where appropriate. “Dilution” is the dilution of the sample in a reaction of 14.5 µL. It was set to 0.069 since the volume of DNA added was always 1 µL. The threshold used for quantification is usually auto calculated. On the scatter plot representing

the fluorescent signals generated by probes, a threshold is auto-calculated to separate amplification dots from non-amplification dots. Auto-calculation may fail when the separation is not obvious, in this situation manual adjustment will apply.

III-3 Result

III-3-1 Primer design

III-3-1-1 Sequence acquisition

All available (as of 2015-11-27) partial *COI* gene sequences of the New Zealand freshwater mussel genus *Echyridella* were retrieved from GenBank (Table III-2). There were 26 sequences for *Echyridella. menziesii*, 5 sequences for *Echyridella. aucklandica*, and 1 sequence for *Echyridella. onekaka.*

Among the 26 sequences for *E. menziesii,* two (AY785398.1 and AF406802.1) were excluded since they were significantly different from the rest (65.7% and 65.8% identical to the consensus sequence while the remaining 24 sequences are 94% identical on average, Figure III-3). These two sequences are likely male-type mtDNA due to the doubly uniparental inheritance that occurs in some bivalve species (Soroka, 2008). Consequently, only 24 sequences from *E. menziesii* were included in a multiple sequence alignment containing 30 sequences from three species (Figure III-4).

Table III-2 Accession numbers of all sequences from GenBank

Excluded two records shown in italic.

Species	Accession number of *COI* sequence in GenBank				
Echyridella. menziesii	AF231747.1	AF305369.1	AF305370.1	*AF406802.1*	AY785394.1
	AY785398.1	HM849074.1	HQ912967.1	HQ912968.1	JN612806.1
	JN612807.1	JN612808.1	JN612809.1	JN612810.1	JN612811.1
	JN612812.1	JN612813.1	JN612814.1	JN612815.1	JN612816.1
	JN612817.1	JN612818.1	JN612819.1	JN612820.1	JN612821.1
	JN612822.1				
Echyridella. aucklandica	HQ912965.1	HQ912966.1	KF866128.1	KF866129.1	KF866130.1
Echyridella. onekaka	HQ912969.1				

Figure III-3. Alignment of 26 *E. menziesii* partial *COI* sequences. Grey lines indicate sites identical to the consensus sequence and coloured blocks indicate sites different from consensus. Sequence no.25 is selected to show statistics on the right side. Note the identical site of No.25 against the majority is only 406 (65.7%).

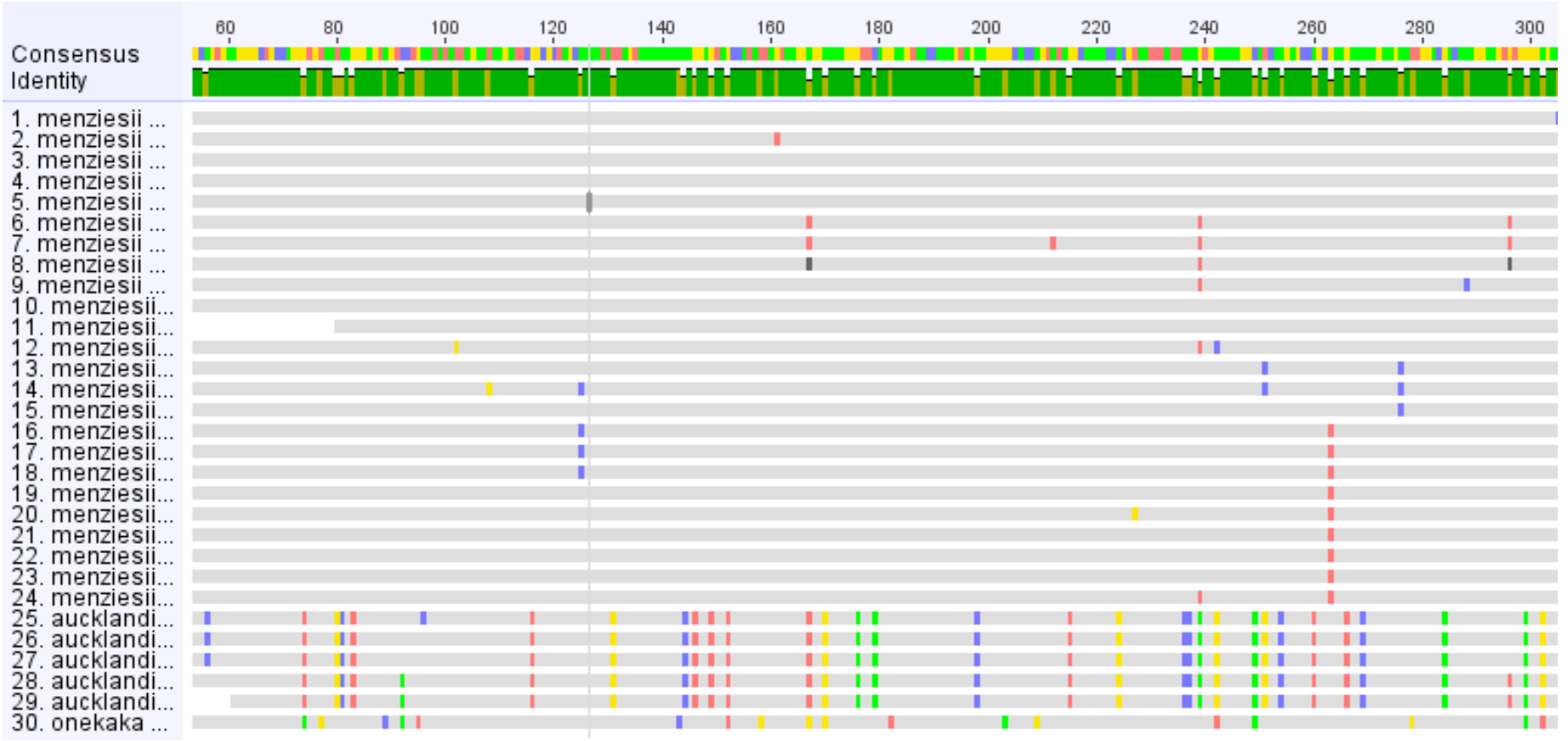


Figure III-4 Partial view of the alignment of 30 sequences. Sequence No.1-24: *E. menziesii*, No. 25-29: *E. aucklandica,* No.30: *E. onekaka*. The conserved bases are shown in grey. Coloured blocks on No.1-24 showed intraspecies heterogenic bases within *E. menziesii* while on No.25-30 showed the heterogenic base of *E. aucklandica* and *E. onekaka* from *E. menziesii.*

III-3-1-2 Multiple sequence alignment of three species

The alignment of all available *Echyridella* partial *COI* gene sequences revealed some useful information about the three species (Figure III-4). Records of *E. menziesii* comprise the majority of the alignment, and they include some intraspecies heterogenetic bases. These heterogenetic bases of *E. menziesii* records correspond with the understanding that species with a wide distribution over variable environments have intraspecies variability (Zardi *et al.*, 2015). The 6 records of *E. aucklandica* has several heterogenetic bases within species, but they also present significant interspecies variability compared with *E. menziesii*. Moreover, for *E. onekaka*, there is only one record since it is a newly identified species and poorly studied (Fenwick & Marshall, 2006).

III-3-1-3 Degenerate primer design

Geneious failed to design standard PCR primers that can amplify sequences of all three species because potential primers have either long homopolymers or low T_m. We then decided to design degenerate primer since it tolerates amplification of heterogenetic bases. A degenerate primer is a mix of similar but not identical oligonucleotide sequences in which some positions contain several possible bases. It gives a population of primers with similar

sequences that cover all possible nucleotide combinations. For example, position 317 in the alignment is G for *E. menziesii* and *E. onekaka*, but A for *E. aucklandica* (Figure III-5). Normal primer design will exclude this position, results in no primer returned from its surrounding region. However, degenerate primer design will consider it as a degenerate base and consequently generate a degenerate primer GTTGGGACTGGGTGRACTG consisting of two primers GTTGGGACTGGGTGAACTG and GTTGGGACTGGGTGGACTG.

In primer design, the term “degeneracy” indicates the maximum number of primers that a degenerate primer sequence can represent. For example, a primer that contains an N and an R has degeneracy of 4 × 2 = 8 because N represents four bases A, T, C, and G while R accounts for two bases A and G. Importantly, the degeneracy should be kept as low as possible in the primers. A high degree of degeneracy reduces the concentration of the primer that perfectly match the target, leading to issues with primer exhaustion as PCR progresses. A high degree of degeneracy also increases the likelihood of amplifying non-target sequences.

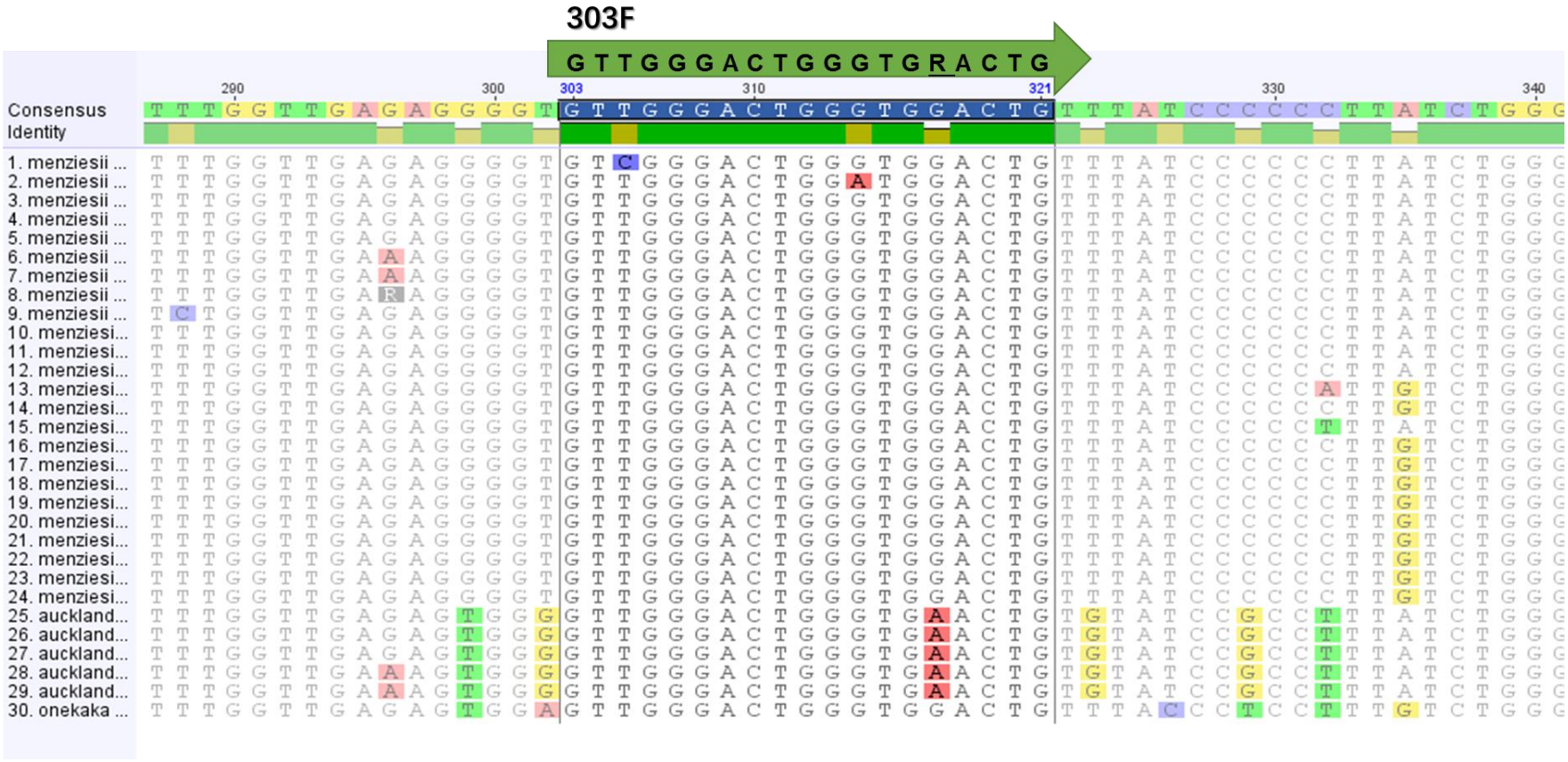


Figure III-5 Degenerate primer 303F (degeneracy value :2) and its targeting sequence in alignment as an example. Note the degenerate base R in the primer represents both G and A in alignment. Minor intraspecies heterogenic bases of *E. menziesii* are ignored

In Geneious, degeneracy was increased gradually to generate primers allowing amplification across species (e.g., degeneracy value of 2 in 303F, Figure III-5). Four forward primers and five reverse primers were generated when degeneracy value was increased to 8. They give seven possible primer pairs (Table III-3). Three primer pairs from five primers were selected since they have low degeneracy value independently and as pairs (Table III-3).

Table III-3 Possible primer pairs.

Product size and degeneracy of each primer and primer pair, chosen primer pair coloured in bold.

No.	Forward Primer (degeneracy)	Reverse Primer (degeneracy)	Product Size	Primer pair Degeneracy
1	57F (1)	208R (4)	152	1x4=4
2	**303F (2)**	**514R (2)**	**213**	**2x2=4**
3	57F (1)	218R (8)	162	1x8=8
4	191F (8)	322R (2)	132	8x2=16
5	**57F (1)**	**322R (2)**	**265**	**1x2=2**
6	199F (8)	322R (2)	124	8x2=16
7	**303F (2)**	**543R (2)**	**241**	**2x2=4**

Five candidate primers were finalised after minor modification (Table III-4). They were either trimmed or extended to get an annealing temperature close to 60℃ since digital PCR use 60℃ as its default annealing temperature. Their T_m were calculated by OligoAnalyzer.

Table III-4 Sequence of candidate primers

Degenerate base shown with underline. Note R=A/G, Y=C/T

Primer	Sequence (5->3)
57F	GAGTTGGGGCAGCCTG
303F	GTTGGGACTGGGTGRACTG
322R	ACAGTYCACCCAGTCCCAA
514R	GCCGTTACCGTAACACTYC
543R	ACACAGGCAAAGARGCAACC

III-3-1-4 Primer Validation *in silica*

Primers were tested *in silica* by predicting their performance and possible nonspecific amplification. Features that are known to affect primer performance, including hairpin, self-dimer, and heterodimer of primer pairs, were checked using OligoAnalyzer.

Possible nonspecific amplifications of primer pairs were also searched using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer-BLAST screens potential PCR primers against user-selected databases to identify existing sequence entries that can cause non-specific amplification (all primer combinations including forward-reverse, forward-forward, and reverse-reverse) (Ye *et al.*, 2012). In this case, the nucleotide database “nr” was chosen to run Primer-BLAST. The nr database has the

greatest number of sequences and is most likely to identify potential non-targets that primers may amplify. Primer-BLAST was set to return unintended targets with 0 to 2 mismatched bases.

Primer-BLAST of the 57F-322R pair showed three perfect match with unintended target. The three hits are freshwater mussels. The distribution of known specimens of these the three species were checked on the MUSSEL Project (http://mussel-project.uwsp.edu). It has been confirmed that one species is from Australia and two from South America (Table III-5). All the remaining non-target showed one or more mismatches (selectively shown in Appendix III).

Table III-5 Unintended target of 57F-322R with no mismatch from Primer-BLAST.

Degenerative base shown in bold. 57F not shown since it hits all three species.

	Distribution	322R ACAGT**Y**CACCCAGTCCCAA
>KX713505.1 Triplodon corrugatus	Amazon Basin of Brazil and Peru, north to the Guyana	ACAGT**C**CACCCAGTCCCAA
>JN612836.1 Hyridella australis	Queensland south to Victoria, eastern Australia	ACAGT**T**CACCCAGTCCCAA
>AF231744.1 Diplodon deceptus	Rio Uruguay and Rio Grande do Sul, Brazil	ACAGT**T**CACCCAGTCCCAA

All unintended targets of 57F-322R have been researched. None of them is known to present in New Zealand. Most the unintended targets are

freshwater mussel species native to other continents including Australia, North America, South America and Asia. The remaining unintended targets come from snails, clams, spiders and worms, which are neither native to New Zealand nor known to present in New Zealand before.

Primer-BLAST of the other two primer pairs were also conducted. The 303F-514R has a degeneracy of four, which means there are four possible combinations of primer pairs to be tested in Primer-BLAST. Same situation applies to 303F-543R. All the combinations of degenerative primer pairs have been tested with each of them showed several perfect matches with unintended targets. All the perfect hits have been confirmed as freshwater mussels. Distribution of theses mussels showed no records in New Zealand.

Since three primer pairs have similar result on Primer-BLAST, 57F-322R which has the least degeneracy (2 compare with 4 of the other two pairs) was selected in future work. The primer pair was then validated in PCR by testing with gDNA samples of three species separately. The products of PCR were confirmed by agarose gel. All the bands showed at the expected product size (265bp). The PCR products were further validated by sequencing, and the sequences proved that the amplification was successful (data not shown).

III-3-1-5 Primer Optimization

Primer pair 57F-322R was chosen for consequent assay development since it has the lowest degree of degeneracy. The PCR condition of this primer pair was optimised using a gradient PCR. Gradient PCR is a method where multiple PCR with same primers and DNA sample are conducted with a gradient of different conditions. In this case a gradient of annealing temperature (55℃ to 65℃,8 temperatures in total) combined with a gradient of $MgCl_2$ concentration (1.5mM, 2.0mM, 2.5mM, 3.0mM) and two primer concentrations (0.2mM, 0.4mM) were tested. Gradient PCR was carried out with No.4 gDNA sample (Appendix I) which had the highest yield from DNA extraction.

All 64 PCR conditions resulted in clear bands on the agarose gel (data not shown), indicating that the assay is not overly sensitive to PCR condition. Further PCRs were performed using the highest $MgCl_2$ concentration (3.0 mM) and primer concentration (0.4 mM) with an annealing temperature of 60°C as the optimised condition.

III-3-2 Species confirmation

With the chosen primer pair (57F-322R), the species of mussel sample can be confirmed through sequencing. Extracted gDNA sample were

amplified using PCR with the optimised condition, and the amplicons were sequenced. Species identity of all the samples were verified (Appendix I), except four that have extraction yield too low for successful PCR. Sample No. 7 (*E.Menziesii*), No. 10 (*E.Aucklandica*), and M12 (*E.Onekaka*) were used as template in further assay validation.

III-3-3 Probe design

Species-specific probes were designed in the sequence alignment of each species using Geneious. All probes were designed within the region amplified by primer pair 57F and 322R. Ten candidate probes for each species were generated with default parameters.

Probes of each species were checked manually across the alignment of three species. The probe that has the most mismatches with the other two non-target species was chosen (Figure III-6). The probe of *E. menziesii* (M165P) has three different bases compare to *E. aucklandica*, and two different bases compare with *E. onekaka*. Probe of *E. onekaka* (O211P) has two distinct bases compare to *E. menziesii*, and three different bases compare with *E. aucklandica*. *E. aucklandica* probe (A251P) has six different bases compare to *E. menziesii* and *E. onekaka*.

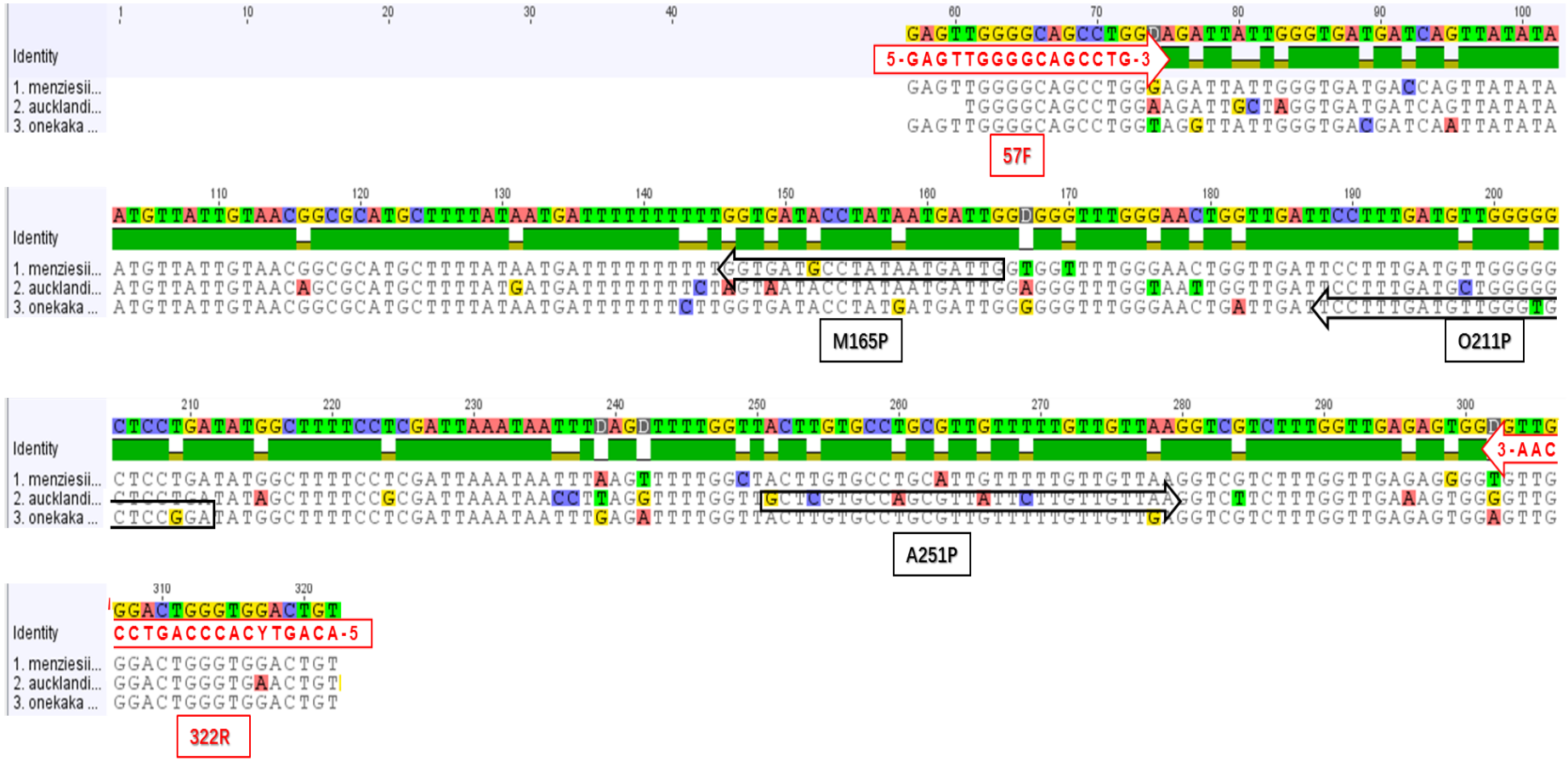


Figure III-6 Amplicons of three species by 57F-322R. Forward and reverse primers shown in red arrows labelled with red box. Probes shown in transparent black arrows labelled with black box. Bases that are different from consensus sequence are coloured

The three chosen probe sequences were analysed use Primer Express® software v3.0 (Applied Biosystems) to calculate the T_m of the synthesised TaqMan probes. Two probes were switched into their reverse complement since they have too many Gs which are not recommended by the Primer Express designing guidelines. Probe for *E. menziesii* (M165P) was designed as an minor groove binder (MGB) probe which has a high T_m and increased specificity with short sequence (Kutyavin *et al.*, 2000) since the original long probe has a too low T_m. All three probes were finalised in Table III-6.

Table III-6 Probe of each species

Probe name	Target species	Probe sequence (5'-3')	Reporter
M165P	E. menziesii	CAATCATTATAGGCATCACC (antisense)	FAM
A251P	E. aucklandica	CCAGCGTTATTCTTGTTGTTAA (sense)	VIC
O211P	E. onekaka	TCCGGAGCACCCAACATCAAAGGA (antisense)	FAM

III-3-4 Assay Validation

III-3-4-1 Validate assays against target species

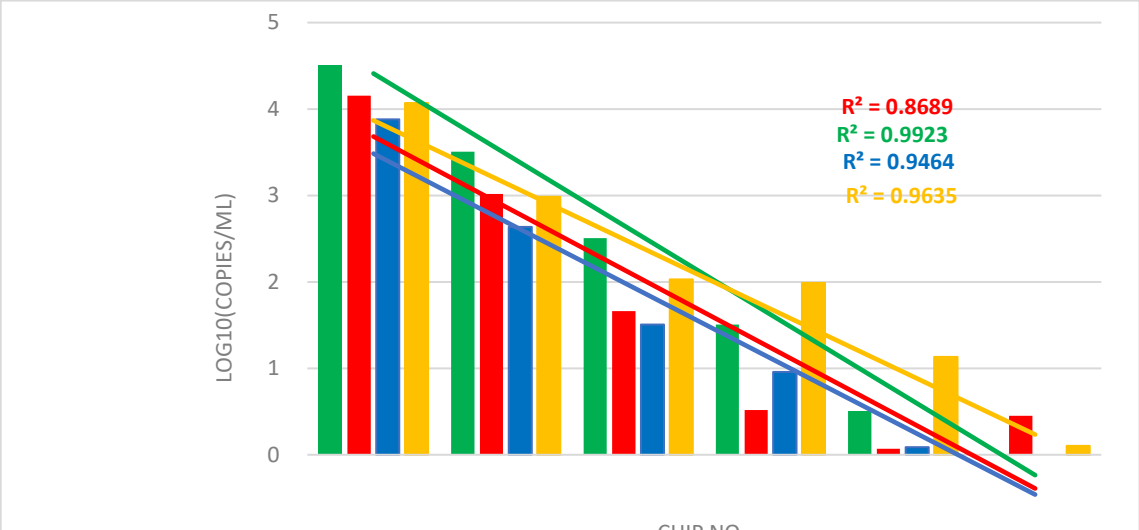
Three single-probe assays all showed amplifications with PCR amplicons (*COI* fragment amplified using primer 57F-543R) of their targeted species. The result of three assays against serial dilutions of PCR amplicons is shown

below (Table III-7). Concentrations are calculated by QuantStudio™ 3D AnalysisSuite™ Software.

Table III-7 Result of 10-fold serial diluted PCR amplicon against three single probe assay in dPCR

		1	2	3	4	5	6
Estimated Template concentration (copies/µL)		32,000	3,200	320	32	3.2	0.32
Result concentration (copies/µL)	E. aucklandica	14,325.00	1,037.90	45.97	3.30	1.18	2.83
	E. onekaka	7,609.50	433.77	32.17	9.09	1.22	0.00
	E. menziesii	11,754.00	967.46	106.70	98.83	13.55	1.29

To better analyse the data, concentrations from expectation and detection are converted to log10. Results are present in a bar chart (Figure III-7). The linear regression of four data sets (one by calculation and three by dPCR) are shown by R^2. It can be found that detection by O211P assay showed the best linear relation while M165P is less linear.



(1)

	1	2	3	4	5	6
Estimated concentration	4.505	3.505	2.505	1.505	0.505	0.000
E.aucklandica	4.156	3.016	1.662	0.519	0.071	0.451
E.onekaka	3.881	2.637	1.507	0.959	0.086	0.000
E.menziesii	4.070	2.986	2.028	1.986	1.132	0.103

Figure III-7 Lower limit dPCR experiment in serial dilution

For the A251P assay, when the estimated target concentration falls below 320 copies/µL, there are fewer than one-tenth of them been detected. It implies that the result is not reliable below this concentration. As a result, target concentration result of A251P under 3200 copies/µL is not reliable.

Concentration generated by O211P is always 1/10 of expected concentration, suggesting fewer targets present in *E. onekaka* sample comparing to the other samples. However, the performance of O211P is more stable since their log10 showed better linear relation and its detection limit is as low as 3.2 copies/µL.

M165P showed the highest result concentration of all probes, suggesting a high sensitivity. However, concentration in chip No.4 showed a clear increase where it shouldn't be. This implies a possibility of nontarget amplification. Thus, concentration lower than 320 copies/µL is not trustworthy.

Three single-probe assays also showed amplifications with gDNA of their targeted species (data not shown).

III-3-4-2 Validate assays against non-target species

Each single-probe assay was run with PCR amplicons and gDNA from non-target species. Digital PCR was run with 1 µL of PCR amplicon of non-target species at an estimated concentration of 3200 copies/µL, which is above the reliable concentration of all three assays examined in section III-3-4-1. Each assay was also examined with 0.5 ng gDNA of the two non-target species.

1. E. aucklandica A251P assay

The A251P assay showed no cross-reactivity. It was noted that the automatically calculated threshold of signal intensity was always above 3,500.

2. Cross-reactivity of *E. onekaka* assay O211P

The O211P assay did not cross-react with *E. aucklandica* but cross-reacted with *E. menziesii* using automatically calculated thresholds. It detected similar concentrations of PCR amplicons from the non-target *E. menziesii* and the target *E. onekaka* at a concentration of 3,200 copies/µL (Figure III-8). When the concentration of amplicons was increased to 32,000 copies/µL, the concentrations of two species detected by O211P assay remain similar (Figure III-8). The result suggests that the O211P assay has similar sensitivity in detecting PCR amplicons of both species.

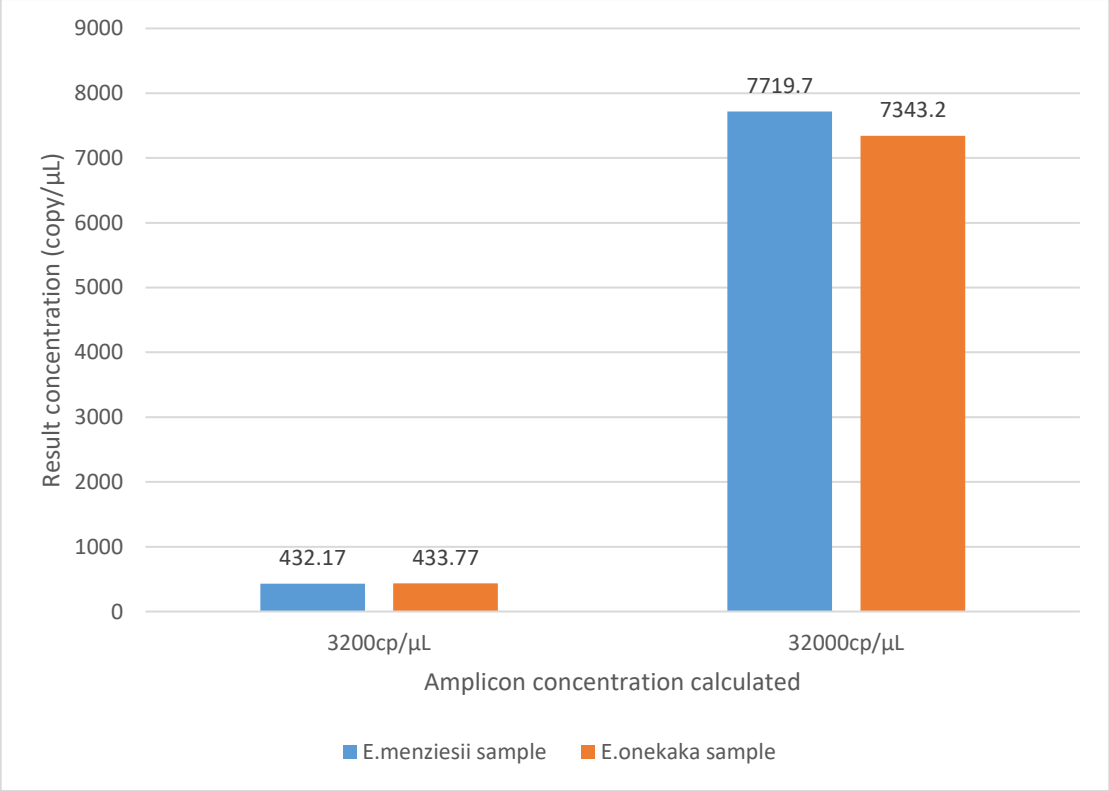


Figure III-8 Comparison of the dPCR result of the O211P assay with PCR amplicon of two species. Both targeted *E. onekaka* and nontargeted *E. menziesii* showed amplification at a similar concentration.

When 0.5ng gDNA was loaded in dPCR instead, the O211P assay detected higher concentration in *E. menziesii* over *E. onekaka* (Figure III-9). The annealing temperature in the dPCR program was increased from 60℃ to 64℃ in an attempt to increase the specificity of the O211P assay. However, both of *E. menziesii* and *E. onekaka* showed increased amplifications without a significant preference on amplifying *E. onekaka*. This indicates that the specificity of O211P assay targeting *E. onekaka* cannot be improved by increasing the annealing temperature.

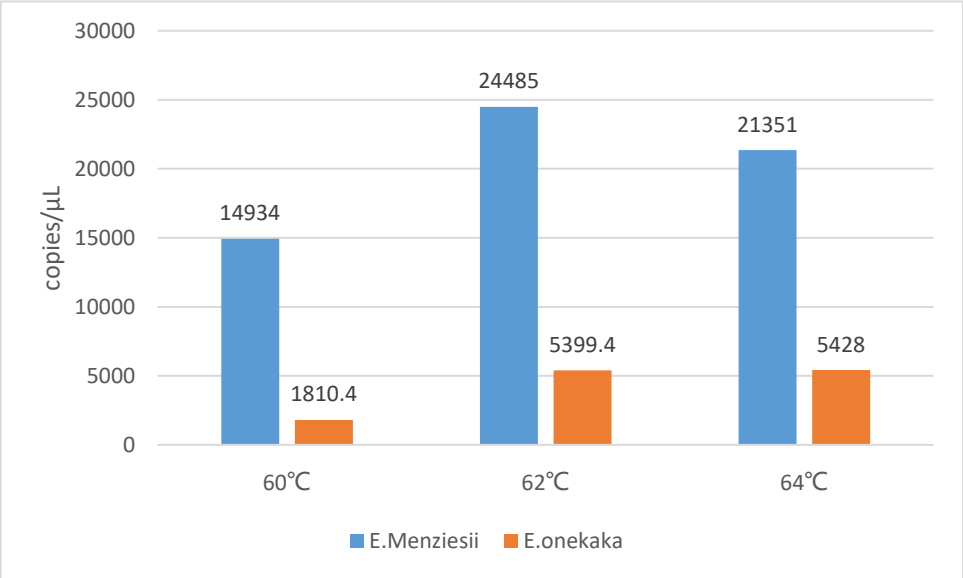


Figure III-9 Onekaka assay react with two species under different annealing temperatures

When analysing result of dPCR, a scatter plot can display the fluorescent signals generated from amplification (Figure III-10). On the scatter plot, each dot represents the fluorescent signal detected in a single well of the chip; blue dots represent amplification signal (above the fluorescence threshold), and

yellow dots represent non-amplification signals. The fluorescence intensity of the FAM reporter dye is plotted against the Y-axis.

It was noticed that the O211P assay showed distinct patterns against the two species on its scatter plots (Figure III-10 andFigure III-11). The intensity of FAM signal showed distinct thresholds. FAM signal of *E. onekaka* sample is always above 3000 (Figure III-10). The dots on the scatter plot are always clearly separated from the nonamplification signals. On the other hand, FAM signals generated by *E. menziesii* using O211P is relatively weak. The fluorescence intensity usually lays within 500-1500 (Figure III-11) which is a clearly different threshold from *E. onekaka*. Moreover, *E. menziesii* signal cluster usually closely adjacent to the nonamplification signal cluster (Figure III-11). The two groups of signals are sometimes too close that the dPCR analysing program may fail to calculate the right threshold automatically. In these situations, the threshold was set manually.

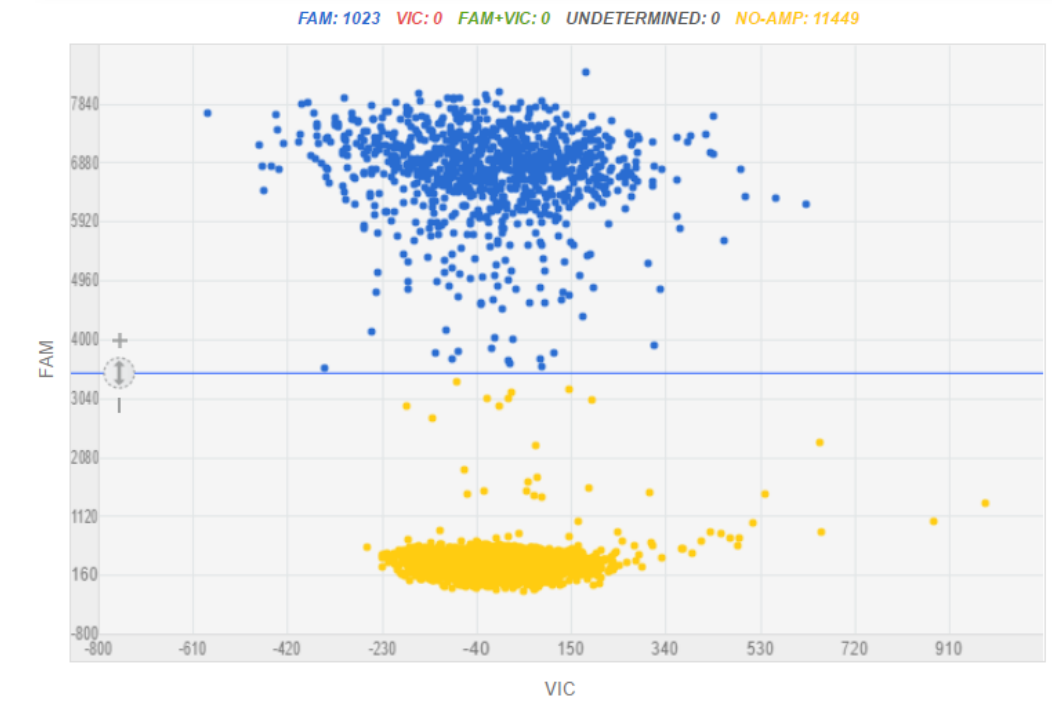


Figure III-10 A typical scatter plot of O211P assay against *E. onekaka* sample.

Blue dots represent FAM signal generated from *E. onekaka* sample, note the threshold is 3422.

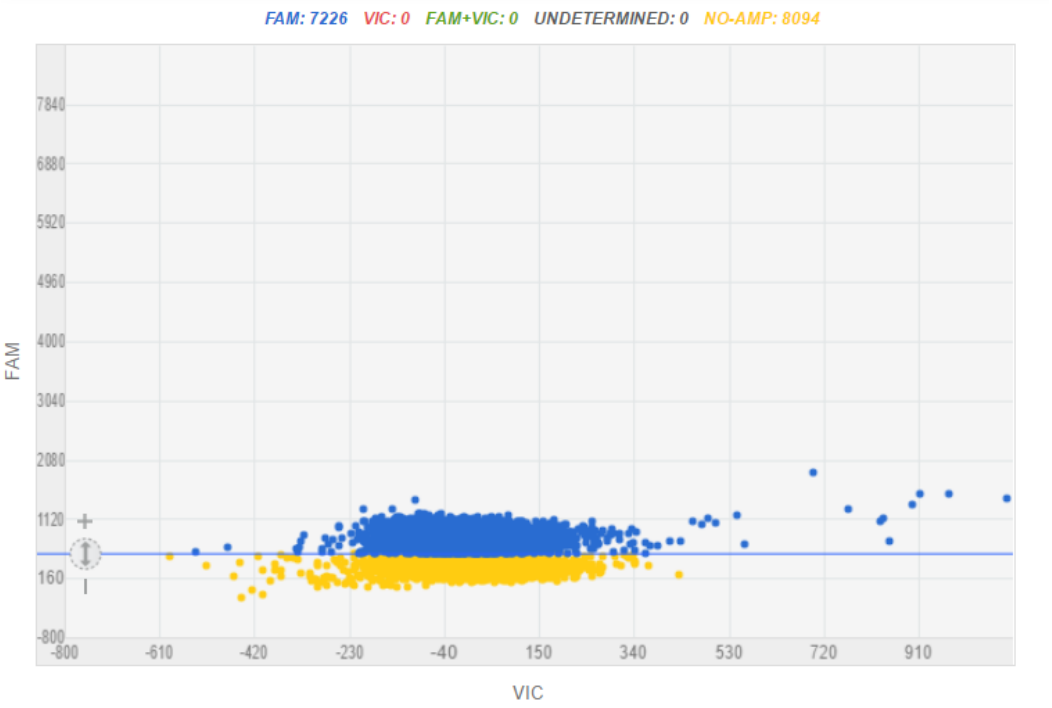


Figure III-11 A typical scatter plot of O211P assay against *E. menziesii* sample.

Blue dots represent FAM signal generated from *E. menziesii* sample, note the threshold is 529.

The unique pattern of *E. onekaka* signal is consistent with all the results of dPCR using O211P assay. Some thresholds of two species against O211P probe are shown in Figure III-12. Thresholds of *E. onekaka* were usually calculated automatically while thresholds of *E. menziesii* were manual adjusted. It can be clear seen that FAM signal from *E. onekaka* has a threshold around 3300 while *E. menziesii* has a threshold around 900. This consistency suggests that in the detection of *E. onekaka* in eDNA sample with the O211P assay, the threshold can be manually set to around 3000 if auto calculation is confused by signals generated by *E. menziesii*.

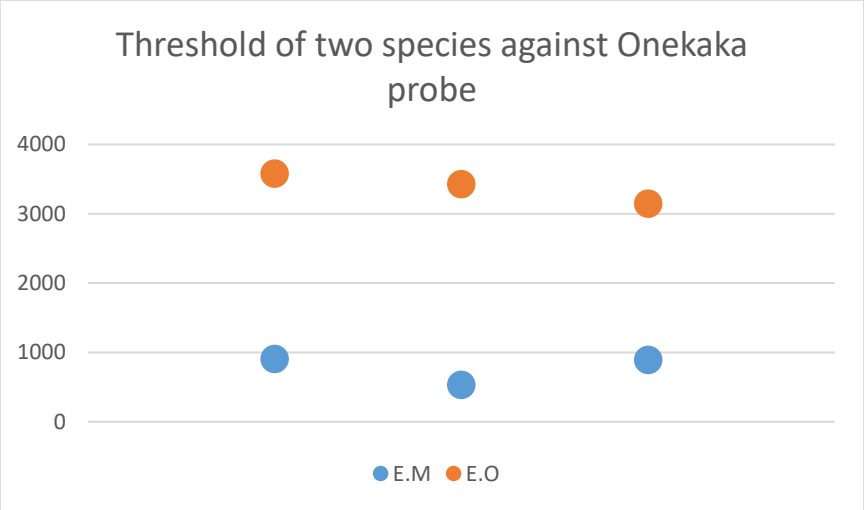


Figure III-12 Threshold of two species against O211P probe. Each dot represents a threshold of a chip with 0.5ng gDNA loaded.

3. Cross-reactivity of *E. menziesii* assay M165P

M165P probe did not cross-react with either PCR amplicons or gDNA of *E. onekaka*. However, amplifications showed up when the M165P assay was

run with PCR amplicons and gDNA of *E. aucklandica*. The signal generated by two species are not distinguishable since their threshold are close. This suggests that M165P is not species specific but detecting *E. menziesii* and *E. aucklandica* simultaneously (Figure III-13).

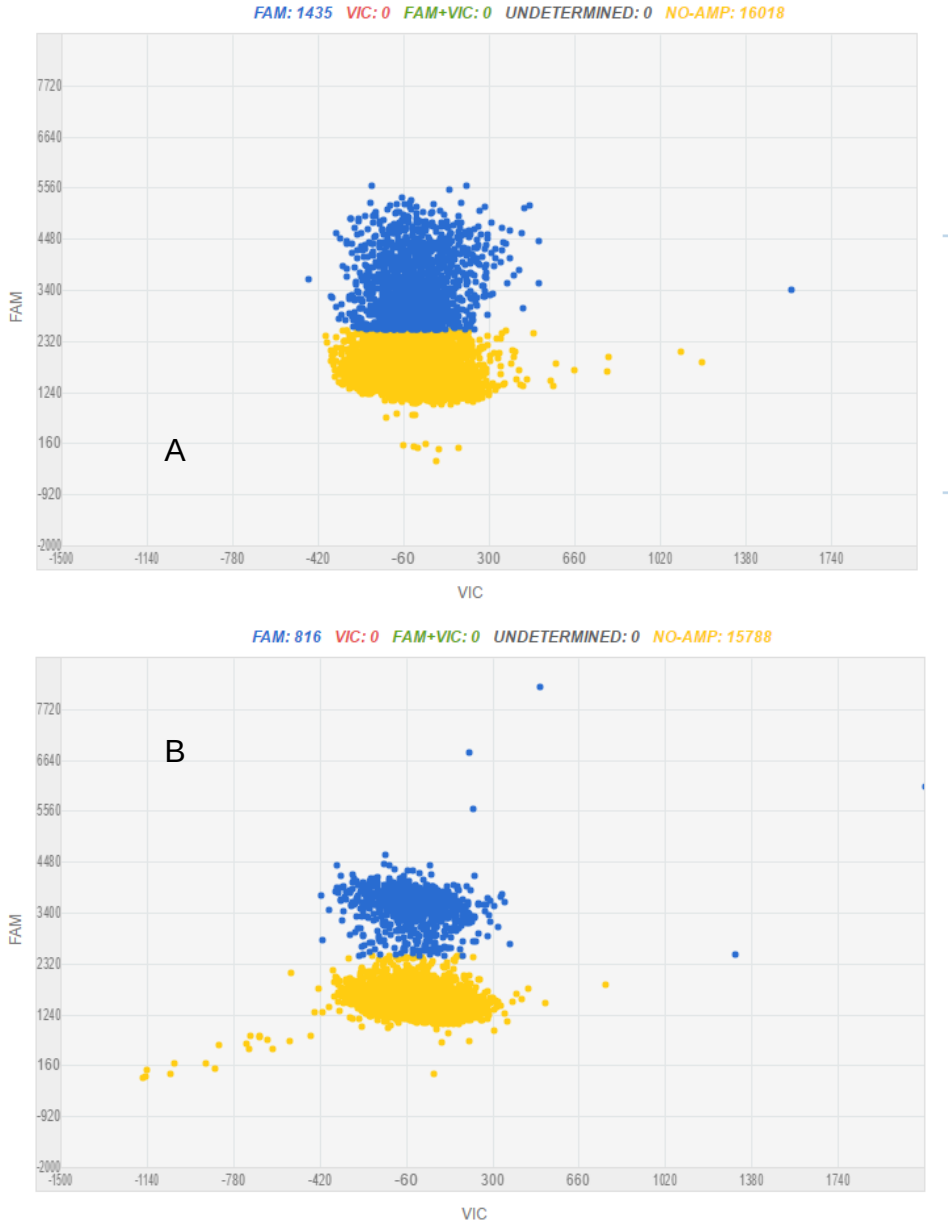


Figure III-13 Cross-reactivity of M165P. A: M165P vs 3200 copy *E. aucklandica* PCR amplicon, threshold:2528; B: M165P vs 3200 copy *E. menziesii* PCR amplicon, threshold: 2458 Thresholds are auto calculated.

III-3-4-3 Validate assay against mixture of target gDNA and nontarget eDNA

To confirm that target can be detected by dPCR in the presence of large amounts of non-target eDNA, 1 µL of PCR amplicons (3,200 copies/µL) was spiked with various amounts of soil eDNA (0 ng, 40 ng, 80 ng, and 120 ng).

1. A251P vs. eDNA

The thresholds of all the chips were auto-calculated. The first chip (0ng soil DNA added) showed a clear threshold of 3500 while the rest chips all showed extremely high threshold (7000 to 9000) which are unreliable. Thus, the thresholds of the rest chips were adjusted close to 3500 manually. Same threshold strategy also applied to the next two assays.

Compared with pure PCR amplicons, A251P detected fewer targets when spiked with eDNA (Figure III-14). Overall, it showed decreased concentrations with the addition of different amount of eDNA. However, the decrease is not significant and the target is still amplifiable even with the highest amount of eDNA (120ng) added (Figure III-14).

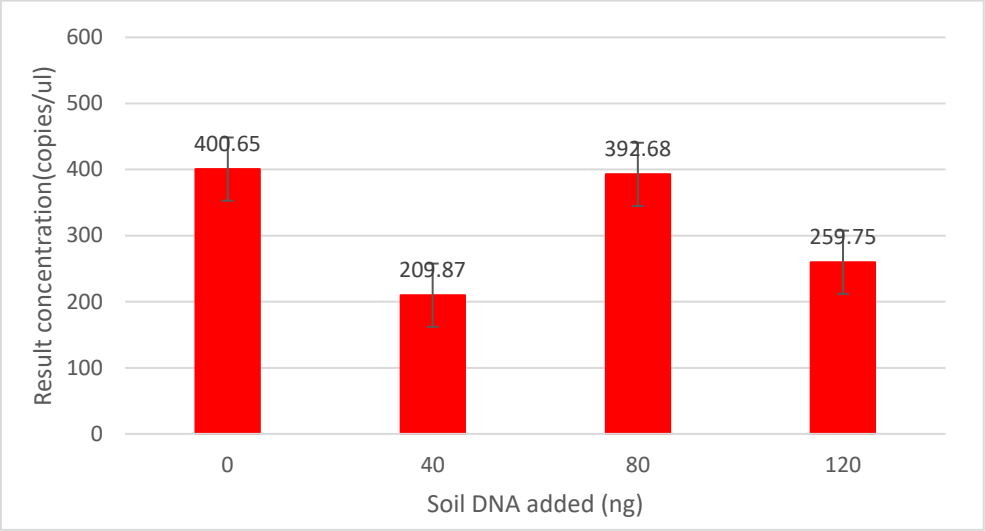


Figure III-14 A251P assay performance. Targeted PCR amplicon was spiked with soil eDNA in three-series amount.

2. O211P vs. eDNA

PCR amplicon spiking with soil DNA showed inhibition on Onekaka probe. However, the inhibition showed no clear relation with the amount of soil DNA added.

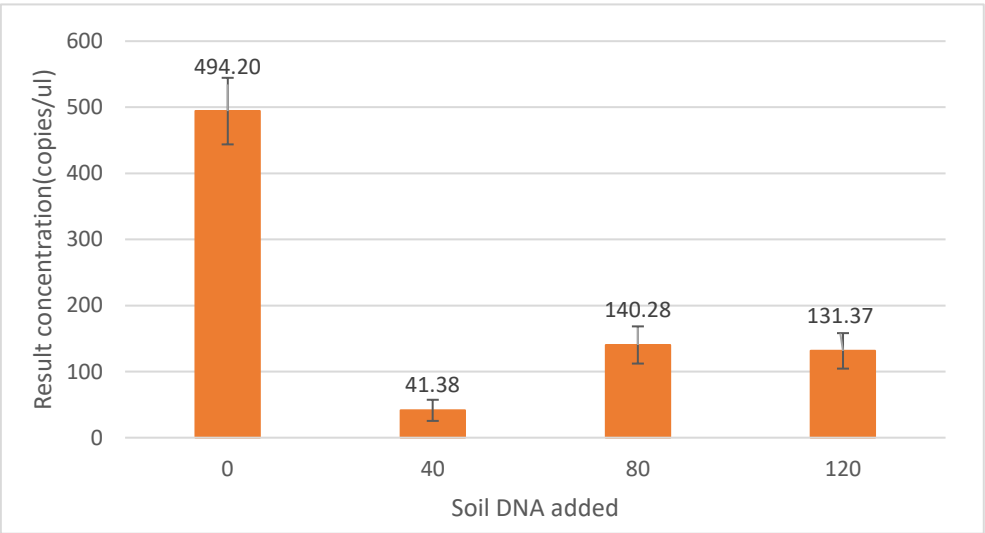


Figure III-15 O211P assay performance. Targeted PCR amplicon was spiked with soil eDNA in three-series amount.

3. M165P vs. eDNA

Spiking soil DNA with PCR amplicons showed inhibition, but amplification still detectable. The result target concentration showed a negative relation with soil DNA added.

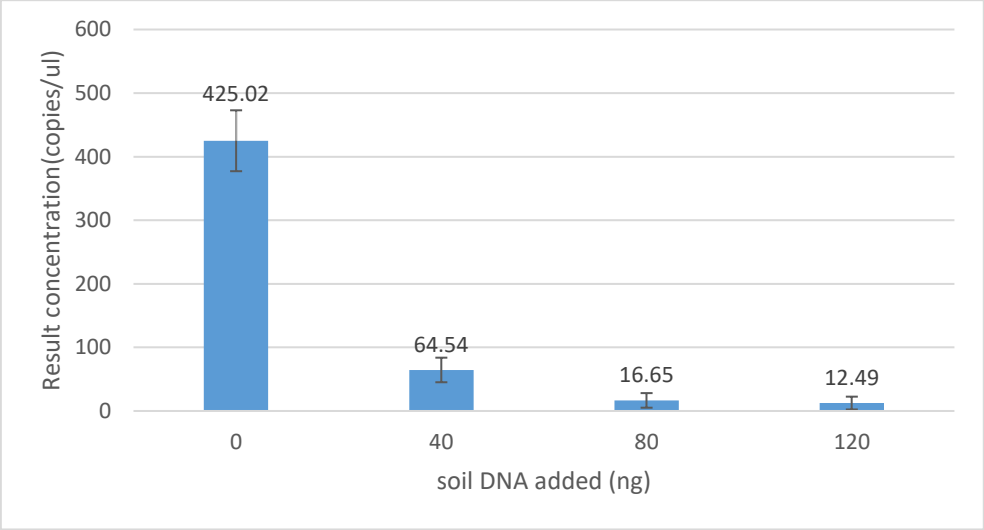


Figure III-16 M165P assay performance. Targeted PCR amplicon was spiked with soil eDNA in three-series amount.

III-3-5 Trial of duplexed assays using Onekaka and Aucklandica probes

From previous validation process, it was found that the O211P probe can detect and distinguish *E. menziesii* and *E. onekaka*, and the A251P probe is specific to *E. aucklandica.* A trial of duplexed assays using Onekaka and Aucklandica probes was conducted regarding identify and quantify *E. aucklandica* and *E. onekaka* by dPCR simultaneously.

In this examination, both probes were added to the dPCR master mix. In each chip, 0.5ng of gDNA from each of the three species were loaded either singly, in pairs or all together. The expected results are shown in Table III-8.

Table III-8 Duplex probe experiment design

Chip No.	gDNA(ng) added			Expected fluorescence in dPCR		
	E. menziesii	E. aucklandica	E. onekaka	FAM <3000	VIC	FAM >3000
1	0.5			√	×	×
2		0.5		×	√	×
3			0.5	×	×	√
4	0.5	0.5		√	√	×
5	0.5		0.5	√	×	√
6		0.5	0.5	×	√	√
7	0.5	0.5	0.5	√	√	√
8				×	×	×

When gDNA from *E. aucklandica* and *E. onekaka* is loaded separately, the scatter plots showed clear separation of dots representing amplifications and non-amplifications (Figure III-17, A, C). Thresholds of these two reactions

were calculated automatically with FAM at around 4000 and VIC at around 3000. These two thresholds were fixed to 4000 (FAM) and 3000 (VIC) to all chips in order to give a comparable display of all scatter plots,

The addition of gDNA from E. menziesii into E. aucklandica and E. onekaka showed interference (Figure III-17, B, D). This influence is not significant for species identification purpose because signal above threshold clearly indicates the detection of target species. However, the interference caused by *E. menziesii* make quantification of the *E. onekaka* targets meaningless since the algorithm calculates FAM signal from both species. The quantification of *E. aucklandica*, on the other hand, will not be influenced by *E. menziesii* since the calculation only employee VIC signal, which is generated by *E. aucklandica* independently.

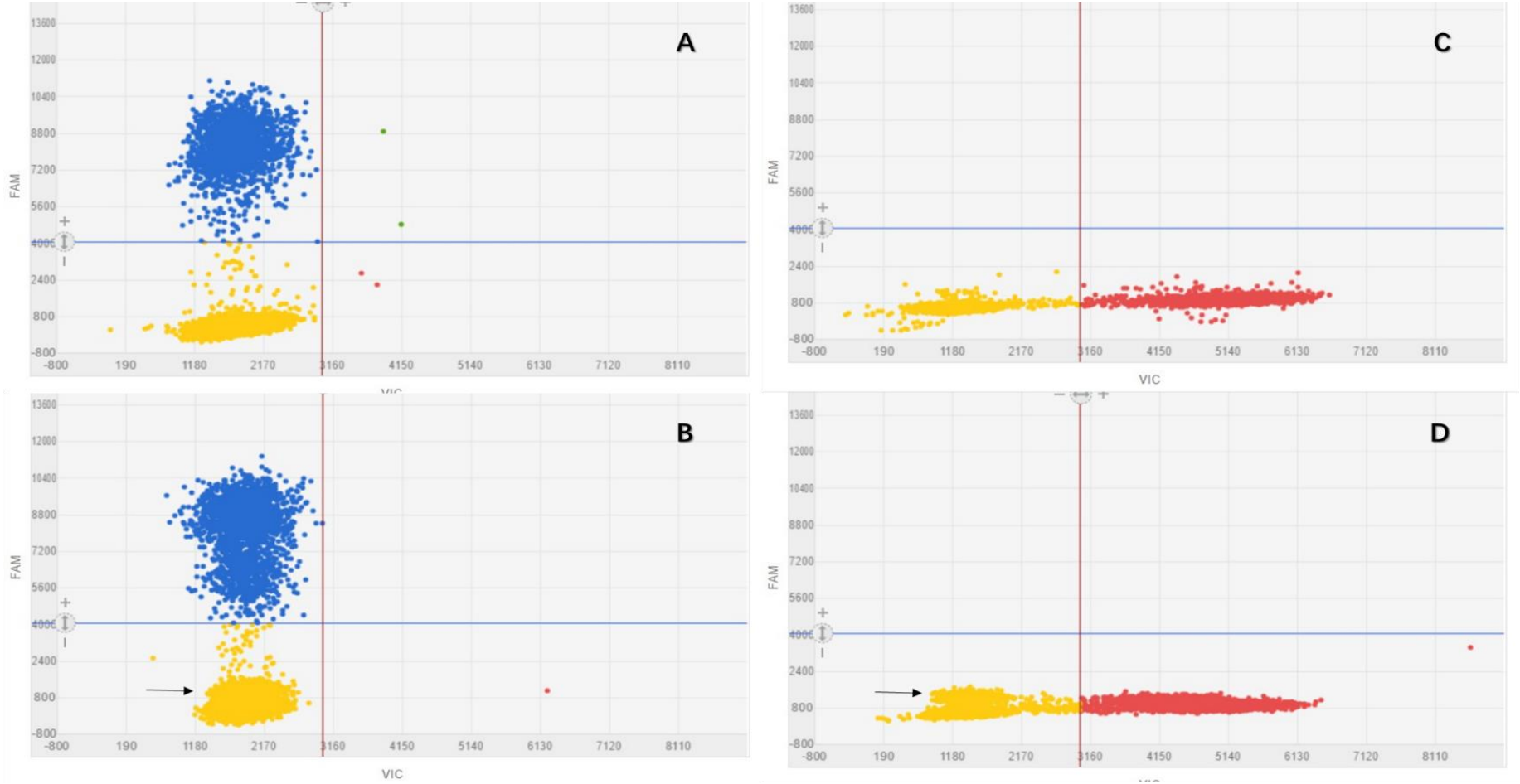


Figure III-17 Comparison of duplex assay result with the addition of *E. menziesii* sample. The black arrow indicates the *E. menziesii* bulk signal.

Sample present: A (E. onekaka); B (E. onekaka + E. menziesii); C (E. aucklandica); D (E. aucklandica + E. menziesii). Threshold FAM:4000 VIC:3000

The duplex assay consisting of A251P and O211P can clearly identify *E. onekaka* and *E. aucklandica* in a sample that gDNA of two species were present (Figure III-18). It implies that if *E. menziesii* is known to be absent, the assay is able to identify and quantify two species simultaneously.

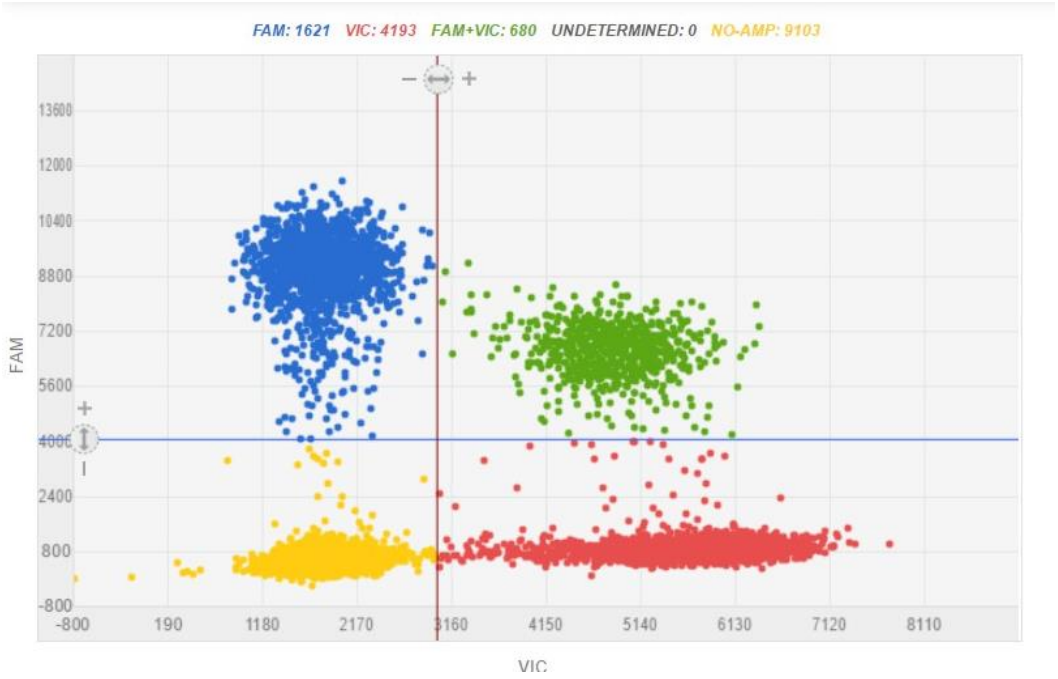


Figure III-18 Duplex probe assay against a combination sample of *E. aucklandica* and *E. onekaka*. Blue dots represent FAM signal generated from *E. onekaka,* and red dots represent VIC signal generated from *E. aucklandica*. Green dots represent the overlapped fluorescence of FAM and VIC detected in each well. Yellow dots indicate wells without amplification. Threshold: FAM:4000 VIC:3000

Furthermore, in the sample which contains gDNA of all three species, the assay can identify *E. aucklandica* and *E. onekaka*, but can only quantify target concentration of *E. aucklandica.*

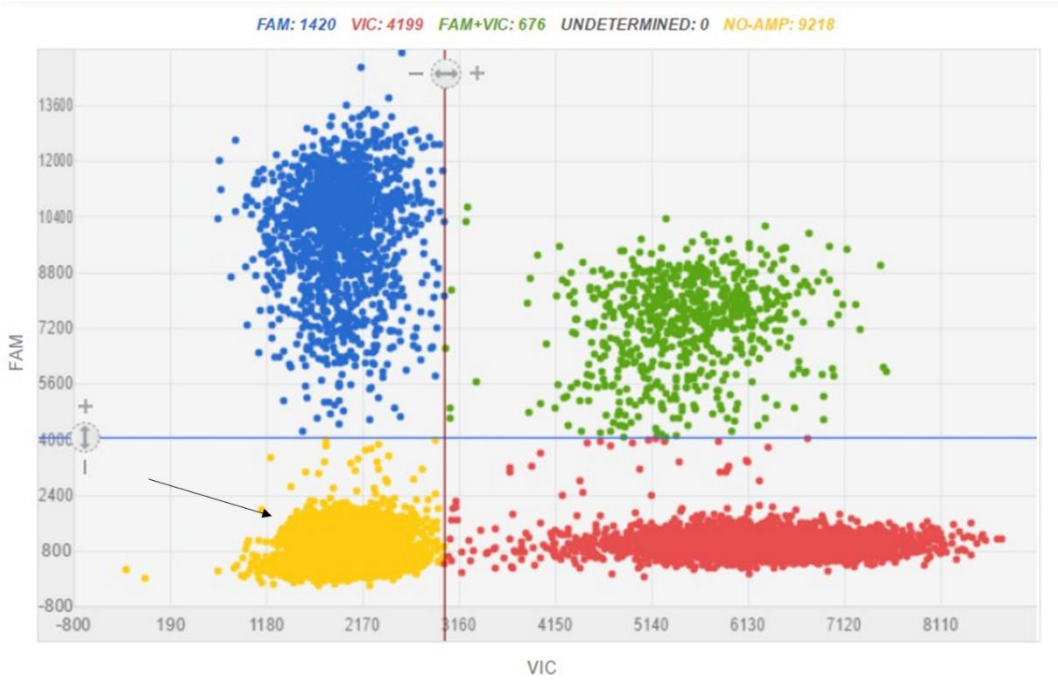


Figure III-19 Duplex assay against mix sample of three species.

The black arrow indicates the *E. menziesii* bulk signal. Threshold FAM:4000 VIC:3000

In summary, the results of this duplex assay trial are as expected in Table III-8. Duplex assay successfully identified the target in all gDNA samples.

III-3-6 Conclusion

In his chapter, three assays for each species were developed and validated (Table III-9). Candidate primers targeting *Echyridella COI* fragment were designed and 57F-322R pair was chosen due to its low degeneracy. Species-specific probes were designed and the probe that has the most mismatches with the other two non-target species was chosen (M165P, A251P, and O211P).

Table III-9 Feature of three assays

Target species	Primer	Probe	Reporter dye	Specificity	eDNA spiking
E. menziesii	57F-322R	M165P	FAM	Cross-react with *E. aucklandica*	Overall Concentration ↓
E. aucklandica	57F-322R	A251P	VIC	No cross reaction	Overall Concentration ↓ but not significant
E. onekaka	57F-322R	O211P	FAM	Cross-react with *E. menziesii*, but *E. onekaka* still identifiable	Overall Concentration ↓

Assays were validated against target PCR amplicons in dPCR. All three assays amplified target PCR amplicons and the lowest reliable result concentration is 3200 copies/µL for *E. aucklandica* probe A251P, 320 copies/µL for *E. menziesii* probe O211P, and 3.2 copies/µL for *E. onekaka* probe O211P.

Then assays were validated against non-target PCR amplicons and gDNA. No amplification of PCR amplicons will prove there is no cross-reactivity of a probe with nontarget mussel species at the assay targeting region. No amplification of nontarget gDNA will prove there is no cross reaction of the probe with any DNA fragment present in the mussel gDNA. In conclusion, the A251P assay is species-specific and does not cross-react with other species, but M165P and O211P have cross-reactivity. M165P cross-reacts with *E. aucklandica* in an indistinguishable manner. O211P cross-reacts with *E. menziesii* but generates distinguishable signals since the signal intensity of *E. onekaka* is above 3000 and of *E. menziesii* is below 1000. The result suggests that A251P and O211P can be multiplexed for detecting *E. aucklandica* and *E. onekaka* simultaneously.

The assays were also validated by spiking with soil DNA. Addition of soil DNA generally inhibited dPCR reaction. A251P is the least affected while M165P is largely inhibited. Moreover, the O211P assay showed a slight increase of result concentration when eDNA added increased from 40 ng to 80ng, which requires more research in the future.

A trial of duplexing O211P and A251P was conducted due to the distinguishable cross-reactivity of O211P assay and species-specific A251P. The duplex assay succeeded in identifying mussel species in a variety of

mixed mussel gDNA sample. However, the duplex assay needs to be further validated with field sample in terms of inhibition and non-specific amplification. Importantly, quantification of *E. aucklandica* is viable in all situation while of *E. onekaka* is dependent on the occurrence of *E. menziesii*.

Chapter IV Sequencing of Mussel mtDNA for Target Capture

IV-1 Introduction

For eDNA-based species detection, false negative refers to the absence of target signal for a sample in which target DNA fragments are present. The absence of target signal can be caused by 1) poor assay sensitivity if the target is present in the reactions, or 2) insufficient assay replication if the target is present in the extracted sample but not loaded into reactions due to limits on the amount of template DNA that each reaction can include. The dPCR-based assays described in Chapter Three allows significantly more template DNA to be used for each assay than qPCR, and this chapter will focus on increasing the probability that any target DNA fragments present are included in the assay through target capture. For this project, target capture is defined as capturing targeted mtDNA fragments from *Echyridella* present in a given eDNA sample. This chapter will explain the utility of target capture techniques in reducing false negatives and present DNA sequences (and the challenges associated with collecting these sequences) that will be used for designing and manufacturing a custom-made target capture tool.

IV-2 Target capture

To increase the certainty that target presents in extracted sample will be loaded into reactions, replication is widely used as a target screening method (Machler*, et al.*, 2016). Screening more of the extracted eDNA allows a smaller uncertainty in detection rates. However, there are no general guidelines for the number of replications considered sufficient for reducing false negative outcomes (Ficetola *et al.*, 2015). In eDNA studies, the number of PCR replicates (technical replicates that increase the overall amount of template eDNA assayed) varies from one (Minamoto et al. 2012) to thirty-three (Wilcox *et al.*, 2013). Due to upper limits on the amount of eDNA template in individual qPCR assays (Technologies, 2010; Machler*, et al.*, 2016), only a (small) aliquot of total extracted eDNA is typically used in qPCR. As a result, even with many replications, the use of unenriched eDNA template in qPCR can result in a false negative result (Figure IV-1A).

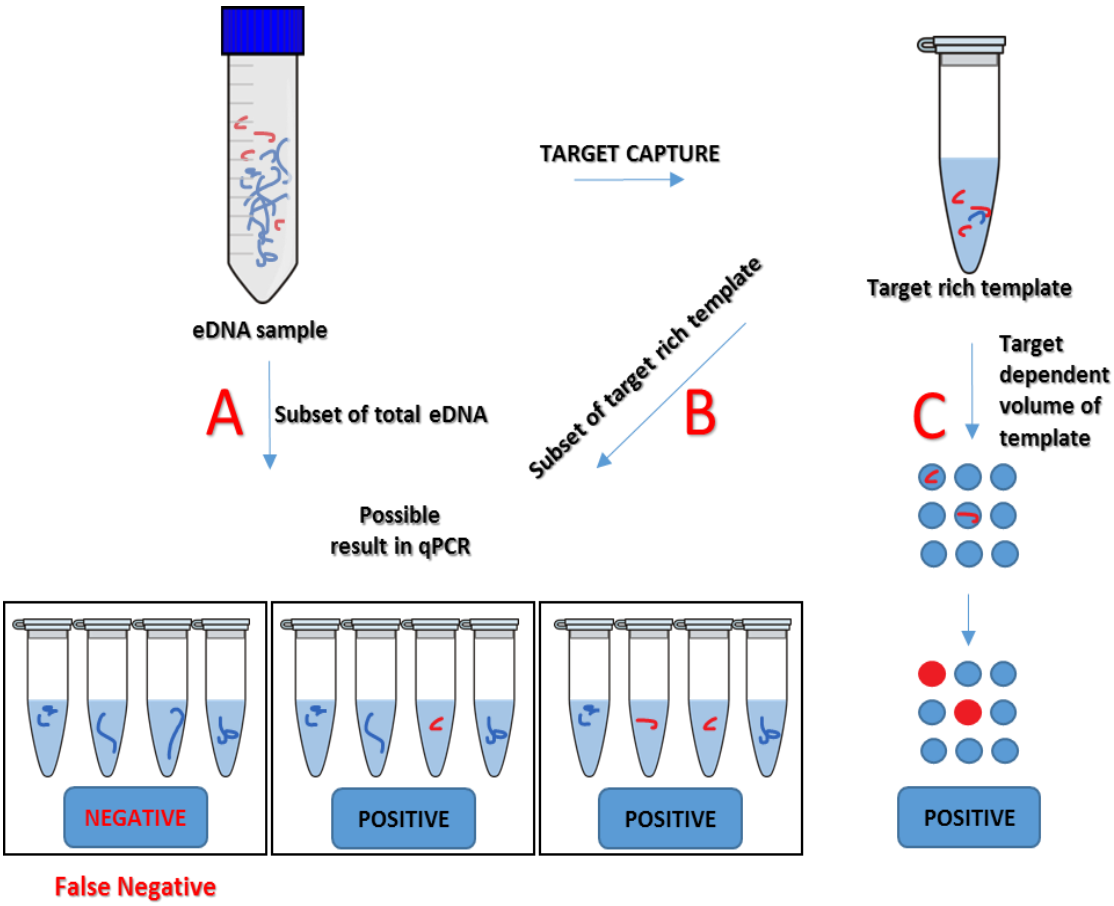


Figure IV-1 Schematic diagram showing how target capture and dPCR reduce false negative detection in eDNA. The uncertainty of detection rate A>B>C.

Target capture increases the effective concentration of target DNA molecules (if they are present at all) in extracted eDNA samples (Figure IV-1B). However, the limitation on maximum template DNA amount in qPCR reaction (e.g., 100 ng) means that not all the extracted eDNA will necessarily be assayed, and false negative may still occur.

The likelihood of false negative can be further reduced using dPCR, which allows more eDNA template (e.g., 330 ng for human genome) to be used in a single assay (Figure IV-1C). Dilution of eDNA sample is

recommended for qPCR because the co-extracted substance in eDNA usually inhibits qPCR (McKee*, et al.*, 2015). This process also co-dilute target DNA, leading to a small amount of eDNA template in qPCR (McKee*, et al.*, 2015; Goldberg*, et al.*, 2016).

There are two strategies for capturing the target: negative selection and positive selection. Negative selection utilises oligonucleotides specially designed for binding non-target molecules. By removing undesired sequences, the effective concentration of desired targets goes up, leading to a higher probability of detection (Vestheim & Jarman, 2008). However, this strategy is only feasible if the identities of the non-target sequences are known and consistent across samples (e.g., ribosomal RNA). For this project, the identities of non-target sequences in eDNA samples are unknown and variable across samples.

Positive selection is where target DNA fragments are captured and enriched due to subsequent removal of non-targets. It relies on the hybridization of target-specific probes to regions of interest on target DNA molecules to selectively enrich sequences. This strategy fits in this project since the targeted *Echyridella* sequences are known and MYbaits is chosen for positive target capture.

Importantly, although dPCR enables absolute quantification, the inclusion of a target capture step potentially nullifies the quantitative aspect of the assay, and the eventual dPCR results can only be interpreted in terms of presence vs. absence. When targets present in eDNA sample have been captured and enriched, it is hard to determine the enrichment efficacy of a target capturing technique. Thus the concentration of enriched target cannot refer back to its original eDNA sample statistically. Due to the breakage of their mathematical relation, quantification of the enriched target cannot lead to a concentration of target present in original eDNA sample. As a result, the method incorporating target capture in this project can only suggest the presence of targeted mussel.

IV-3 MYbaits

MYbaits is a customised commercial product from MYcroarray (Ann Arbor, Michigan, USA) that enriches target DNA fragments positively for sequencing and other DNA-based applications such as qPCR and dPCR. MYbaits kits are custom sequence capture probe libraries (aka “baits”), which are customised biotinylated RNA oligos complementary to target sequences. With a vast excess of the baits mixed in with DNA samples (aka “ponds”), an in-solution hybridization of baits and target occurs (Figure IV-2).

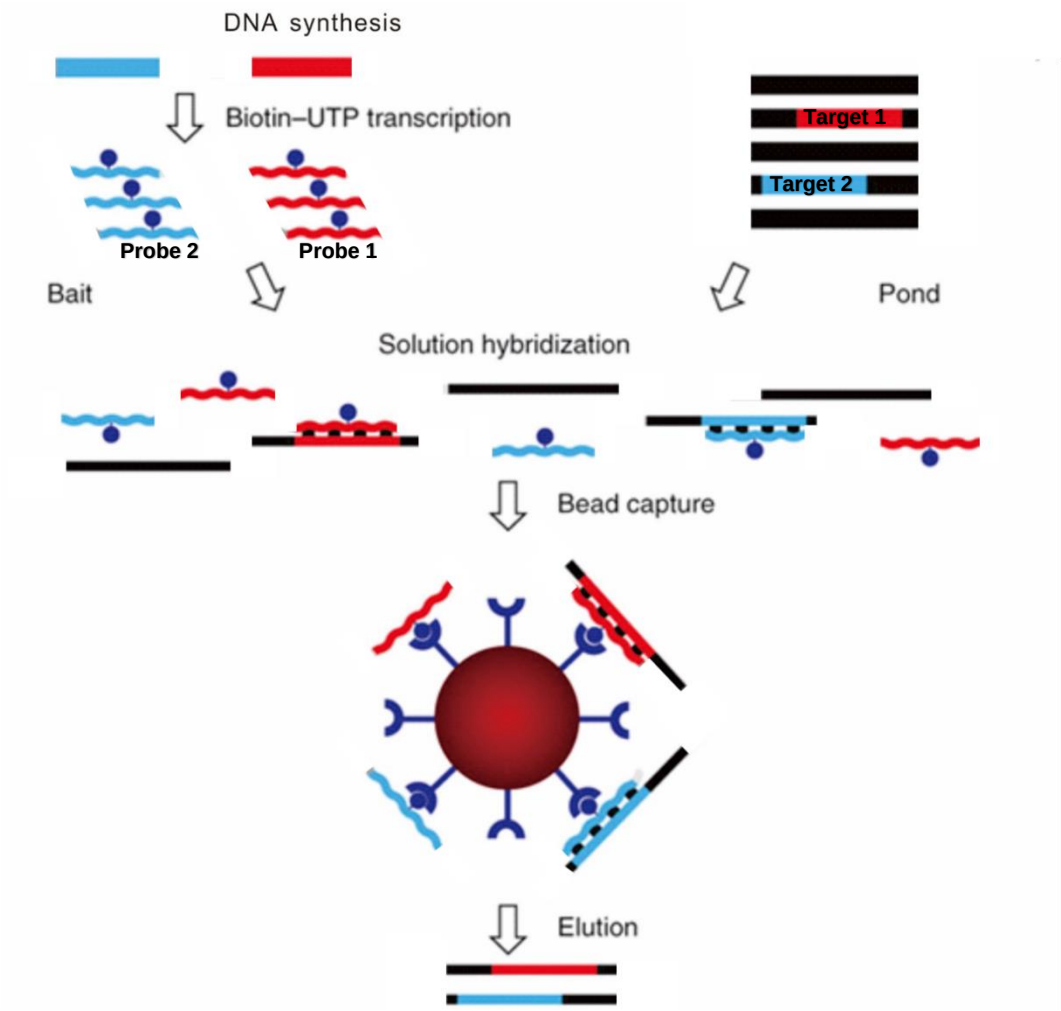


Figure IV-2 Overview of the positive selection of MYbaits. Illustrated are steps involved in the preparation of biotinylated RNA capture probes (bait; top left), Two targets and their respective baits are shown in red and blue. The excess of single-stranded non-self-complementary RNA (wavy lines) drives the hybridization, followed by bead capture and elution. Reproduced from Gnirke *et al.* (2009)

To manufacture a custom MYbaits library for capturing *Echyridella* mtDNA fragments, a multiple sequence alignment of mtDNA from targeted mussel species is required to identify regions suitable for bait design. Multiple sequence alignment, as opposed to representative sequences from all three species, is essential due to the discovery that *E. menziesii* has many intraspecies heterogenetic bases (see Figure III-3 in III-3-1-1 Sequence

acquisition). A multiple sequence alignment containing all available sequences of these three species will better capture and represent all inter- and intraspecies heterogeneities. Moreover, the multiple sequence alignment needs to cover not only the 200 bp target for dPCR but also 1,000 bp flanking both sides of available partial cytochrome c oxidase subunit I (*COI*) gene sequences. Typical length of DNA fragment detected by eDNA assays ranges from 62 bp to 650 bp (Ma *et al.*, 2016). Having baits targeting regions flanking the 200 bp target region increase the likelihood of capturing targets in eDNA samples. A longer reference sequence alignment will also enable MYbaits scientists to design suitable baits.

Building the reference sequence alignment requires sequencing the mtDNA of these species since there are no records of complete mtDNA sequence of the *Echyridella* genus in GenBank. The available sequences are mostly partial *COI* gene that covers the same 500~600 bps region. The only *COI* record (Accession: AY785394.1) longer than 600 bp is a sequence of *Hyridella menziesii*, which is 1,011 bp and partially covers *COI* gene and cytochrome c oxidase subunit II (*COII*) gene.

IV-4 Consensus primers

Although it is possible to sequence the complete mtDNA of *Echyridella* using shotgun genomic sequencing, it was decided to sequence only the

targeted region by Sanger sequencing with consensus primers designed from close relatives due to the following reasons:

1. The MYbaits baits will only target 2-3 kbp centred around the target region for dPCR.

2. It is possible to design consensus primer since there are numerous mtDNA sequences of *Unionoida* for designing and sufficient mussel DNA sample to validate primers.

3. The cost of sequencing complete mitochondrial genomes is too high for the scope of this project.

Consensus primers are primers designed from conserved regions across several closely related species that should amplify corresponding regions of another species within the same clade (P1-R and P2-R in Figure IV-3). Since designing consensus primers requiring species sharing a monophyletic group with target species, this project will firstly construct a phylogenetic tree based on the *COI* sequence of all freshwater mussel species, then choose the most closely related species for designing consensus primers. The mtDNA of selected species will be aligned to identify conserved regions, and primers will be designed from those regions. PCR will be run with primer pairs producing the longest amplicon (i.e. P1-F and P1-R in Figure IV-3), followed by

sequencing using both forward and reverse primers. During sequencing, it is possible that primers may fail to generate a high-quality sequence. When this situation occurs, new primers will be designed for sequencing (e.g., P2-F in Figure IV-3). The design will either base on the alignment of close species or newly generated sequences.

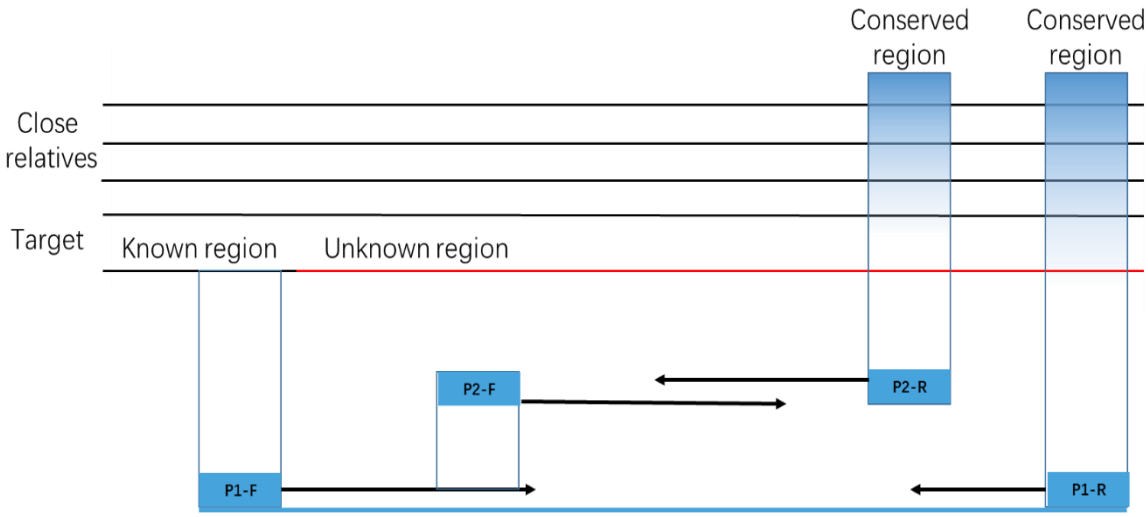


Figure IV-3 Schematic diagram of consensus primer design and sequencing.

Blue lines indicate PCR amplicons and black arrowed lines indicates sequencing result. P1-F is designed from a known region of the target while P1-R is designed from a conserved region of closed relatives. Their amplicons may only generate short sequences that require additional primers for sequencing. P2-F is designed from the newly acquired sequence and P2-R is designed from the conserved region.

IV-5 Results

IV-5-1 Phylogenetic tree

From GenBank, 1,765 sequences affiliated with *Unionoida*, which includes the targeted genus *Echyridella*, and containing the *COI* gene were retrieved (Appendix II). All available complete mitochondrial genomes of *Unionidae* were also downloaded to act as a scaffold for multiple sequence alignment. All sequences were aligned by Geneious and then trimmed to a 517 bp block corresponding to available *Echyridella COI* sequences. A phylogenetic tree based on this partial *COI* alignment was built using the Neighbour-joining algorithm constructed in Geneious (Figure IV-4A).

From this comprehensive phylogenetic tree, it should be noted that *Echyridella* appears to be a monophyletic genus unique to New Zealand (Figure IV-4B), which means all native freshwater mussels in New Zealand evolved from one common ancestor. This observation is congruent with Marshall*, et al.* (2014), which reported that there are three New Zealand freshwater mussel species forming a monophyletic clade.

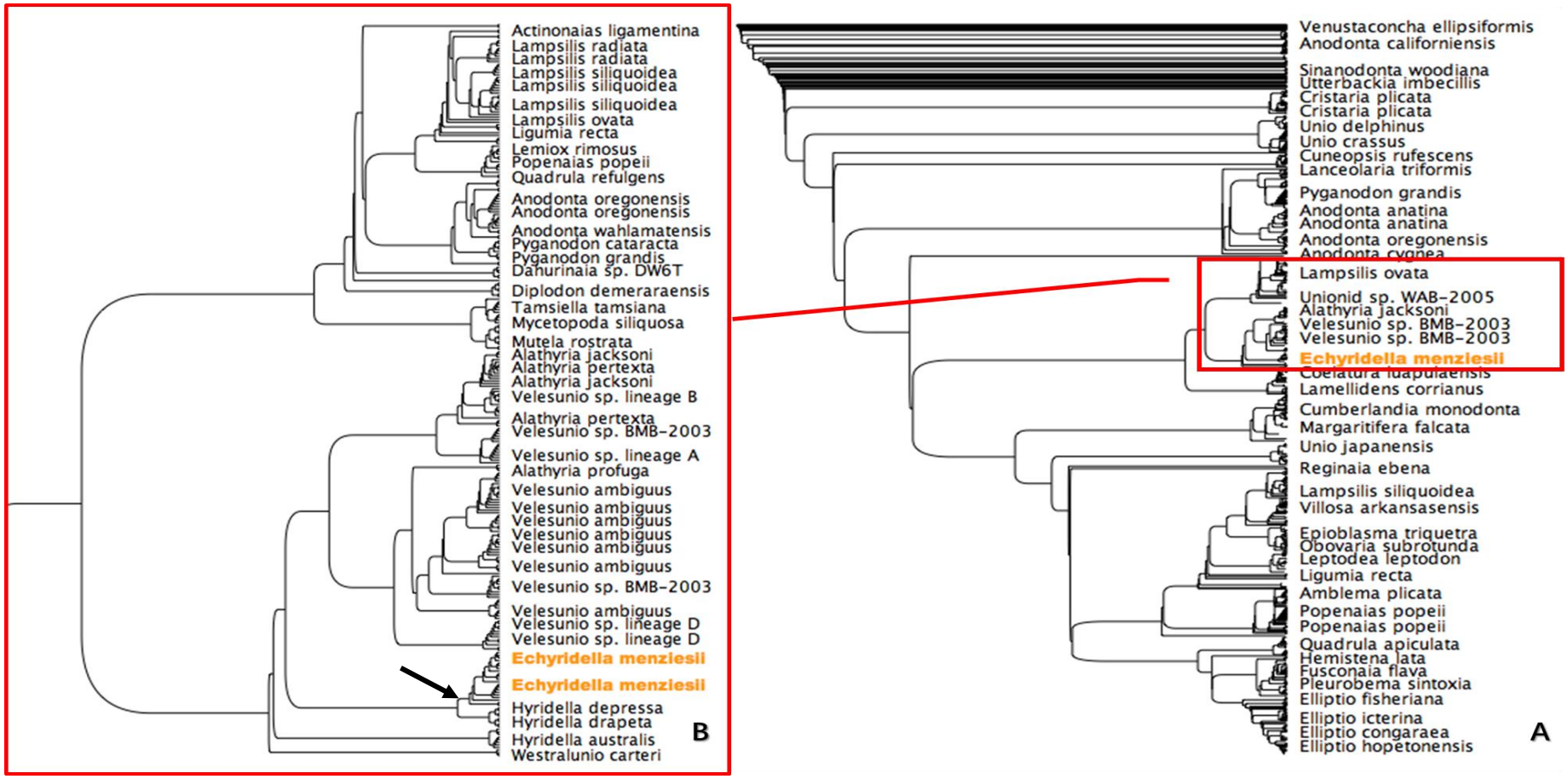


Figure IV-4 A: Phylogenetic tree of freshwater mussel order *Unionoida* B: the selected large monophyletic clade consisting of 278 sequences including targeted genus *Echyridella.* Black arrow indicates monophyletic clade of *Echyridella*. Labels are selectively shown due to too condensed organisation

In addition, this partial *COI* gene-based phylogenetic tree suggests that this ancestor diverged into two groups in which one became the modern *E. aucklandica,* and the other one became the most recent ancestor for *E. menziesii* and *E. onekaka* (Figure IV-5). Moreover, *E. menziesii* shows two diverging clades, implying a possibility of two subgroups in this taxon.

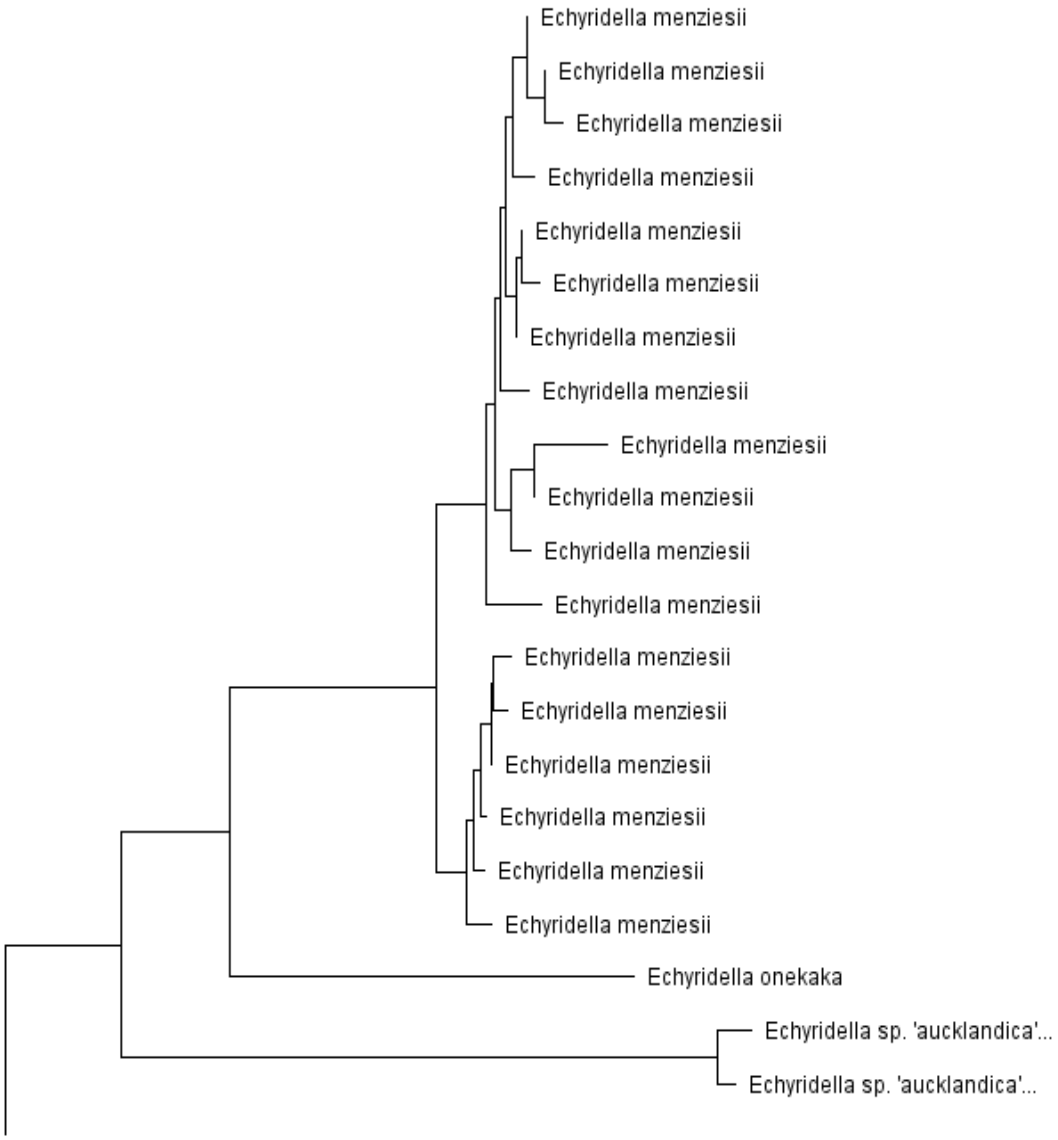


Figure IV-5 Partial phylogenetic tree of Unionoida showing the genus *Echyridella* in a single clade. Note *E. aucklandica* diverged first, followed by the divergence of *E. onekaka*. The *E. menziesii* also showed two clades indicating a possibility of two subgroups.

IV-5-2 Consensus primer design

To design consensus primers, a large monophyletic clade consisting of 278 records was selected from the phylogenetic tree (Figure IV-4B). I anticipated to select records that have long sequences for primer design. However, none of the 278 sequences were from the complete mitochondrial genome, and only 18 of them contained partial *COI* and *COII* gene, while the remaining sequences were all partial *COI* gene overlapping know sequences for *Echyridella*. They were unhelpful for designing consensus primer to extend the known *COI* gene sequences for *Echyridella*.

Consequently, all records with keywords “COII” and “complete mitochondrial” were highlighted first, and a large clade containing many highlighted records and sequences of *Echyridella* was selected. There were 76 sequences highlighted in this clade. All 76 sequences originated from either complete mitochondrial genomes or records containing both *COI* and *COII* genes. A second phylogenetic tree was built with these 76 sequences (Figure IV-6A).

On the second tree, the only long *Echyridella* sequence resided in its own clade with a large sister clade (Figure IV-6B). Species within this large clade were ordered by their genetic distance from their most common ancestor. To

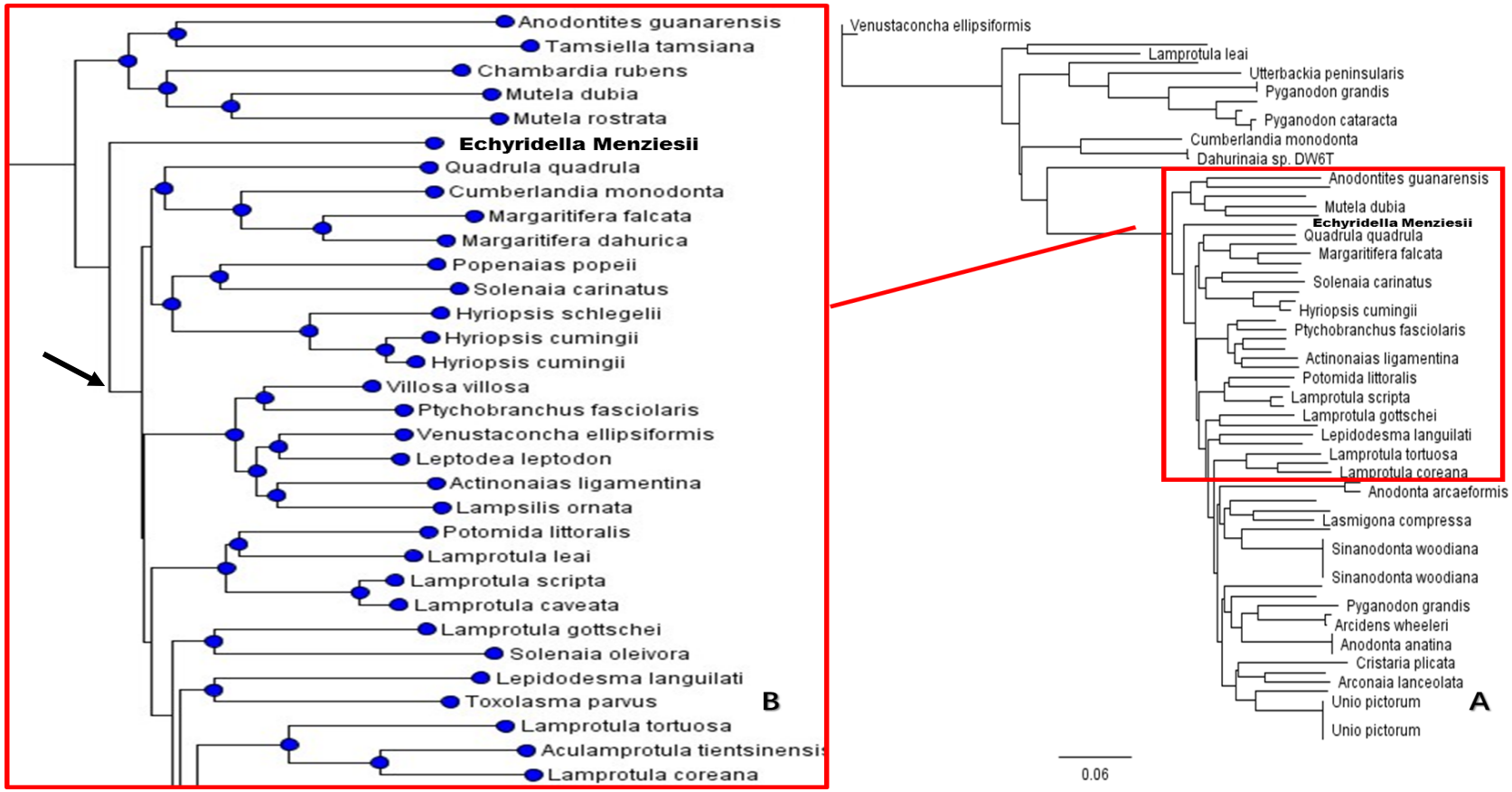


Figure IV-6 A: second tree consisting of 76 long sequences. B: The selected 32 sequences. Black arrow indicates the large sister clade. Note the 26 sequences in the sister clade on the bottom shared the most recent common ancestor with *Echyridella. menziesii* with least divergent distance, and 5 sequences on the top are from the only sister clade to the 26 sequences. The only long sequence of *Echyridella. menziesii* is shown in bold.

design consensus primers from the most closely related species, 32 sequences were selected from this sister clade (Figure IV-6B). They included 26 sequences that shared the most recent common ancestor with *Echyridella. menziesii* with least divergent distance, 5 sequences from the only sister clade to the 26 sequences, and the only long sequence of *Echyridella. menziesii*.

Of the 32 sequences, 22 originated from complete mitochondrial genomes. I extracted partial mitochondrial genome of these 22 sequences containing two genes upstream and downstream of *COI* and *COII* genes. However, two possible gene orders upstream of the *COII* gene were identified (Figure IV-7). It was unknown which gene order applies to *Echyridella*.

13 of them showed one gene order:

Mitochondrially encoded NADH dehydrogenase 3 gene (*ND3/MT-ND3*) → *COII* → *COI*

The remaining 9 records showed another gene order:

Mitochondrially encoded NADH dehydrogenase 2 gene (*ND2/MT-ND2*) → mitochondrially encoded tRNA serine 1(*trnS1/TRNS1*) gene → mitochondrially encoded tRNA histidine (*trnH/TRNH*) gene → *COII* → *COI*

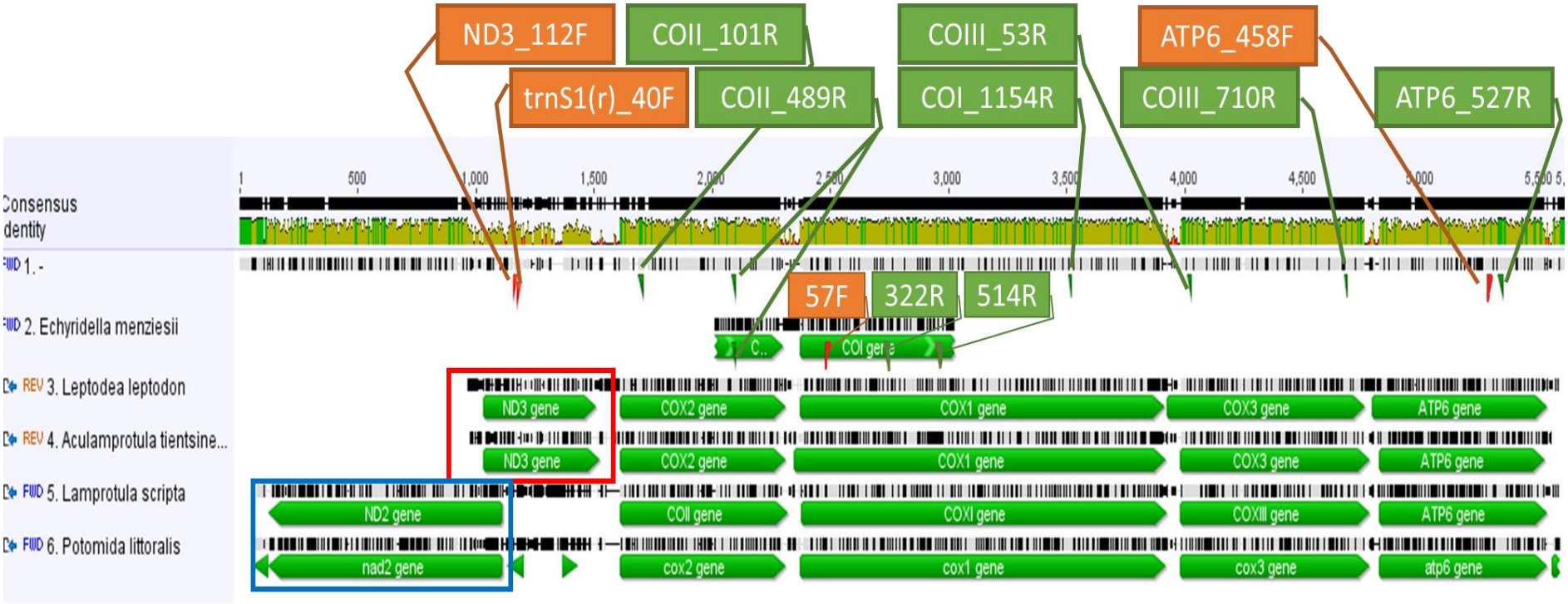


Figure IV-7 Diagram showing representatives sequences showing two gene orders and consensus primers designed

Some genes are shown in multiple names: ND2 (nad2); COII (COXII/COX2); COI (COXI/COX1); COIII (COXIII/COX3); ATP6(atp6)

No.1 consensus sequences of alignment of 23 closely related species. No. 2 Longest available *E. menziesii* mtDNA sequence from GenBank. No 3-4 *ND3-COII-COI* gene order with *ND3* gene shown in the red box. No 5-6 ND2-tRNA-COII-COI gene order with ND2-tRNA shown in the blue box. Primers are lying as indicated. All forward primers are shown in orange and reverse shown in green. Note COII_489R is designed from COII of *E. menziesii*. And 57F,322R and 514R are primers designed in chapter three.

For the region downstream of *COI* gene, where the second half *COI,* cytochrome c oxidase subunit III (*COIII),* and ATP synthase F_o subunit 6 (*ATP6/MT-ATP6)* of *Echyridella* was unknown, primers based on the alignment of all 22 sequences were designed. For the region upstream of COII gene, separate alignments for each gene order were made, and primers for each alignment were designed.

We anticipate high-quality sequencing results and designed primers that are 500-600 bp apart on the alignment (Figure IV-7). For the COI downstream region, which is 2,500 bp long, four reverse primers and one forward primer were designed. For the COII gene upstream region, for which the gene order needs to be determined first, two forward primers were designed. For the order with *ND2* gene and *trnS1* gene, one primer was designed for *trnS1*. For the order with *ND3* gene upstream, one forward primer was designed. Two reverse primers for the COII gene were also designed, one is a consensus primer based on the alignment of all 22 sequences, the other one is specifically designed from the available partial COII sequence of *Echyridella. Menziesii.*

All the primers were designed using Primer3 integrated into Geneious. The heterogenetic bases in the alignment of 23 sequences resulted in these primers all being degenerative primers. However, too many degenerative bases will reduce the efficiency of PCR since the concentration of the primer variant that perfectly matches the target is low (12.5% of total concentration for an 8-fold degenerate primer), leading to issues with primer exhaustion as PCR progresses. Too many degenerative bases also reduce PCR specificity since the likelihood of amplifying non-target sequences increases. Thus, all designed primers were edited manually to allow degenerative bases only at locations where there were at least four identical heterogenetic bases (Table IV-1). An example is shown in Figure IV-8.

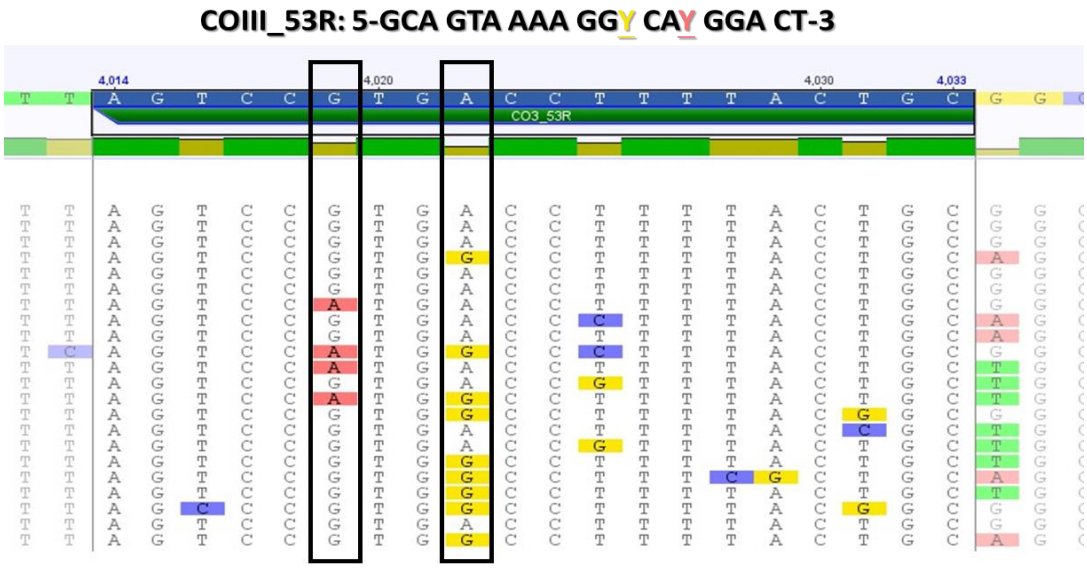


Figure IV-8 Example of a consensus primer in which degenerative bases only appear at the site with at least four heterogenetic bases. Heterogenetic bases in the alignment shown in black boxes. Degenerative bases are shown underlined in primer sequence with corresponded colour.

Table IV-1 Consensus primers designed

No.	Primer name	Sequence (5->3)	T_m	Degeneracy
1	ND3_112F	CCT TTT GAG TGT GGK TTT GA	52.1	2
2	trnS1(r)_40F	GAA GCC ACA TAT TAG CTC GGC	55.9	0
3	COII_101R	ACY AAI ACA AGA ACA AAC ATA RC	51.1	4
4	COII_489R	AGC ATG AAC TAC GTC CAT TGA	54.0	0
5	COI_1154R	GCA AAA ACR GCY CCY AT	54.0	8
6	COIII_53R	GCA GTA AAA GGY CAY GGA CT	51.9	4
7	COIII_710R	GCA GCA GCC TCA AAM C	55.1	2
8	ATP6_458F	TRA TTC GTC CIA TTA CTT TRG G	52.1	4
9	ATP6_527R	ACI TGY AAY ATI ARA TGI CCY A	56.0	16

IV-5-3 Sequencing

For the region upstream of *COI* and *COII*, the gene order needs to be identified before sequencing. ND3_112F and trnS1(r)_40F were paired with 514R and used for PCR on all *Echyridella* DNA samples. All PCR products were analysed on an agarose gel. PCR using ND3_112F showed a clear band at around 1,700 bp as expected, whereas PCR using trnS1(r)_40F didn't show any band at the expected size but many unspecific bands at smaller sizes. Thus, it was determined that the gene upstream of *COI* and *COII* gene in the mtDNA of *Echyridella* is *ND3*.

Sanger sequencing was conducted after PCR. Amplicons of all samples were sequenced using ND3_112F and 514R. However, ND3_112F only generated a sequence of 300bp long (Figure IV-9). Even with 1100bp generated by 514R, the 1,700 bp amplicon was not fully sequenced, leaving a

gap of 300 bp. Thus, other primers including 322R and COII_489R were used to sequence the gap region. Moreover, COII_489R, which was designed from the sequence of *E. menziesii*, can only sequence amplicon of *E. menziesii* samples. Thus, COII_101R was used alternatively to sequence samples of other two species.

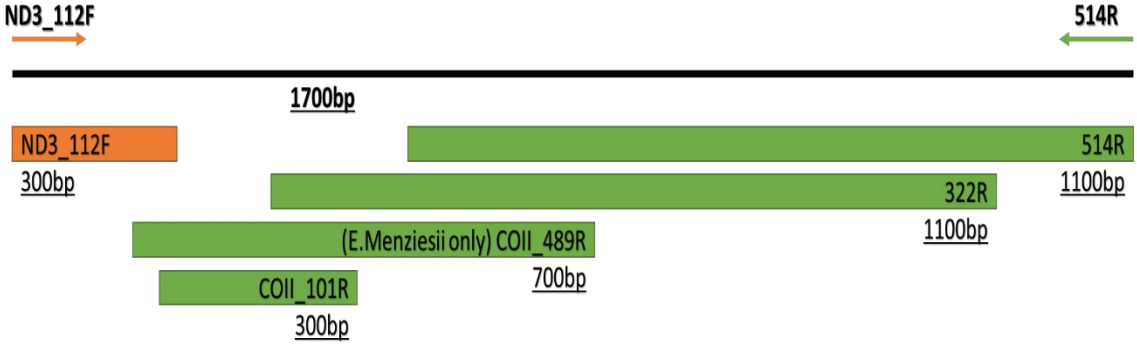


Figure IV-9 Schematic diagram showing how amplicon of ND3_112F & 514R were sequenced.

The forward primer is shown in orange arrow, and reverse primers are shown in green arrow. Sequences generate by forward primer is indicated by orange strip while sequences produced by reverse primers are indicated by green strips. The length of each sequence is labelled with underlines.

For the region downstream of *COI*, ATP6_527R was firstly selected for PCR with 57F since this would generate the longest amplicon. Amplicons were detected at the right size, but the amplification is not consistent since the result cannot be repeated with the same sample under the same condition. Thus, all reverse primers downstream of *COI* (COI_1154R, COIII_53R, COIII_710R) were tested in PCR. They all showed a similar problem (Table IV-2). A PCR additive, BSA (bovine serum albumin), proved to be beneficial to

PCR (Farell & Alexandre, 2012) and was used with 57F & COIII_710R in PCR. As a result, a 2300 bp long region can be amplified consistently.

Table IV-2 Primer pairs tried in PCR for downstream region

No.	Forward primer	Reverse primer	Product size (bp)	Amplification at the right size	Consistency
1	57F	ATP6_527R	3000	√	×
2	303F	ATP6_527R	2600	√	×
3	57F	COI_1154R	1100	√	×
4	57F	COIII_53R	1700	√	×
5	57F	COIII_710R	2300	√	√

Eight samples were selectively amplified by 57F & COIII_710R, followed by purification and sequencing with the two primers. However, three amplicons failed the sequencing and each primer only worked with five amplicons in which only four amplicons have been sequenced by both primers. The sequences generated by 57F range from 173bp to 695bp while COIII_710R can sequence 245bp to 1000bp (Figure IV-10). The result cannot produce a complete sequence of 2300bp long. Thus, another primer is required for sequencing. This work will be done in the future since the time in this research is limited.

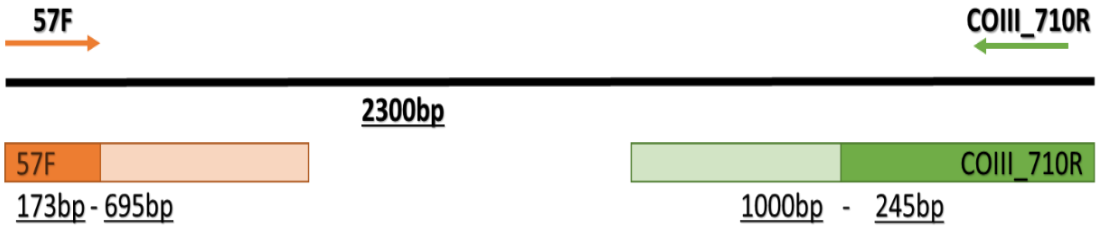


Figure IV-10 Schematic diagram showing how amplicon of 57F & COIII_710R has been sequenced.

The forward primer is shown in orange arrow, and reverse primers are shown in green arrow. Sequences generate by forward primer is indicated by orange strip while sequences produced by reverse primers are indicated by green strips. The length of each sequence is labelled with underlines.

IV-6 Conclusion

In conclusion, with the ultimate goal of detecting *Echyridella* in eDNA, target enrichment is essential. In this chapter, MYbaits was selected to capture the target in eDNA which requires a reference multiple sequence alignment. To obtain reference sequences, the following steps were carried out: (i) building a phylogenetic tree consisting of 1,765 freshwater mussel species, (ii) selecting 76 sequences to create a multiple sequence alignment for designing consensus primers, (iii) designing 9 consensus primers, (iv) conducting PCR to identify the gene order upstream of COI and COII for *Echyridella*, (v) sequencing PCR amplicons, and (vi) generating a multiple sequence alignment of sequenced sample (Appendix IV). The region upstream of *COI* and *COII* gene was fully sequenced while the region downstream of *COI* requires additional effort.

IV-7 Material and Methods

IV-7-1 PCR

Standard PCR protocols were as described in Material and Methods in Chapter Three. The thermal cycle programme (especially the annealing temperature) was changed according to the primer pair being used (average T_m of both primers). Elongation time was also adjusted to the product length at an estimate rate of 500bp per 30 seconds. Where BSA was added, the concentration was 0.4 mg/mL.

IV-7-2 Gel electrophoresis and purification

Agarose gel electrophoresis was previously used to verify the expected size of the amplifyied DNA target. In this chapter, it was also used to separate the product from the template DNA, unincorporated nucleotides, polymerase and buffer salts. Protocol of gel electrophoresis is the same as Chapter Three.

Purification of the PCR product from the gel was conducted after the agarose gel electrophoresis. The amplicons of the correct size were cut out of the gel with a scalpel while illuminated with UV-light. The UltraClean® 15 DNA Purification Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) was used for purification of the gel fragment following the protocol provided. The DNA was

resuspended in MiliQ water, and its concentration was measured with NanoDrop and Qubit.

IV-7-3 Sequencing

Purified PCR amplicons were sent to the Waikato DNA Sequencing Facility (http://sci.waikato.ac.nz/research/facilities/dna) for sequencing on an ABI 3730XL DNA sequencer.

References

Farell, E. M., & Alexandre, G. (2012). Bovine serum albumin further enhances the effects of organic solvents on increased yield of polymerase chain reaction of GC-rich templates. *BMC Research Notes, 5*, 257-257.

Ficetola, G. F., Pansu, J., Bonin, A., Coissac, E., Giguet-Covex, C., De Barba, M., Gielly, L., Lopes, C. M., Boyer, F., Pompanon, F., Raye, G., & Taberlet, P. (2015). Replication levels, false presences and the estimation of the presence/absence from eDNA metabarcoding data. *Mol Ecol Resour, 15*(3), 543-56.

Gnirke, A., Melnikov, A., Maguire, J., Rogov, P., LeProust, E. M., Brockman, W., Fennell, T., Giannoukos, G., Fisher, S., Russ, C., Gabriel, S., Jaffe, D. B., Lander, E. S., & Nusbaum, C. (2009). Solution hybrid selection with ultra-long oligonucleotides for massively parallel targeted sequencing. *Nat Biotech, 27*(2), 182-189.

Goldberg, C. S., Turner, C. R., Deiner, K., Klymus, K. E., Thomsen, P. F., Murphy, M. A., Spear, S. F., McKee, A., Oyler‐McCance, S. J., Cornman, R. S., Laramie, M. B., Mahon, A. R., Lance, R. F., Pilliod, D. S., Strickler, K. M., Waits, L. P., Fremier, A. K., Takahara, T., Herder, J. E., & Taberlet, P. (2016). Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods in Ecology and Evolution, 7*(11), 1299-1307.

Ma, H., Stewart, K., Lougheed, S., Zheng, J., Wang, Y., & Zhao, J. (2016). Characterization, optimization, and validation of environmental DNA (eDNA) markers to detect an endangered aquatic mammal. *Conservation Genetics Resources, 8*(4), 561-568.

Machler, E., Deiner, K., Spahn, F., & Altermatt, F. (2016). Fishing in the Water: Effect of Sampled Water Volume on Environmental DNA-Based Detection of Macroinvertebrates. *Environ Sci Technol, 50*(1), 305-12.

Marshall, B. A., Fenwick, M. C., & Ritchie, P. A. (2014). New Zealand Recent Hyriidae (Mollusca: Bivalvia: Unionida). *Molluscan Research, 34*(3), 181-200.

McKee, A. M., Spear, S. F., & Pierson, T. W. (2015). The effect of dilution and the use of a post-extraction nucleic acid purification column on the accuracy, precision, and inhibition of environmental DNA samples. *Biological Conservation, 183*, 70-76.

Vestheim, H., & Jarman, S. N. (2008). Blocking primers to enhance PCR amplification of rare sequences in mixed samples–a case study on prey DNA in Antarctic krill stomachs. *Frontiers in Zoology, 5*(1), 12.

Wilcox, T. M., McKelvey, K. S., Young, M. K., Jane, S. F., Lowe, W. H., Whiteley, A. R., & Schwartz, M. K. (2013). Robust Detection of Rare Species Using Environmental DNA: The Importance of Primer Specificity. *Plos One, 8*(3).

Chapter V Conclusion and future direction

V-1 Conclusion

The aim of this research is to develop quantitative assays for detecting and identifying three New Zealand endemic freshwater mussel in eDNA. To achieve this, species-specific dPCR assays were designed and validated in chapter III. The concept of target capture was then introduced in chapter IV. To design and manufacture a custom-made target capture tool MYbaits, the extended unknown region of *COI* gene was sequenced.

In chapter III, three TaqMan probes (M165P for *E. menziesii*, A251P for *E. aucklandica*, and O211P for *E. onekaka*) were developed along with genus-specific PCR primers for *Echyridella*. The resulting dPCR assays can reliably detect target PCR amplicons above certain concentrations (3200 copies/µL for *E. aucklandica* probe A251P, 320 copies/µL for *E. menziesii* probe O211P, and 3.2 copies/µL for *E. onekaka* probe O211P).

Moreover, assays can be influenced by high concentrations of non-targeted soil eDNA. The influence on A251P is less significant since the target concentration spiked with soil eDNA is at least half of target concentration (209.87 copies/µl) without spiking (400.65 copies/µl). But significant inhibition

was observed as for O211P and M165P. When spiked with soil eDNA, the target concentration (41.38 copies/µl) can be one-tenth of the non-spiking concentration (494.20 copies/µl) for O211P, and one-fortieth for M165P (425.02 copies/µl vs 12.49 copies/µl).

Cross-reactivity was also tested. The A251P assay is species-specific with no cross-reaction with other species, but M165P and O211P have cross-reactivity. M165P cross-reacts with *E. aucklandica* in an indistinguishable pattern since their fluorescence thresholds are close. However, the cross-reactivity of O211P with *E. menziesii* is distinguishable. The auto-calculated fluorescence threshold of O211P against *E. onekaka* sample is always above 3000 but thresholds of *E. menziesii* sample is below 1000. This means signals generated by O211P with a threshold above 3000 by can be considered as detection of E. onekaka. As a result, a trial assay duplexing two probes A251P and O211P was conducted against mixed mussel gDNA and succeed in detecting *E. aucklandica* and *E. onekaka* simultaneously.

In chapter IV, we introduced target capture as a target enrichment method to reduce false negative. Errors may happen when targets are present in the extracted sample but not loaded into reactions, leading to a low detection rate or a false negative result. Target capture can enrich the target

and consequently leads to a higher possibility of loading target into dPCR. Using positive selection strategy, the non-target eDNA content can also be reduced. Thus we decided to use a commercially available product MYbaits to capture target.

However, the sequence available is insufficient for customizing MYbaits. Thus, I retrieved 1745 sequences of *Unionoida*, build a phylogenetic three, and found 32 closely related species of *Echyridella*. Consensus primers were designed based on the 32 sequences and were used in subsequent sequencing.

About 1.7kb long mtDNA sequence of three *Echyridella* species were sequenced. It triples the known 500~600 bp data set and extended the available species from one to three. Moreover, two different gene orders upstream of *COI* gene among *Unionoida* species has been discovered. The order of *Echyridella* has been identified as *ND3* → *COII* → *COI*.

There are still some ongoing works of sequencing the downstream region of *COI* gene. Once completed, the sequencing result will support future research in target capture using MYbaits. Moreover, the result can also provide useful information for other research once upload online.

V-2 Future Direction

Assay testing has three stage: *in silico, in vitro, in situ* (Goldberg*, et al.*, 2016). This thesis tested three assays *in silico* and *in vitro* use gDNA.

Testing in situ will be the future focus. For testing in situ, positive controls and negative controls are necessary. The positive control is eDNA sample collected from sites where the mussel species are known to present. The negative control, on the other hand, is eDNA sample collected from sites where none of the three species are present. However, it is difficult to establish that a site has no mussels. Thus, a negative control can potentially be defined as a sample collected from sites upstream/downstream from a positive site until no target signal is detected.

For future direction, the target DNA production rate and persistence will also need to be tested. This can be done in a controlled condition by putting a certain number of mussels into a water tank and detecting the concentration of target DNA in water across time. The persistence of target can be evaluated by removing the mussels from the tank.

Assays should be validated with real freshwater eDNA in serial dilution. It examines the specificity of assay when conducting dPCR in eDNA, and the sensitivity of assay with various target concentration that may occur in eDNA.

They will be run against its own positive sample and non-target DNA to check specificity and sensitivity. Cross-reactivity will also be examined with eDNA. Lastly the duplex assay will be examined.

In addition to assay validation, the assay will also be tested through a series of distance from the target source. By doing this, the detecting range of assay is evaluated. It is expected the detected target will decrease with the increase of distance from the mussel source. When the target concentration falls below a certain level then MYbaits will be applied to sample for target capture. The specificity of each assay needs to be confirmed by sequencing.

To date, there are no molecular assays for identifying the three New Zealand freshwater mussel species directly. We have little knowledge of the presently known three species in terms of their comprehensive biogeography, physiology, and evolution. With the molecular assays developed in this study, identification and survey of freshwater mussels will be faster with less effort. Characterization of the mussel distribution, habitat preferences would be more efficient. By comparing different detecting result, it is possible to reveal factors that contributed to the unique distribution of *E. aucklandica* and *E. onekaka.*

With this assay, our understanding of not only New Zealand freshwater mussels but also the status of New Zealand freshwater ecosystem will be

improved. Survey and identification of mussel is essential for further studies regarding environmental influences and species interactions for sustaining and restoring mussel biodiversity and their ecological functions. For example, in studying the relation of decline of host fish Koaro and decline of E. menziesii, and assessing the water quality using mussel as bioindicator. These assays may also benefits answering questions such as how their most recent common ancestor arrived in New Zealand, what caused the divergence into three species, and why some *E. aucklandica* share the same habitat with *E. menziesii* without interbreeding.

Refernce

Anthony, J. L., & Downing, J. A. (2001). Exploitation trajectory of a declining fauna: a century of freshwater mussel fisheries in North America. *Canadian Journal of Fisheries and Aquatic Sciences, 58*(10), 2071-2090.

Bar, T., Kubista, M., & Tichopad, A. (2012). Validation of kinetics similarity in qPCR. *Nucleic Acids Research, 40*(4), 1395-1406.

Barnhart, C., & Neves, D. (2005). Overview of North American freshwater mussels. Mussels Toxicity Testing Workshop Hosted by U.S. EPA, Chicago, Illinois,2005.

Barnhart, M. C., Haag, W. R., & Roston, W. N. (2008). Adaptations to host infection and larval parasitism in Unionoida. *Journal of the North American Benthological Society, 27*(2), 370-394.

Bhadury, P., Austen, M. C., Bilton, D. T., Lambshead, P. D., Rogers, A. D., & Smerdon, G. R. (2006). Molecular detection of marine nematodes from environmental samples: overcoming eukaryotic interference. *Aquatic Microbial Ecology, 44*(1), 97-103.

Bizouarn, F. (2014). Introduction to digital PCR. *Methods Mol Biol, 1160*, 27-41.

Bogan, A. E. (1993). Freshwater bivalve extinctions (Mollusca: Unionoida): a search for causes. *American Zoologist, 33*(6), 599-609.

Breton, S., Beaupré, H. D., Stewart, D. T., Hoeh, W. R., & Blier, P. U. (2007). The unusual system of doubly uniparental inheritance of mtDNA: isn't one enough? *Trends in Genetics, 23*(9), 465-474.

Breton, S., Beaupré, H. D., Stewart, D. T., Piontkivska, H., Karmakar, M., Bogan, A. E., Blier, P. U., & Hoeh, W. R. (2009). Comparative Mitochondrial Genomics of Freshwater Mussels (Bivalvia: Unionoida) With Doubly Uniparental Inheritance of mtDNA: Gender-Specific Open Reading Frames and Putative Origins of Replication. *Genetics, 183*(4), 1575.

Bringolf, R. B., Cope, W. G., Eads, C. B., Lazaro, P. R., Barnhart, M. C., & Shea, D. (2007). Acute and chronic toxicity of technical‐grade pesticides to glochidia and juveniles of freshwater mussels (unionidae). *Environmental Toxicology and Chemistry, 26*(10), 2086-2093.

Butterworth, J. (2008). *Lake Rotokakahi: the kakahi (Hyridella menziesi) in a general framework of lake health* (

Cao, Y., Stodola, A., Douglass, S., Shasteen, D., Cummings, K., & Holtrop, A. (2015). Modelling and mapping the distribution, diversity and abundance of freshwater mussels (Family Unionidae) in wadeable streams of Illinois, U.S.A. *Freshwater Biology, 60*(7), 1379-1397.

Clearwater, S. J., Thompson, K. J., & Hickey, C. W. (2014). Acute Toxicity of Copper, Zinc, and Ammonia to Larvae (Glochidia) of a Native Freshwater Mussel Echyridella menziesii in New Zealand. *Archives of Environmental Contamination and Toxicology, 66*(2), 213-226.

Collier, K. J., Clearwater, S. J., Neijenhuis, P. H. M. W., & Wood, S. A. (2017). Factors influencing biodeposit production by the New Zealand freshwater mussel Echyridella menziesii. *New Zealand Journal of Marine and Freshwater Research*, 1-15.

Collins, R. A., Armstrong, K. F., Holyoake, A. J., & Keeling, S. (2013). Something in the water: biosecurity monitoring of ornamental fish imports using environmental DNA. *Biological Invasions, 15*(6), 1209-1215.

Cope, W. G., Bringolf, R. B., Buchwalter, D. B., Newton, T. J., Ingersoll, C. G., Wang, N., Augspurger, T., Dwyer, F. J., Barnhart, M. C., & Neves, R. J. (2008). Differential exposure, duration, and sensitivity of unionoidean bivalve life stages to environmental contaminants. *Journal of the North American Benthological Society, 27*(2), 451-462.

Curole, J. P., & Kocher, T. D. (2002). Ancient Sex-Specific Extension of the Cytochrome c Oxidase II Gene in Bivalves and the Fidelity of Doubly-Uniparental Inheritance. *Molecular Biology and Evolution, 19*(8), 1323-1328.

Darling, J. A., & Mahon, A. R. (2011). From molecules to management: Adopting DNA-based methods for monitoring biological invasions in aquatic environments. *Environmental Research, 111*(7), 978-988.

Deagle, B. E., Jarman, S. N., Coissac, E., Pompanon, F., & Taberlet, P. (2014). DNA metabarcoding and the cytochrome c oxidase subunit I marker: not a perfect match. *Biology Letters, 10*(9).

Deiner, K., & Altermatt, F. (2014). Transport distance of invertebrate environmental DNA in a natural river. *PloS one, 9*(2), e88786.

Dejean, T., Valentini, A., Miquel, C., Taberlet, P., Bellemain, E., & Miaud, C. (2012). Improved detection of an alien invasive species through environmental DNA barcoding: the example of the American bullfrog Lithobates catesbeianus. *Journal of Applied Ecology, 49*(4), 953-959.

Dejean, T., Valentini, A., Duparc, A., Pellier-Cuit, S., Pompanon, F., Taberlet, P., & Miaud, C. (2011). Persistence of Environmental DNA in Freshwater Ecosystems. *PLoS ONE, 6*(8), 1-4.

Dell, R. (1953). The Fresh-water Mollusca of New Zealand Part 1—The Genus Hyridella. *Transactions and Proceedings of the Royal Society of New Zealand, 81*.

Dhanasekaran, S., Doherty, T. M., & Kenneth, J. (2010). Comparison of different standards for real-time PCR-based absolute quantification. *Journal of Immunological Methods, 354*(1–2), 34-39.

Doi, H., Uchii, K., Takahara, T., Matsuhashi, S., Yamanaka, H., & Minamoto, T. (2015). Use of Droplet Digital PCR for Estimation of Fish Abundance and Biomass in Environmental DNA Surveys. *PLoS ONE, 10*(3), e0122763.

Ellis, M. (1936). Erosion silt as a factor in aquatic environments. *Ecology, 17*(1), 29-42.

Ellis, R. J. (1997). *Biomonitoring of Waikato river water quality using the freshwater mussel Hyridella menziesi.* thesis.

Farell, E. M., & Alexandre, G. (2012). Bovine serum albumin further enhances the effects of organic solvents on increased yield of polymerase chain reaction of GC-rich templates. *BMC Research Notes, 5*, 257-257.

Fenwick, M. C., & Marshall, B. A. (2006). A new species of Echyridella from New Zealand, and recognition of Echyridella lucasi (Suter, 1905)(Mollusca: Bivalvia: Hyriidae). *Molluscan Research, 26*(2), 69-76.

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

Ficetola, G. F., Pansu, J., Bonin, A., Coissac, E., Giguet-Covex, C., De Barba, M., Gielly, L., Lopes, C. M., Boyer, F., Pompanon, F., Raye, G., & Taberlet, P. (2015). Replication levels, false presences and the estimation of the presence/absence from eDNA metabarcoding data. *Mol Ecol Resour, 15*(3), 543-56.

Foote, A. D., Thomsen, P. F., Sveegaard, S., Wahlberg, M., Kielgast, J., Kyhn, L. A., Salling, A. B., Galatius, A., Orlando, L., & Gilbert, M. T. P. (2012). Investigating the Potential Use of Environmental DNA (eDNA) for Genetic Monitoring of Marine Mammals. *Plos One, 7*(8).

Gnirke, A., Melnikov, A., Maguire, J., Rogov, P., LeProust, E. M., Brockman, W., Fennell, T., Giannoukos, G., Fisher, S., Russ, C., Gabriel, S., Jaffe, D. B., Lander, E. S., & Nusbaum, C. (2009). Solution hybrid selection with ultra-long oligonucleotides for massively parallel targeted sequencing. *Nat Biotech, 27*(2), 182-189.

Goldberg, C. S., Sepulveda, A., Ray, A., Baumgardt, J., & Waits, L. P. (2013). Environmental DNA as a new method for early detection of New Zealand mudsnails (Potamopyrgus antipodarum). *Freshwater Science, 32*(3), 792-800.

Goldberg, C. S., Turner, C. R., Deiner, K., Klymus, K. E., Thomsen, P. F., Murphy, M. A., Spear, S. F., McKee, A., Oyler‐McCance, S. J., Cornman, R. S., Laramie, M. B., Mahon, A. R., Lance, R. F., Pilliod, D. S., Strickler, K. M., Waits, L. P., Fremier, A. K., Takahara, T., Herder, J. E., & Taberlet, P. (2016). Critical considerations for the application of

environmental DNA methods to detect aquatic species. *Methods in Ecology and Evolution, 7*(11), 1299-1307.

Gordon, M. E., & Layzer, J. B. (1993). Glochidial host of Alasmidonta atropurpurea (Bivalvia: Unionoidea, Unionidae). *Transactions of the American Microscopical Society*, 145-150.

Graf, D. L., & Cummings, K. S. (2007). Review of the systematics and global diversity of freshwater mussel species (Bivalvia: Unionoida). *Journal of Molluscan Studies, 73*(4), 291-314.

Grainger, N., Collier, K. J., Hitchmough, R., Harding, J. S., Smith, B., Sutherland, D., & New Zealand. Department of, C. (2014). *Conservation status of New Zealand freshwater invertebrates, 2013* (Vol. 8.; 8). Wellington, New Zealand: Publishing Team, Department of Conversation.

Gray, J., & Dieffenbach, E. (1843). Catalogue of the species of Mollusca and their shells, which have hitherto been recorded as found at New Zealand, with the description of some lately discovered species. In *Travels in New Zealand; with contributions to the geography, geology, botany and natural history of that country* (Vol 2, pp. 228-256): J. Murray London.

Green, H. C., & Field, K. G. (2012). Sensitive detection of sample interference in environmental qPCR. *Water research, 46*(10), 3251-3260.

Group, C. P. W., Hollingsworth, P. M., Forrest, L. L., Spouge, J. L., Hajibabaei, M., Ratnasingham, S., van der Bank, M., Chase, M. W., Cowan, R. S., Erickson, D. L., Fazekas, A. J., Graham, S. W., James, K. E., Kim, K.-J., Kress, W. J., Schneider, H., van AlphenStahl, J., Barrett, S. C. H., van den Berg, C., Bogarin, D., Burgess, K. S., Cameron, K. M., Carine, M., Chacón, J., Clark, A., Clarkson, J. J., Conrad, F., Devey, D. S., Ford, C. S., Hedderson, T. A. J., Hollingsworth, M. L., Husband, B. C., Kelly, L. J., Kesanakurti, P. R., Kim, J. S., Kim, Y.-D., Lahaye, R., Lee, H.-L., Long, D. G., Madriñán, S., Maurin, O., Meusnier, I., Newmaster, S. G., Park, C.-W., Percy, D. M., Petersen, G., Richardson, J. E., Salazar, G. A., Savolainen, V., Seberg, O., Wilkinson, M. J., Yi, D.-K., & Little, D. P. (2009). A DNA barcode for land plants. *Proceedings of the National Academy of Sciences, 106*(31), 12794-12797.

Gusman, A., Lecomte, S., Stewart, D. T., Passamonti, M., & Breton, S. (2016). Pursuing the quest for better understanding the taxonomic distribution of the system of doubly uniparental inheritance of mtDNA. *PeerJ, 4*, e2760.

Hamilton, D. P., Collier, K. J., & Howard-Williams, C. (2016). Lake Restoration in New Zealand. *Ecological Management & Restoration, 17*(3), 191-199.

Handy, S. M., Hutchins, D. A., Cary, S. C., & Coyne, K. J. (2006). Simultaneous enumeration of multiple raphidophyte species by quantitative real-time PCR: capabilities and limitations. *Limnology and Oceanography: Methods, 4*(6), 193-204.

Hebert, P. D. N., Ratnasingham, S., & de Waard, J. R. (2003a). Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London B: Biological Sciences, 270*(Suppl 1), S96-S99.

Hebert, P. D. N., Cywinska, A., Ball, S. L., & deWaard, J. R. (2003b). Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences, 270*(1512), 313-321.

Helfrich, L. A., Neves, R. J., & Chapman, H. (2005). Sustaining America's Aquatic Biodiversity. Freshwater Mussel Biodiversity and Conservation.

Hickey, C. W., Roper, D. S., & Buckland, S. J. (1995). Metal concentrations of resident and transplanted freshwater mussels Hyridella menziesi (Unionacea: Hyriidae) and sediments in the Waikato River, New Zealand. *Science of The Total Environment, 175*(3), 163-177.

Hindson, B. J., Ness, K. D., Masquelier, D. A., Belgrader, P., Heredia, N. J., Makarewicz, A. J., Bright, I. J., Lucero, M. Y., Hiddessen, A. L., Legler, T. C., Kitano, T. K., Hodel, M. R., Petersen, J. F., Wyatt, P. W., Steenblock, E. R., Shah, P. H., Bousse, L. J., Troup, C. B., Mellen, J. C., Wittmann, D. K., Erndt, N. G., Cauley, T. H., Koehler, R. T., So, A. P., Dube, S., Rose, K. A., Montesclaros, L., Wang, S., Stumbo, D. P., Hodges, S. P., Romine, S., Milanovich, F. P., White, H. E., Regan, J. F., Karlin-Neumann, G. A., Hindson, C. M., Saxonov, S., & Colston, B. W. (2011). High-Throughput Droplet Digital PCR System for Absolute Quantitation of DNA Copy Number. *Analytical Chemistry, 83*(22), 8604-8610.

Hoeh, W. R., Stewart, D. T., & Guttman, S. I. (2002). High Fidelity of Mitochondrial Genome Transmission under the Doubly Uniparental Mode of Inheritance in Freshwater Mussels (Bivalvia: Unionoidea). *Evolution, 56*(11), 2252-2261.

Hoeh, W. R., Stewart, D. T., Sutherland, B. W., & Zouros, E. (1996). Multiple Origins of Gender-Associated Mitochondrial DNA Lineages in Bivalves (Mollusca: Bivalvia). *Evolution, 50*(6), 2276-2286.

Hornbach, D. J., Kurth, V. J., & Hove, M. C. (2010). Variation in Freshwater Mussel Shell Sculpture and Shape Along a River Gradient. *American Midland Naturalist, 164*(1), 22-36.

Hunter, M. E., Dorazio, R. M., Butterfield, J. S. S., Meigs-Friend, G., Nico, L. G., & Ferrante, J. A. (2017). Detection limits of quantitative and digital PCR assays and their influence

in presence–absence surveys of environmental DNA. *Molecular Ecology Resources, 17*(2), 221-229.

Jerde, C. L., Mahon, A. R., Chadderton, W. L., & Lodge, D. M. (2011). "Sight-unseen" detection of rare aquatic species using environmental DNA. *Conservation Letters, 4*(2), 150-157.

Kuenzler, E. J. (1961). Structure and energy flow of a mussel population in a Georgia salt marsh. *Limnology and Oceanography, 6*(2), 191-204.

Kutyavin, I. V., Afonina, I. A., Mills, A., Gorn, V. V., Lukhtanov, E. A., Belousov, E. S., Singer, M. J., Walburger, D. K., Lokhov, S. G., Gall, A. A., Dempcy, R., Reed, M. W., Meyer, R. B., & Hedgpeth, J. (2000). 3′-Minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. *Nucleic Acids Research, 28*(2), 655-661.

Levinton, J. (1995). Bioturbators as ecosystem engineers: control of the sediment fabric, inter-individual interactions, and material fluxes. In *Linking Species & Ecosystems* (pp. 29-36): Springer.

Levy-Booth, D. J., Campbell, R. G., Gulden, R. H., Hart, M. M., Powell, J. R., Klironomos, J. N., Pauls, K. P., Swanton, C. J., Trevors, J. T., & Dunfield, K. E. (2007). Cycling of extracellular DNA in the soil environment. *Soil Biology and Biochemistry, 39*(12), 2977-2991.

Linnaeus, C. v. (1758). Systema naturae, 10th edn, vol. 1. *Stockholm: L. Salvii*.

Liu, W. T., Marsh, T. L., Cheng, H., & Forney, L. J. (1997). Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Applied and Environmental Microbiology, 63*(11), 4516-4522.

Lopes-Lima, M., Teixeira, A., Froufe, E., Lopes, A., Varandas, S., & Sousa, R. (2014). Biology and conservation of freshwater bivalves: past, present and future perspectives. *Hydrobiologia, 735*(1), 1-13.

Lopes-Lima, M., Sousa, R., Geist, J., Aldridge, D. C., Araujo, R., Bergengren, J., Bespalaya, Y., Bódis, E., Burlakova, L., Van Damme, D., Douda, K., Froufe, E., Georgiev, D., Gumpinger, C., Karatayev, A., Kebapçi, Ü., Killeen, I., Lajtner, J., Larsen, B. M., Lauceri, R., Legakis, A., Lois, S., Lundberg, S., Moorkens, E., Motte, G., Nagel, K.-O., Ondina, P., Outeiro, A., Paunovic, M., Prié, V., von Proschwitz, T., Riccardi, N., Rudzīte, M., Rudzītis, M., Scheder, C., Seddon, M., Şereflişan, H., Simić, V., Sokolova, S., Stoeckl, K., Taskinen, J., Teixeira, A., Thielen, F., Trichkova, T., Varandas, S., Vicentini, H., Zajac, K., Zajac, T., & Zogaris, S. (2017). Conservation status of freshwater mussels in Europe: state of the art and future challenges. *Biological Reviews, 92*(1), 572-607.

Lydeard, C., Cowie, R. H., Ponder, W. F., Bogan, A. E., Bouchet, P., Clark, S. A., Cummings, K. S., Frest, T. J., Gargominy, O., & Herbert, D. G. (2004). The global decline of nonmarine mollusks. *BioScience, 54*(4), 321-330.

Ma, H., Stewart, K., Lougheed, S., Zheng, J., Wang, Y., & Zhao, J. (2016). Characterization, optimization, and validation of environmental DNA (eDNA) markers to detect an endangered aquatic mammal. *Conservation Genetics Resources, 8*(4), 561-568.

Machler, E., Deiner, K., Spahn, F., & Altermatt, F. (2016). Fishing in the Water: Effect of Sampled Water Volume on Environmental DNA-Based Detection of Macroinvertebrates. *Environ Sci Technol, 50*(1), 305-12.

Marshall, B. A., Fenwick, M. C., & Ritchie, P. A. (2014). New Zealand Recent Hyriidae (Mollusca: Bivalvia: Unionida). *Molluscan Research, 34*(3), 181-200.

McDowall, B. (2002). Decline of the kakahi–identifying cause and effect. *Water and Atmosphere 10 (4)*, 8-9.

McKee, A. M., Spear, S. F., & Pierson, T. W. (2015). The effect of dilution and the use of a post-extraction nucleic acid purification column on the accuracy, precision, and inhibition of environmental DNA samples. *Biological Conservation, 183*, 70-76.

McMichael, D. F. (Compiler) (1958). *The nature and origin of the New Zealand freshwater mussel fauna*. Transactions of the Royal Society of New Zealand.

Minamoto, T., Yamanaka, H., Takahara, T., Honjo, M. N., & Kawabata, Z. i. (2012). Surveillance of fish species composition using environmental DNA. *Limnology, 13*(2), 193-197.

Mioduchowska, M., Kaczmarczyk, A., Zając, K., Zając, T., & Sell, J. (2016). Gender-Associated Mitochondrial DNA Heteroplasmy in Somatic Tissues of the Endangered Freshwater Mussel Unio crassus (Bivalvia: Unionidae): Implications for Sex Identification and Phylogeographical Studies. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, 325*(9), 610-625.

Moore, S. (2013). Echyridella menziesii. The IUCN Red List of Threatened Species. Version 2015.2. .

Naimo, T. J. (1995). A review of the effects of heavy metals on freshwater mussels. *Ecotoxicology, 4*(6), 341-362.

Navarro, J., & Thompson, R. (1997). Biodeposition by the horse mussel Modiolus modiolus (Dillwyn) during the spring diatom bloom. *Journal of Experimental Marine Biology and Ecology, 209*(1-2), 1-13.

Nowak, J., & Kozlowski, M. (2013). *Mussels: ecology, life habits and control*. Hauppauge, New York: Nova Science Publishers, Inc.

Ogram, A., Sayler, G. S., & Barkay, T. (1987). The extraction and purification of microbial DNA from sediments. *Journal of microbiological methods, 7*(2), 57-66.

Olson, Z. H., Briggler, J. T., & Williams, R. N. (2012). An eDNA approach to detect eastern hellbenders (Cryptobranchus a. alleganiensis) using samples of water. *Wildlife Research, 39*(7), 629-636.

Passamonti, M., & Ghiselli, F. (2009). Doubly Uniparental Inheritance: Two Mitochondrial Genomes, One Precious Model for Organelle DNA Inheritance and Evolution. *DNA Cell Biol, 28*.

Passamonti, M., Ricci, A., Milani, L., & Ghiselli, F. (2011). Mitochondrial genomes and Doubly Uniparental Inheritance: new insights from Musculista senhousia sex-linked mitochondrial DNAs (Bivalvia Mytilidae). *BMC Genomics, 12*(1), 442.

Phillips, N. (2007). *Review of the potential for biomanipulation of phytoplankton abundance by freshwater mussels (kakahi) in the Te Arawa lakes* (Environment Bay of Plenty.

Piaggio, A. J., Engeman, R. M., Hopken, M. W., Humphrey, J. S., Keacher, K. L., Bruce, W. E., & Avery, M. L. (2014). Detecting an elusive invasive species: a diagnostic PCR to detect Burmese python in Florida waters and an assessment of persistence of environmental DNA. *Molecular Ecology Resources, 14*(2), 374-380.

Pietramellara, G., Ascher, J., Borgogni, F., Ceccherini, M., Guerri, G., & Nannipieri, P. (2009). Extracellular DNA in soil and sediment: fate and ecological relevance. *Biology and Fertility of Soils, 45*(3), 219-235.

Pilliod, D. S., Goldberg, C. S., Arkle, R. S., & Waits, L. P. (2013). Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. *Canadian Journal of Fisheries and Aquatic Sciences, 70*(8), 1123-1130.

Pilliod, D. S., Goldberg, C. S., Arkle, R. S., & Waits, L. P. (2014). Factors influencing detection of eDNA from a stream-dwelling amphibian. *Molecular Ecology Resources, 14*(1), 109-116.

Ranjard, L., Poly, F., Lata, J.-C., Mougel, C., Thioulouse, J., & Nazaret, S. (2001). Characterization of bacterial and fungal soil communities by automated ribosomal intergenic spacer analysis fingerprints: biological and methodological variability. *Applied and environmental microbiology, 67*(10), 4479-4487.

Raut, A. D., Kapadnis, B. P., Shashidhar, R., Bandekar, J. R., Vaishampayan, P., & Shouche, Y. S. (2007). Nonspecific PCR amplification of the 16S rRNA gene segment in different bacteria by use of primers specific for Campylobacter, Arcobacter, and Helicobacter spp. *Journal of clinical microbiology, 45*(4), 1376-1377.

Rees, H. C., Maddison, B. C., Middleditch, D. J., Patmore, J. R. M., & Gough, K. C. (2014). REVIEW: The detection of aquatic animal species using environmental DNA – a

review of eDNA as a survey tool in ecology. *Journal of Applied Ecology, 51*(5), 1450-1459.

Rogers-Lowery, C. L., & Dimock Jr, R. V. (2006). Encapsulation of attached ectoparasitic glochidia larvae of freshwater mussels by epithelial tissue on fins of naive and resistant host fish. *The Biological Bulletin, 210*(1), 51-63.

Rondon, M. R., August, P. R., Bettermann, A. D., Brady, S. F., Grossman, T. H., Liles, M. R., Loiacono, K. A., Lynch, B. A., MacNeil, I. A., & Minor, C. (2000). Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. *Applied and environmental microbiology, 66*(6), 2541-2547.

Roper, D. S., & Hickey, C. W. (1994). Population structure, shell morphology, age and condition of the freshwater mussel Hyridella menziesi (Unionacea: Hyriidae) from seven lake and river sites in the Waikato River system. *Hydrobiologia, 284*(3), 205-217.

SantaLucia, J. (2007). Physical principles and visual-OMP software for optimal PCR design. *PCR Primer Design*, 3-33.

Schriewer, A., Wehlmann, A., & Wuertz, S. (2011). Improving qPCR efficiency in environmental samples by selective removal of humic acids with DAX-8. *Journal of Microbiological Methods, 85*(1), 16-21.

Smart, A. S., Weeks, A. R., Rooyen, A. R. v., Moore, A., McCarthy, M. A., & Tingley, R. (2016). Assessing the cost‐efficiency of environmental DNA sampling. *Methods in Ecology and Evolution, 7*(11), 1291-1298.

Śmietanka, B., Wenne, R., & Burzyński, A. (2016). Complete male mitochondrial genomes of European Mytilus edulis mussels. *Mitochondrial DNA Part A, 27*(3), 1634-1635.

Soroka, M. (2008). Doubly uniparental inheritance of mitochondrial DNA in the freshwater bivalve Anodonta woodiana (Bivalvia: Unionidae). *Folia Biol (Krakow), 56*(1-2), 91-5.

Sousa, R., Novais, A., Costa, R., & Strayer, D. L. (2014). Invasive bivalves in fresh waters: impacts from individuals to ecosystems and possible control strategies. *Hydrobiologia, 735*(1), 233-251.

Stankovic, S., & Jovic, M. (2012). Health risks of heavy metals in the mediterranean mussels as seafood. *Environmental chemistry letters, 10*(2), 119-130.

Stankovic, S., Jovic, M., Stankovic, A. R., & Katsikas, L. (2012). Heavy metals in seafood mussels. Risks for human health. In *Environmental chemistry for a sustainable world* (pp. 311-373): Springer.

Stansbery, D. H. (1970). *Eastern Freshwater Mollusks: The Mississippi and St. Lawrence River Systems. I* (Institute of Malacology.

Strayer, D. L. (1999). Effects of alien species on freshwater mollusks in North America. *Journal of the North American Benthological Society, 18*(1), 74-98.

Strayer, D. L. (2008). *Freshwater mussel ecology: a multifactor approach to distribution and abundance* (Vol. 1). Berkeley: University of California Press.

Strayer, D. L. (2014). Understanding how nutrient cycles and freshwater mussels (Unionoida) affect one another. *Hydrobiologia, 735*(1), 277-292.

Strayer, D. L., & Smith, D. R. (2003). *A guide to sampling freshwater mussel populations* (Vol. no. 8; 8). Bethesda, Md: American Fisheries Society.

Strayer, D. L., Caraco, N. F., Cole, J. J., Findlay, S., & Pace, M. L. (1999). Transformation of freshwater ecosystems by bivalves. *BioScience, 49*(1), 19-27.

Strayer, D. L., Downing, J. A., Haag, W. R., King, T. L., Layzer, J. B., Newton, T. J., & Nichols, S. J. (2004). Changing perspectives on pearly mussels, North America's most imperiled animals. *BioScience, 54*(5), 429-439.

Suter, H. (1913). *Manual of the New Zealand mollusca: With an atlas of quarto plates* (Vol. 1: J. Mackay, Government Printer.

Taberlet, P., Coissac, E., Hajibabaei, M., & Rieseberg, L. H. (2012). Environmental DNA. *Molecular ecology, 21*(8), 1789-1793.

Takahara, T., Minamoto, T., Yamanaka, H., Doi, H., & Kawabata, Z. i. (2012). Estimation of Fish Biomass Using Environmental DNA. *Plos One, 7*(4).

Technologies, A. (2010). Critical Factors for Successful Real-Time PCR. *Sample and Assay Technologies, 19*(40), 64-64.

Thomsen, P. F., & Willerslev, E. (2015). Environmental DNA–an emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation, 183*, 4-18.

Thomsen, P. F., Kielgast, J. O. S., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., Orlando, L., & Willerslev, E. (2012). Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology, 21*(11), 2565-2573.

Trdan, R. J., & Hoeh, W. R. (1982). Eurytopic host use by two congeneric species of freshwater mussel (Pelecypoda: Unionidae: Anodonta). *American Midland Naturalist*, 381-388.

Turner, C. R., Barnes, M. A., Xu, C. C. Y., Jones, S. E., Jerde, C. L., & Lodge, D. M. (2014). Particle size distribution and optimal capture of aqueous macrobial eDNA. *Methods in Ecology and Evolution, 5*(7), 676-684.

Vaughn, C. C., & Hakenkamp, C. C. (2001). The functional role of burrowing bivalves in freshwater ecosystems. *Freshwater Biology, 46*(11), 1431-1446.

Vestheim, H., & Jarman, S. N. (2008). Blocking primers to enhance PCR amplification of rare sequences in mixed samples–a case study on prey DNA in Antarctic krill stomachs. *Frontiers in Zoology, 5*(1), 12.

Walker, J. M., Curole, J. P., Wade, D. E., Chapman, E. G., Bogan, A. E., Watters, G. T., & Hoeh, W. R. (2006). Taxonomic distribution and phylogenetic utility of gender-associated mitochondrial genomes in the Unionoida (Bivalvia). *MALACOLOGIA-PHILADELPHIA-, 48*(1/2), 265.

Walker, K. F., Jones, H. A., & Klunzinger, M. W. (2014). Bivalves in a bottleneck: taxonomy, phylogeography and conservation of freshwater mussels (Bivalvia: Unionoida) in Australasia. *Hydrobiologia, 735*(1), 61-79.

Walker, K. F., Byrne, M., Hickey, C. W., & Roper, D. S. (2001). Freshwater mussels (Hyriidae) of Australasia. In *Ecology and evolution of the freshwater mussels Unionoida* (pp. 5-31): Springer.

Watters, G. T. (Compiler) (1999). *Freshwater mussels and water quality: a review of the effects of hydrologic and instream habitat alterations*. Proceedings of the First Freshwater Mollusk Conservation Society Symposium.

Watters, G. T., & O'dee, S. (Compiler) (2000). *Glochidial release as a function of water temperature: beyond bradyticty and tachyticty*. Proceedings of the conservation, captive care, and propagation of freshwater mussels symposium. Ohio Biological Survey, Ohio.

Welker, M., & Walz, N. (1998). Can mussels control the plankton in rivers?—a planktological approach applying a Lagrangian sampling strategy. *Limnology and Oceanography, 43*(5), 753-762.

Wilcox, T. M., McKelvey, K. S., Young, M. K., Jane, S. F., Lowe, W. H., Whiteley, A. R., & Schwartz, M. K. (2013). Robust Detection of Rare Species Using Environmental DNA: The Importance of Primer Specificity. *Plos One, 8*(3).

Williams, J. D., Warren Jr, M. L., Cummings, K. S., Harris, J. L., & Neves, R. J. (1993). Conservation status of freshwater mussels of the United States and Canada. *Fisheries, 18*(9), 6-22.

Wood, S. A., Smith, K. F., Banks, J. C., Tremblay, L. A., Rhodes, L., Mountfort, D., Cary, S. C., & Pochon, X. (2013). Molecular genetic tools for environmental monitoring of New Zealand's aquatic habitats, past, present and the future. *New Zealand Journal of Marine and Freshwater Research, 47*(1), 90-119.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., & Madden, T. L. (2012). Primer-BLAST: A tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics, 13*, 134-134.

Zardi, G. I., Nicastro, K. R., McQuaid, C. D., Castilho, R., Costa, J., Serrão, E. A., & Pearson, G. A. (2015). Intraspecific genetic lineages of a marine mussel show behavioural divergence and spatial segregation over a tropical/subtropical biogeographic transition. *BMC Evolutionary Biology, 15*(1), 100.

Zouros, E. (2013). Biparental Inheritance Through Uniparental Transmission: The Doubly Uniparental Inheritance (DUI) of Mitochondrial DNA. *Evolutionary Biology, 40*(1), 1-31.

Zouros, E., Ball, A. O., Saavedra, C., & Freeman, K. R. (1994). Mitochondrial DNA inheritance. *Nature, 368*.

Appendix

Appendix I. List of mussel samples in this thesis

Species were confirmed by *COI* sequence (57F-322R)

Sample No.	Species	Sample Type	Description
1	E. menziesii	Mussel specimen	7/9/2015 Lake Rotorua, Parkcliff Rd Reserve
2	E. menziesii		7/9/2015 Rototiti Lake, Okawa Bay Boatramp
3	E. menziesii		13/8/2015 Lake Rotoehu, Kennedy Bay Boatramp
4	E. menziesii		7/9/2015 Lake Tarawera, Boatshed Bay
5	E. menziesii		7/9/2015 Lake Rotokakahi, Boatramp
6	E. menziesii		28/4/2016 1253_8 E1791222N577122 Waikato River, Waitomo Valley Road
7	E. menziesii		27/4/2016 707_9 E1818543N5779863 Owairaka Stream, Wairoka Valley Road
8	E. menziesii		13/9/12 Manurima Stream
9	E. aucklandica		28/4/2016 1253_8 E1791222N577122 Waikato River, Waitomo Valley Road
10	E. aucklandica		27/4/2016 707_9 E1818543N5779863 Owairaka Stream, Wairoka Valley Road
M1	-	Foot and mantle tissue	1/9/2015 Unknown location
M2	E. onekaka		
M3	E. onekaka		
M4	E. menziesii		
M5	E. menziesii		
M6	E. onekaka		
M7	E. onekaka		

M8	E. onekaka		
M9	E. onekaka		
M10	-		
M11	E. onekaka		
M12	E. onekaka		
M13	E. onekaka		
M14	E. menziesii		
M15	E. onekaka		
M16	E. onekaka		
PC1	E. menziesii		
PC2	E. menziesii		
PC3	E. menziesii		
PC4	E. menziesii		
PC5	-		
PC6	-		

Appendix II. Accession numbers of 1,765 *Unionoida* sequences

AB040823	AF156496	AF231732	AF232811	AF385095	AY094373	AY211584
AB040824	AF156497	AF231733	AF232812	AF385096	AY094374	AY211585
AB040825	AF156498	AF231734	AF232813	AF385097	AY211550	AY211586
AB040826	AF156499	AF231735	AF232815	AF385098	AY211551	AY211587
AB040827	AF156500	AF231736	AF232816	AF385099	AY211552	AY211588
AB040828	AF156501	AF231737	AF232817	AF385100	AY211553	AY211589
AB040829	AF156502	AF231738	AF232818	AF385101	AY211554	AY211590
AB040830	AF156503	AF231739	AF232819	AF385102	AY211555	AY211591
AB040831	AF156504	AF231740	AF232820	AF385103	AY211556	AY211592
AB040832	AF156505	AF231741	AF232821	AF385104	AY211557	AY211593
AF049499	AF156506	AF231742	AF232822	AF385105	AY211558	AY211594
AF049500	AF156507	AF231743	AF232823	AF385106	AY211559	AY211595
AF049501	AF156508	AF231744	AF232824	AF385107	AY211560	AY211596
AF049502	AF156509	AF231745	AF232825	AF385108	AY211561	AY211597
AF049503	AF156510	AF231746	AF303309	AF385109	AY211562	AY211598
AF049504	AF156511	AF231747	AF303313	AF385110	AY211563	AY386969
AF049505	AF156512	AF231748	AF303321	AF385111	AY211564	AY386970
AF049506	AF156513	AF231749	AF303323	AF385112	AY211565	AY386971
AF049507	AF156514	AF231750	AF303330	AF385113	AY211566	AY386972
AF049508	AF156515	AF231751	AF303331	AF385114	AY211567	AY386973
AF049509	AF156516	AF231752	AF303333	AF406794	AY211568	AY386974
AF049510	AF156517	AF231753	AF303334	AF406795	AY211569	AY386975
AF049511	AF156518	AF231754	AF303336	AF406796	AY211570	AY386976
AF049512	AF156519	AF231755	AF303337	AF406797	AY211571	AY386977
AF049513	AF156520	AF231756	AF303339	AF406798	AY211572	AY386978
AF049514	AF156521	AF231757	AF303349	AF406799	AY211573	AY386979
AF049515	AF156522	AF232801	AF305367	AF406800	AY211574	AY386980
AF049516	AF156523	AF232802	AF305368	AF406801	AY211575	AY386981
AF049517	AF156524	AF232803	AF305369	AF406802	AY211576	AY386982
AF049518	AF156525	AF232804	AF305370	AF406803	AY211577	AY386983
AF049519	AF156526	AF232805	AF305371	AF406804	AY211578	AY386984
AF049520	AF156527	AF232806	AF305372	AF406805	AY211579	AY386985
AF049521	AF156528	AF232807	AF385091	AF468683	AY211580	AY386986
AF049522	AF231729	AF232808	AF385092	AF494102	AY211581	AY386987
AF120652	AF231730	AF232809	AF385093	AF514296	AY211582	AY386988
AF120653	AF231731	AF232810	AF385094	AY094372	AY211583	AY386989

AY386990	AY493473	AY613820	AY655004	AY785401	DQ298528	DQ648097
AY386991	AY493474	AY613821	AY655005	DQ060168	DQ298529	DQ648098
AY386992	AY493475	AY613822	AY655006	DQ060169	DQ298530	DQ648099
AY386993	AY493476	AY613823	AY655007	DQ060170	DQ298531	DQ648100
AY386994	AY493477	AY613824	AY655008	DQ060171	DQ298532	DQ648101
AY386995	AY493478	AY613825	AY655009	DQ060173	DQ298533	DQ648102
AY386996	AY493479	AY613826	AY655010	DQ060174	DQ298534	DQ648103
AY386997	AY493480	AY613827	AY655011	DQ060175	DQ298535	DQ648104
AY386998	AY493481	AY613828	AY655012	DQ060176	DQ298536	DQ648105
AY386999	AY493482	AY613829	AY655013	DQ220724	DQ298537	DQ648106
AY387000	AY493484	AY613830	AY655014	DQ220725	DQ298538	DQ648108
AY387001	AY493486	AY613831	AY655015	DQ220726	DQ298539	DQ648109
AY387002	AY493488	AY613832	AY655016	DQ264394	DQ340804	DQ648110
AY387003	AY493489	AY613833	AY655017	DQ272359	DQ383427	DQ648111
AY387004	AY493490	AY613834	AY655018	DQ272360	DQ383428	DQ648112
AY387005	AY493491	AY613835	AY655019	DQ272361	DQ383429	DQ648113
AY387006	AY493492	AY613836	AY655020	DQ272362	DQ383430	DQ648114
AY387007	AY493493	AY613837	AY655021	DQ272363	DQ383431	DQ648115
AY387008	AY493494	AY613838	AY655022	DQ272364	DQ383432	DQ648116
AY387009	AY493496	AY613839	AY655023	DQ272365	DQ383433	DQ648117
AY387010	AY493497	AY613840	AY655024	DQ272366	DQ383434	DQ648118
AY387011	AY493498	AY613841	AY655025	DQ272367	DQ479944	DQ648119
AY387012	AY493499	AY613842	AY655026	DQ272368	DQ479945	DQ648120
AY387013	AY493500	AY613843	AY785383	DQ272369	DQ479946	DQ648121
AY387014	AY493503	AY613844	AY785384	DQ272370	DQ479947	EF033252
AY387015	AY493504	AY613845	AY785385	DQ272371	DQ479948	EF033253
AY387016	AY493505	AY613846	AY785386	DQ272372	DQ479949	EF033254
AY387017	AY493506	AY654990	AY785387	DQ272373	DQ648083	EF033255
AY387018	AY493507	AY654991	AY785388	DQ272374	DQ648084	EF033256
AY387019	AY522851	AY654992	AY785389	DQ272375	DQ648085	EF033257
AY387020	AY522857	AY654993	AY785390	DQ272376	DQ648086	EF033258
AY493462	AY579122	AY654994	AY785391	DQ272377	DQ648087	EF033259
AY493463	AY579123	AY654995	AY785392	DQ272378	DQ648088	EF033260
AY493464	AY579124	AY654996	AY785393	DQ272379	DQ648089	EF033261
AY493465	AY579125	AY654997	AY785394	DQ272380	DQ648090	EF033262
AY493466	AY579126	AY654998	AY785395	DQ272381	DQ648091	EF033263
AY493467	AY579127	AY654999	AY785396	DQ272382	DQ648092	EF033264
AY493468	AY579128	AY655000	AY785397	DQ272383	DQ648093	EF033265
AY493469	AY579129	AY655001	AY785398	DQ298524	DQ648094	EF033266
AY493470	AY579130	AY655002	AY785399	DQ298526	DQ648095	EF033267
AY493472	AY579131	AY655003	AY785400	DQ298527	DQ648096	EF033268

EF033269	EF507812	EU327354	EU698925	GU070953	GU085305	HQ153534
EF033270	EF507813	EU327355	EU698926	GU070961	GU085306	HQ153535
EF033271	EF507814	EU327356	EU698927	GU070964	GU085307	HQ153536
EF033290	EF507815	EU327357	EU698928	GU070965	GU085309	HQ153537
EF033291	EF507816	EU429676	EU698929	GU070968	GU085310	HQ153538
EF033292	EF571387	EU429677	EU698930	GU070969	GU085311	HQ153539
EF033293	EF571389	EU429680	EU698931	GU070970	GU085312	HQ153540
EF033294	EF571390	EU429684	EU698932	GU070971	GU085313	HQ153541
EF033295	EF571391	EU429685	EU698933	GU070975	GU085314	HQ153542
EF033296	EF571392	EU698893	EU698934	GU070976	GU085315	HQ153543
EF033297	EF571393	EU698894	EU698935	GU070980	GU085316	HQ153544
EF033298	EF571394	EU698895	EU698936	GU070981	GU085317	HQ153545
EF033299	EF571395	EU698896	EU698937	GU070983	GU085318	HQ153546
EF033300	EF571396	EU698897	EU698938	GU070984	GU085319	HQ153547
EF033301	EF571397	EU698898	EU698939	GU070986	GU085320	HQ153548
EF033302	EF571398	EU698899	EU698940	GU070988	GU085321	HQ153549
EF033303	EF571399	EU698900	EU698941	GU070989	GU085322	HQ153550
EF033304	EF571401	EU698901	EU698942	GU085280	GU085323	HQ153551
EF033305	EF571402	EU698902	EU698943	GU085281	GU085324	HQ153552
EF033306	EF571403	EU698903	EU698944	GU085282	GU085326	HQ153553
EF033307	EF571404	EU698904	EU698945	GU085283	GU085327	HQ153554
EF033308	EF571405	EU698905	EU698946	GU085284	GU085328	HQ153555
EF033309	EF571406	EU698906	EU698947	GU085285	GU085329	HQ153556
EF418016	EF571407	EU698907	EU698948	GU085287	GU085332	HQ153557
EF418017	EF571412	EU698908	EU698949	GU085288	GU085333	HQ153558
EF418018	EF571413	EU698909	EU698950	GU085289	GU230742	HQ153559
EF418019	EF571417	EU698910	EU735754	GU085290	GU230743	HQ153560
EF418020	EF571419	EU698911	EU735760	GU085291	GU230744	HQ153561
EF418021	EF571420	EU698912	EU735761	GU085292	GU230745	HQ153562
EF418022	EF571421	EU698913	EU735763	GU085293	GU230746	HQ153563
EF418023	EF571422	EU698914	GQ149468	GU085294	GU230747	HQ153564
EF418024	EF571423	EU698915	GQ149469	GU085295	GU230748	HQ153565
EF488184	EF571428	EU698916	GQ149471	GU085296	GU230749	HQ153566
EF488185	EF571429	EU698917	GQ149472	GU085297	GU230750	HQ153567
EF488186	EF571436	EU698918	GQ149473	GU085298	HM852948	HQ153568
EF488187	EF571439	EU698919	GQ149474	GU085299	HQ153528	HQ153569
EF488188	EF571442	EU698920	GQ149475	GU085300	HQ153529	HQ153570
EF488189	EU327350	EU698921	GU070948	GU085301	HQ153530	HQ153571
EF507805	EU327351	EU698922	GU070949	GU085302	HQ153531	HQ153572
EF507810	EU327352	EU698923	GU070951	GU085303	HQ153532	HQ153573
EF507811	EU327353	EU698924	GU070952	GU085304	HQ153533	HQ153574

HQ153575	HQ153616	HQ828397	HQ828743	JN243904	JQ437390	KC291716
HQ153576	HQ153617	HQ828544	HQ828753	JN243905	JQ437391	KC291717
HQ153577	HQ153618	HQ828546	HQ828761	JN612806	JQ437392	KC408744
HQ153578	HQ153619	HQ828556	HQ828763	JN612807	JQ437393	KC408745
HQ153579	HQ153620	HQ828560	HQ828764	JN612808	JQ691662	KC408746
HQ153580	HQ153621	HQ828570	HQ828765	JN612811	JQ861221	KC408747
HQ153581	HQ186048	HQ828571	HQ828771	JN612812	JQ861222	KC408748
HQ153582	HQ186055	HQ828589	HQ828772	JN612813	JQ861223	KC408749
HQ153583	HQ186067	HQ828593	HQ828778	JN612814	JQ861224	KC408750
HQ153584	HQ186068	HQ828597	HQ828779	JN612815	JQ861225	KC408751
HQ153585	HQ186071	HQ828601	HQ828794	JN612817	JQ861226	KC408752
HQ153586	HQ186075	HQ828606	HQ912965	JN612818	JQ861227	KC408753
HQ153587	HQ186079	HQ828610	HQ912966	JN612821	JQ861228	KC408754
HQ153588	HQ186098	HQ828611	HQ912967	JN612822	JQ861230	KC408755
HQ153589	HQ186102	HQ828614	HQ912968	JN612832	JQ861231	KC408756
HQ153590	HQ186122	HQ828618	HQ912969	JN612833	JQ861233	KC408757
HQ153591	HQ186141	HQ828623	HQ912970	JN612836	JQ861234	KC408758
HQ153592	HQ186142	HQ828625	HQ912971	JQ253872	JQ861235	KC408759
HQ153593	HQ186156	HQ828628	JF700152	JQ253873	JQ861236	KC408760
HQ153594	HQ186164	HQ828636	JF700153	JQ253874	JQ861238	KC408761
HQ153595	HQ186178	HQ828640	JF700192	JQ253875	JQ861239	KC408762
HQ153596	HQ186200	HQ828670	JN243884	JQ253876	JQ861240	KC408763
HQ153597	HQ186206	HQ828674	JN243885	JQ253877	JQ861241	KC408764
HQ153598	HQ186215	HQ828676	JN243886	JQ253878	JQ861242	KC408765
HQ153599	HQ186219	HQ828677	JN243887	JQ253879	JQ861243	KC408766
HQ153600	HQ612178	HQ828679	JN243888	JQ253880	JQ861244	KC408767
HQ153601	HQ828260	HQ828683	JN243889	JQ253881	JX101478	KC408768
HQ153602	HQ828263	HQ828684	JN243890	JQ253882	JX101479	KC408769
HQ153603	HQ828279	HQ828689	JN243891	JQ253883	JX101480	KC408770
HQ153604	HQ828291	HQ828694	JN243892	JQ253884	JX101481	KC408771
HQ153605	HQ828301	HQ828699	JN243893	JQ253885	JX101482	KC408772
HQ153606	HQ828312	HQ828703	JN243894	JQ253886	JX101483	KC408773
HQ153607	HQ828323	HQ828705	JN243895	JQ253887	JX101484	KC408774
HQ153608	HQ828345	HQ828724	JN243896	JQ253888	JX101485	KC408775
HQ153609	HQ828360	HQ828726	JN243897	JQ253889	JX101486	KC408776
HQ153610	HQ828364	HQ828730	JN243898	JQ253890	JX101487	KC408777
HQ153611	HQ828366	HQ828732	JN243899	JQ253891	JX101488	KC408778
HQ153612	HQ828381	HQ828735	JN243900	JQ253892	JX101489	KC408779
HQ153613	HQ828383	HQ828739	JN243901	JQ253894	JX101490	KC408780
HQ153614	HQ828384	HQ828740	JN243902	JQ253895	JX101491	KC408781
HQ153615	HQ828391	HQ828741	JN243903	JQ435822	KC150028	KC408782

KC408783 KC583482 KC703907 KC703969 KF035184 KF035257 KF647473
KC408784 KC583484 KC703908 KC847114 KF035185 KF035262 KF647482
KC408785 KC583485 KC703909 KF035133 KF035186 KF035263 KF647484
KC408786 KC583486 KC703911 KF035134 KF035187 KF035264 KF647496
KC408787 KC583488 KC703914 KF035135 KF035188 KF035265 KF647497
KC408788 KC583496 KC703919 KF035136 KF035189 KF035266 KF647508
KC408789 KC583499 KC703924 KF035137 KF035191 KF035267 KF647515
KC408790 KC583501 KC703925 KF035139 KF035194 KF035268 KF647521
KC408791 KC583505 KC703926 KF035140 KF035195 KF035269 KF647528
KC408792 KC583507 KC703927 KF035141 KF035196 KF035270 KF672864
KC408793 KC583510 KC703928 KF035142 KF035197 KF035273 KF672865
KC408794 KC583513 KC703930 KF035143 KF035198 KF035274 KF672866
KC408795 KC583516 KC703931 KF035144 KF035199 KF035276 KF672867
KC408796 KC583518 KC703932 KF035145 KF035200 KF035277 KF672868
KC408797 KC703870 KC703933 KF035147 KF035201 KF035279 KF672869
KC408798 KC703871 KC703934 KF035148 KF035202 KF035280 KF672870
KC408799 KC703876 KC703935 KF035149 KF035204 KF647333 KF672871
KC408800 KC703878 KC703936 KF035151 KF035209 KF647334 KF672872
KC408801 KC703879 KC703938 KF035152 KF035211 KF647361 KF672873
KC429106 KC703880 KC703939 KF035154 KF035213 KF647363 KF672874
KC429107 KC703881 KC703940 KF035156 KF035214 KF647367 KF672875
KC429108 KC703885 KC703942 KF035158 KF035215 KF647371 KF672876
KC429109 KC703886 KC703944 KF035160 KF035216 KF647373 KF672877
KC583447 KC703887 KC703945 KF035162 KF035217 KF647382 KF672878
KC583450 KC703888 KC703947 KF035163 KF035222 KF647385 KF672879
KC583452 KC703889 KC703948 KF035164 KF035223 KF647389 KF672880
KC583453 KC703890 KC703949 KF035165 KF035224 KF647391 KF672881
KC583456 KC703891 KC703950 KF035166 KF035225 KF647416 KF672882
KC583458 KC703892 KC703951 KF035168 KF035226 KF647417 KF672883
KC583459 KC703893 KC703952 KF035170 KF035228 KF647423 KF672884
KC583460 KC703894 KC703953 KF035171 KF035230 KF647425 KF672885
KC583462 KC703895 KC703954 KF035173 KF035231 KF647431 KF672886
KC583465 KC703896 KC703956 KF035174 KF035232 KF647432 KF672887
KC583470 KC703899 KC703957 KF035176 KF035235 KF647436 KF672888
KC583472 KC703900 KC703960 KF035177 KF035236 KF647443 KF672889
KC583474 KC703901 KC703962 KF035178 KF035237 KF647444 KF672890
KC583475 KC703902 KC703964 KF035179 KF035240 KF647446 KF672891
KC583476 KC703903 KC703965 KF035180 KF035241 KF647449 KF672892
KC583478 KC703904 KC703966 KF035181 KF035244 KF647458 KF672893
KC583480 KC703905 KC703967 KF035182 KF035249 KF647462 KF672894
KC583481 KC703906 KC703968 KF035183 KF035254 KF647466 KF672895

KF672896 KJ081164 KJ434479 KJ434524 KJ716947 KM262547 KP779725
KF672897 KJ081165 KJ434480 KJ434525 KM262507 KM262548 KP779733
KF672898 KJ081166 KJ434481 KJ434527 KM262508 KM262549 KP779735
KF672899 KJ081167 KJ434483 KJ434528 KM262509 KM262550 KP779736
KF672900 KJ081168 KJ434485 KJ434529 KM262510 KM262551 KP779738
KF672901 KJ081169 KJ434486 KJ434530 KM262511 KM268534 KP779742
KF672902 KJ081170 KJ434487 KJ434531 KM262512 KM272949 KP779778
KF672903 KJ081171 KJ434488 KJ434533 KM262513 KM656075 KP779779
KF701431 KJ081172 KJ434490 KJ525912 KM262514 KM656076 KP779789
KF701432 KJ081173 KJ434491 KJ525913 KM262515 KM656077 KP779791
KF701433 KJ081174 KJ434492 KJ525914 KM262516 KM656078 KP779799
KF701434 KJ081175 KJ434493 KJ525915 KM262517 KP184896 KP779801
KF701435 KJ081176 KJ434494 KJ525916 KM262518 KP184897 KP779842
KF701436 KJ081177 KJ434495 KJ525917 KM262519 KP184898 KP779843
KF701437 KJ081178 KJ434496 KJ525918 KM262520 KP184899 KP779844
KF701438 KJ081179 KJ434497 KJ525919 KM262521 KP184900 KP779845
KF701439 KJ081180 KJ434498 KJ525920 KM262522 KP184901 KP779846
KF701440 KJ081181 KJ434499 KJ525921 KM262523 KP184902 KP779847
KF701441 KJ081182 KJ434500 KJ525922 KM262524 KP184903 KP779848
KF701442 KJ081183 KJ434501 KJ525923 KM262525 KP184904 KP779849
KF701443 KJ161500 KJ434502 KJ525924 KM262526 KP184905 KP779850
KF701444 KJ161505 KJ434503 KJ526779 KM262527 KP184906 KP779851
KF701445 KJ161508 KJ434504 KJ526791 KM262528 KP184907 KP779852
KF701446 KJ161509 KJ434505 KJ526792 KM262529 KP184908 KP779853
KF701447 KJ161510 KJ434506 KJ716925 KM262530 KP184909 KP779854
KF701448 KJ161511 KJ434507 KJ716927 KM262531 KP184910 KP779855
KF701449 KJ161512 KJ434508 KJ716928 KM262532 KP184911 KP779856
KF701450 KJ161513 KJ434509 KJ716929 KM262533 KP184912 KP779860
KF701451 KJ161514 KJ434510 KJ716930 KM262534 KP184913 KP779861
KF701452 KJ161520 KJ434511 KJ716931 KM262535 KP184914 KP779862
KF701453 KJ161522 KJ434513 KJ716932 KM262536 KP184915 KP779863
KF701454 KJ161532 KJ434514 KJ716933 KM262537 KP184916 KP779867
KF701455 KJ161551 KJ434515 KJ716934 KM262538 KP184917 KP779868
KF701456 KJ402053 KJ434516 KJ716935 KM262539 KP184918 KP779869
KF701457 KJ402054 KJ434517 KJ716937 KM262540 KP779663 KP779870
KF701458 KJ434469 KJ434518 KJ716941 KM262541 KP779664 KP779871
KF701459 KJ434470 KJ434519 KJ716942 KM262542 KP779676 KP779873
KJ081160 KJ434472 KJ434520 KJ716943 KM262543 KP779682 KP779874
KJ081161 KJ434473 KJ434521 KJ716944 KM262544 KP779688 KP779875
KJ081162 KJ434475 KJ434522 KJ716945 KM262545 KP779702 KP779877
KJ081163 KJ434477 KJ434523 KJ716946 KM262546 KP779710 KP779878

KP779879
KP779881
KP779882
KP779885
KP779887
KP779888
KP779889
KP779890
KP779892
KP779893
KP779896
KP779898
KP779899
KP779900
KP795021
KP795022
KP795023
KP795024
KP795025
KP795026
KP795027
KP795028
KP795029
KP795030
KP795031
KP795032
KP795033
KP795034
KP795035
KP795036
KP795037
KP795038
KP843087
KR011044
KR011047
KR011048
KT285617
KT285618
KT285619
KT285620
KT285621
KT285622
KT285623
KT285624
KT285625
KT285626
KT285627
KT285628
KT285629
KT285632
KT285633
KT285634
KT285636
KT285637
KT285638
KT285639
KT285640
KT285641
KT285642
KT285643
KT285644
KT285645
KT285646
KT285647
KT285648
KT285649
KT285650
KT285651
KT285652
KT285654
KT285655
KT285656
KT285657
KT285658
KT285659
KT285660
KT362704
KT362705

Appendix III. Representative Primer-BLAST result

Representative Primer-BLAST results of 57F(GAGTTGGGGCAGCCTG) and 322R (ACAGT**Y**CACCCAGTCCCAA) with 0-2 mismatches

>KX713505.1 Triplodon corrugatus voucher BivAToL-380 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial

product length = 266

Forward primer 1 GAGTTGGGGCAGCCTG 16

Template 70 85

Reverse primer 1 ACAGTCCACCCAGTCCCAA 19

Template 335 317

>KJ434533.1 Solenaia rivularis isolate 68SR6 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial

```
product length = 266
Forward primer  1    GAGTTGGGGCAGCCTG  16
Template        22   ................  37

Reverse primer  1    ACAGTCCACCCAGTCCCAA  19
Template        287  ..........T.......  269
```

>KJ434518.1 Solenaia triangularis isolate 212ST2 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial

```
product length = 266
Forward primer  1    GAGTTGGGGCAGCCTG  16
Template        22   ................  37

Reverse primer  1    ACAGTCCACCCAGTCCCAA  19
Template        287  ..........T.......  269
```

>JN612836.1 Hyridella australis voucher HA_Glo_M_273285_02 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial

```
product length = 266
Forward primer  1   GAGTTGGGGCAGCCTG  16
Template        4   ................  19

Reverse primer  1    ACAGTCCACCCAGTCCCAA  19
Template        269  .....T............  251
```

>HQ153605.1 Lampsilis radiata isolate wuspCOX78 cytochrome oxidase subunit I (coxI) gene, partial cds; mitochondrial

```
product length = 266
Forward primer  1  GAGTTGGGGCAGCCTG  16
Template        1  ................  16

Reverse primer  1    ACAGTCCACCCAGTCCCAA  19
Template        266  ..T.............  248
```

>EF507810.1 Epioblasma triquetra isolate St. Croix River 2 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial

```
product length = 266
Forward primer  1   GAGTTGGGGCAGCCTG  16
Template        27  ................  42

Reverse primer  1    ACAGTCCACCCAGTCCCAA  19
Template        292  ..C.............  274
```

>AF231744.1 Diplodon deceptus cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial gene for mitochondrial product

```
product length = 266
Forward primer  1   GAGTTGGGGCAGCCTG  16
Template        64  ................  79
```

```
Reverse primer  1    ACAGTCCACCCAGTCCCAA  19
Template        329  .....T.............  311
```

>KT285655.1 Quadrula mortoni voucher FLMNH441171 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial

```
product length = 266
Forward primer  1    GAGTTGGGGCAGCCTG  16
Template        70   ................  85

Reverse primer  1    ACAGTCCACCCAGTCCCAA  19
Template        335  ..C.....G..........  317
```

>KT285649.1 Quadrula houstonensis voucher FLMNH441135 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial

```
product length = 266
Forward primer  1    GAGTTGGGGCAGCCTG  16
Template        70   ................  85

Reverse primer  1    ACAGTCCACCCAGTCCCAA  19
Template        335  ..C.....G..........  317
```

>KT285618.1 Amblema plicata voucher FLMNH441152 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial

```
product length = 266
Forward primer  1    GAGTTGGGGCAGCCTG  16
```

```
Template        70  ...............  85

Reverse primer  1    ACAGTCCACCCAGTCCCAA  19
Template        335  ..T.....A.........  317
```

>KT285617.1 Amblema neislerii voucher FLMNH437977 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial

```
product length = 266
Forward primer  1   GAGTTGGGGCAGCCTG  16
Template        70  ...............  85

Reverse primer  1    ACAGTCCACCCAGTCCCAA  19
Template        335  ..T.....A.........  317
```

>KU946941.1 Potomida littoralis isolate Plit138 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial

```
product length = 266
Forward primer  1   GAGTTGGGGCAGCCTG  16
Template        71  ...............  86

Reverse primer  1    ACAGTCCACCCAGTCCCAA  19
Template        336  ..T.............C.  318
```

>KP795033.1 Ensidens sagittarius voucher UMMZ:304651 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial

```
product length = 266
Forward primer  1    GAGTTGGGGCAGCCTG  16
Template        69   ................  84

Reverse primer  1    ACAGTCCACCCAGTCCCAA  19
Template        334  ..T..T............  316
```

>KP270876.1 Reptantia sp. MCZ IZ 45643 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial

```
product length = 266
Forward primer  1    GAGTTGGGGCAGCCTG  16
Template        71   ................  86

Reverse primer  1    ACAGTCCACCCAGTCCCAA  19
Template        336  ........T.........G  318
```

>KF261326.1 Nacella concinna isolate H13N cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial

```
product length = 266
Forward primer  1    GAGTTGGGGCAGCCTG  16
Template        85   ................  100

Reverse primer  1    ACAGTCCACCCAGTCCCAA  19
Template        350  .....T........T....  332
```

>KJ434508.1 Lamprotula caveata isolate 116LAC6 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial

```
product length = 266
Forward primer  1    GAGTTGGGGCAGCCTG  16
Template        22   ................  37

Reverse primer  1    ACAGTCCACCCAGTCCCAA  19
Template        287  ..C.............C.   269
```

>JQ964734.1 Colobostylus redfieldianus voucher ANSP:JBS514-01 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial

```
product length = 266
Forward primer  1    GAGTTGGGGCAGCCTG  16
Template        85   ................  100

Reverse primer  1    ACAGTCCACCCAGTCCCAA  19
Template        350  .....T........T....  332
```

>HM230410.1 Cyclonaias tuberculata voucher UAM1490 cytochrome oxidase subunit 1 (cox1) gene, partial cds; mitochondrial

```
product length = 266
Forward primer  1    GAGTTGGGGCAGCCTG  16
Template        52   ................  67
```

```
Reverse primer  1    ACAGTCCACCCAGTCCCAA  19
Template        317  ..C.....G.........  299
```

>JN243889.1 Castalia ambigua voucher ANSP:416341 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial

```
product length = 266
Forward primer  1    GAGTTGGGGCAGCCTG  16
Template        69   ................  84

Reverse primer  1    ACAGTCCACCCAGTCCCAA  19
Template        334  .....T..A.........  316
```

>GU085318.1 Quadrula pustulosa voucher photo PP3 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial

```
product length = 266
Forward primer  1    GAGTTGGGGCAGCCTG  16
Template        40   ................  55

Reverse primer  1    ACAGTCCACCCAGTCCCAA  19
Template        305  ..C.....G.........  287
```

>EF033266.1 Hamiota subangulata isolate H1720 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial

```
product length = 266
Forward primer  1    GAGTTGGGGCAGCCTG  16
Template        109  ................  124

Reverse primer  1    ACAGTCCACCCAGTCCCAA  19
Template        374  ..C..........T....  356
```

>EF033255.1 Toxolasma glans isolate H1709 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial

```
product length = 266
Forward primer  1    GAGTTGGGGCAGCCTG  16
Template        109  ................  124

Reverse primer  1    ACAGTCCACCCAGTCCCAA  19
Template        374  ..T.....A.........  356
```

>KY067440.1 Ptychorhynchus ptisteri haplotype female mitochondrion, complete genome

```
product length = 266
Forward primer  1     GAGTTGGGGCAGCCTG  16
Template        1437  ................  1422

Reverse primer  1     ACAGTCCACCCAGTCCCAA  19
```

```
Template        1172  ..........T..T....  1190
```

>AY787245.1 Astralium rhodostomum haplotype NEC8 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial

```
product length = 266
Forward primer  1    GAGTTGGGGCAGCCTG  16
Template        54   ................  69

Reverse primer  1     ACAGTCCACCCAGTCCCAA  19
Template        319   ..T..T............  301
```

>AF156526.1 Villosa vanuxemensis UMMZ 265714 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial gene for mitochondrial product

```
product length = 266
Forward primer  1    GAGTTGGGGCAGCCTG  16
Template        69   ................  84

Reverse primer  1     ACAGTCCACCCAGTCCCAA  19
Template        334   ..C..........T....  316
```

>AY655023.1 Toxolasma texasiensis voucher UAUC80 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial

```
product length = 266
```

```
Forward primer  1    GAGTTGGGGCAGCCTG  16
Template        43   ................  58

Reverse primer  1     ACAGTCCACCCAGTCCCAA  19
Template        308   ..C.....A..........  290
```

>AF385104.1 Lampsilis subangulata UAUC#116 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial gene for mitochondrial product

```
product length = 266
Forward primer  1    GAGTTGGGGCAGCCTG  16
Template        43   ................  58

Reverse primer  1     ACAGTCCACCCAGTCCCAA  19
Template        308   ..C...........T....  290
```

>AF385101.1 Lampsilis australis UAUC#128 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial gene for mitochondrial product

```
product length = 266
Forward primer  1    GAGTTGGGGCAGCCTG  16
Template        43   ................  58

Reverse primer  1     ACAGTCCACCCAGTCCCAA  19
```

```
Template        308  ..C..T.............  290
```

>AF385100.1 Lampsilis australis UAUC#643 cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial gene for mitochondrial product

```
product length = 266
Forward primer  1    GAGTTGGGGCAGCCTG  16
Template        43   ................  58

Reverse primer  1    ACAGTCCACCCAGTCCCAA  19
Template        308  ..C...........T....  290
```

Appendix IV. Sequencing result

Multiple sequence alignment contains 15 sequences of *E. menziesii* samples,1 sequence of *E. aucklandica*, and 1 sequences of *E. onekaka.* The latter two species don't have interspecies heterogenetic bases within the sequenced area thus shown with one sequence only.

```
                    1         10        20        30        40        50        60
                    |         |         |         |         |         |         |
      menziesii_1   ------------------------------------------------------------
      menziesii_2   ------------------------------------------------------------
      menziesii_3   ------------------------------------------------------------
      menziesii_4   ------------------------------------------------------------
      menziesii_5   ------------------------------------------------------------
      menziesii_6   -TCCTTTTGAGTGTGGGTTTGATCCGGTTGGGTCTTCTCGTGTGCCTTTTTCTTTACGGT
      menziesii_7   -TCCTTTTGAGTGTGGGTTTGATCCGGTTGGGTCTTCTCGTGTGCCTTTTTCTTTACGGT
      menziesii_8   CTCCTTTTGAGTGTGGGTTTGATCCGGTTGGGTCTTCTCGTGTGCCTTTTTCTTTACGGT
      menziesii_M4  ----------TGGGTTTGATCCGGTTGGGTCTTCTCGTGTACCTTTTTCTTTACGGT
      menziesii_M5  ------------------------------------------------------------
      menziesii_M14 ------------------------------------------------------------
      menziesii_PC1 ------------------------------------------------------------
      menziesii_PC2 ------------------------------------------------------------
      menziesii_PC3 ------------------------------------------------------------
      menziesii_PC4 ------------------------------------------------------------
aucklandica_consensus ------------------------------------------------------------
    onekaka_consensus ------------------------------------------------------------


      menziesii_1   ------------------------------------------------------------
      menziesii_2   ------------------------------------------------------------
```

```
          menziesii_3   ------------------------------------------------------------
          menziesii_4   ------------------------------------------------------------
          menziesii_5   ------------------------------------------------------------
          menziesii_6   TTTTTTTGCTTGCGGTTATTTTTGTGGTTTTTGACGTGGAGATTGTGTTGTTATTTCCTA
          menziesii_7   TTTTTTTGCTTGCGGTTATTTTTGTGGTTTTTGACGTGGAGATTGTGTTGTTATTTCCTA
          menziesii_8   TTTTTTTGCTTGCGGTTATTTTTGTGGTTTTTGACGTGGAGATTGTGTTGTTATTTCCTA
         menziesii_M4   TTTTTTTGCTTGCGGTTATTTTTGTGGTTTTTGACGTGGAGATTGTGTTGTTATTTCCTA
         menziesii_M5   ------------------------------------------------------------
        menziesii_M14   ------------------------------------------------------------
        menziesii_PC1   ------------------------------------------------------------
        menziesii_PC2   ------------------------------------------------------------
        menziesii_PC3   ------------------------------------------------------------
        menziesii_PC4   ------------------------------------------------------------
aucklandica_consensus   ------------------------------------------------------------
    onekaka_consensus   ------------------------------------------------------------


          menziesii_1   ------------------------------------------------------------
          menziesii_2   ------------------------------------------------------------
          menziesii_3   ------------------------------------------------------------
          menziesii_4   ------------------------------------------------------------
          menziesii_5   ------------------------------------------------------------
          menziesii_6   TTGTCTTAGGTGTGGGGGGATGTTGGTTGTGGGTTAATAGGTATTTAGTGTTGTTTGTGT
          menziesii_7   TTGTCTTAGGTGTGGGGGGGTGTTGGTTGTGGGTTAATAGGTATTTAGTGTTGCTTGTGT
          menziesii_8   TTGTCTTAGGTGTGGGGGGGTGTTGGTTGTGGGTTAATAGGTATTTAGTGTTGCTTGTGT
         menziesii_M4   TTGTCTTAGGTGTGGGGGGATGTTGGTTGTGGGTTAATAGATATTTAGTGTTGTTTGTGT
         menziesii_M5   ------------------------------------------------------------
        menziesii_M14   ------------------------------------------------------------
        menziesii_PC1   ------------------------------------------------------------
        menziesii_PC2   ------------------------------------------------------------
        menziesii_PC3   ------------------------------------------------------------
```

```
        menziesii_PC4   ------------------------------------------------------------
aucklandica_consensus   ------------------------------------------------------------
    onekaka_consensus   ------------------------------------------------------------

          menziesii_1   ------------------------------------------------------------
          menziesii_2   ------------------------------------------------------------
          menziesii_3   ------------------------------------------------------------
          menziesii_4   ------------------------------------------------------------
          menziesii_5   ------------------------------------------------------------
          menziesii_6   TCTTGGGGATTTTATTGGTTGGGGTTGTTCATGAGTGGCGTGAGGGGTCTTTGGAGTGAG
          menziesii_7   TCTTGGGGATTTTATTGGTTGGGGTTGTTCATGAGTGGCGTGAGGGGTCTTTGGAGTGAG
          menziesii_8   TCTTGGGGATTTTATTGGTTGGGGTTGTTCATGAGTGGCGTGAGGGGTCTTTGGAGTGAG
         menziesii_M4   TCTTGGGGATTTTATTGGTTGGGGTTGTTCATGAGTGGCGTGAGGGGTCTTTGGAGTGAG
         menziesii_M5   ------------------------------------------------------------
        menziesii_M14   ------------------------------------------------------------
        menziesii_PC1   ------------------------------------------------------------
        menziesii_PC2   ------------------------------------------------------------
        menziesii_PC3   ------------------------------------------------------------
        menziesii_PC4   ------------------------------------------------------------
aucklandica_consensus   ------------------------------------------------------------
    onekaka_consensus   ------------------------------------------------------------

          menziesii_1   ------------------------------------------------------------
          menziesii_2   ------------------------------------------------------------
          menziesii_3   ------------------------------------------------------------
          menziesii_4   ------------------------------------------------------------
          menziesii_5   ------------------------------------------------------------
          menziesii_6   GAGAGTAGGTTTGAGTTTGGTGGGTGATTTGATAATTGAGGACAGTATATGAGCTTATGA
          menziesii_7   GAGAGTAGGTTTGAGTTTGGTGGGTGATTTGATAATGGAGGACAGTATATGAGCTTATGA
          menziesii_8   GAGAGTAGGTTTGAGTTTGGTGGGTGATTTGATAATGGAGGACAGTATATGAGCTTATGA
```

```
          menziesii_M4   GAGAGTAGGTTTGAGTTTAGTGGGTGATTTGATAATGGAGGACAGTATATGAGCTTATGA
          menziesii_M5   ------------------------------------------------------------
         menziesii_M14   ------------------------------------------------------------
          menziesii_PC1  ------------------------------------------------------------
          menziesii_PC2  ------------------------------------------------------------
          menziesii_PC3  ------------------------------------------------------------
          menziesii_PC4  ------------------------------------------------------------
    aucklandica_consensus ------------------------------------------------------------
        onekaka_consensus ------------------------------------------------------------

          menziesii_1    ------------------------------------------------------------
          menziesii_2    ------------------------------------------------------------
          menziesii_3    ------------------------------------------------------------
          menziesii_4    ------------------------------------------------------------
          menziesii_5    ------------------------------------------------------------
          menziesii_6    GGTCAGTTGAGGTTTCAGGATAGTAGAAGTTGGTTGGGGTCGGAGCTGTGTTTTTTCCAT
          menziesii_7    GGTCAGTTGAGGTTTCAGGATAGTAGAAGTTGGTTGGGGTCGGAGCTGTGTTTTTTCCAT
          menziesii_8    GGTCAGTTGAGGTTTCAGGATAGTAGAAGTTGGTTGGGGTCGGAGCTGTGTTTTTTCCAT
          menziesii_M4   GGTCAGTTAAGGTTTCAGGACAGTAGAAGTTGGTTGGGGTCGGAGCTGTGTTTTTTCCAT
          menziesii_M5   ------------------------------------------------------------
         menziesii_M14   ------------------------------------------------------------
          menziesii_PC1  ------------------------------------------------------------
          menziesii_PC2  ------------------------------------------------------------
          menziesii_PC3  ------------------------------------------------------------
          menziesii_PC4  ------------------------------------------------------------
    aucklandica_consensus ------------------------------------------------------------
        onekaka_consensus ------------------------------------------------------------

          menziesii_1    ------------------------------------------------------------
          menziesii_2    ------------------------------------------------------------
```

```
          menziesii_3   ------------------------------------------------------------
          menziesii_4   ------------------------------------------------------------
          menziesii_5   ------------------------------------------------------------
          menziesii_6   GATCATGCTATGTTCGTTCTTATTTTGGTTGCTTCTTTTGTTGGTTATATGATGGTGTGT
          menziesii_7   GATCATGCTATGTTCGTTCTTATTTTGGTTGCTTCTTTTGTTGGTTATATGATGGTGTGC
          menziesii_8   GATCATGCTATGTTCGTTCTTATTTTGGTTGCTTCTTTTGTTGGTTATATGATGGTGTGC
         menziesii_M4   GATCATGCTATGTTCGTTCTTATTCTGGTTGCTTCTTTTGTTGGTTATATGATGGTTTGC
         menziesii_M5   ------------------------------------------------------------
        menziesii_M14   ------------------------------------------------------------
        menziesii_PC1   ------------------------------------------------------------
        menziesii_PC2   ------------------------------------------------------------
        menziesii_PC3   ------------------------------------------------------------
        menziesii_PC4   ------------------------------------------------------------
aucklandica_consensus   ------------------------------------------------------------
    onekaka_consensus   ------------------------------------------------------------

          menziesii_1   ------------------------------------------------------------
          menziesii_2   ------------------------------------------------------------
          menziesii_3   ------------------------------------------------------------
          menziesii_4   ------------------------------------------------------------
          menziesii_5   ------------------------------------------------------------
          menziesii_6   TTATTGAAGAGAAGGTTATTGTCCCGTTTTCTTGTTGAGGCGCA-AAGTTTGGAGGCTGC
          menziesii_7   TTATTGAAGAGAAGGTTATTGTCCCGTTTTCTTGTTGAGGCGCA-AAGTTTGGAGGCTGC
          menziesii_8   TTATTGAAGAGAAGGTTATTGTCCCGTTTTCTTGTTGAGGCGCA-AAGTTTGGAGGCTGC
         menziesii_M4   TTATTGAAGAGAAGGTTATTGTCCCGTTTTCTTGTTGAGGCGCA-AAGTTTGGAGGCTGC
         menziesii_M5   --------------GTTATTGTCCCGTTTTCTTGTTGAGGCGCA-AAGTTTGGAGGCTGC
        menziesii_M14   ------------AGGTTATTGTCCCGTTTTCTTGTTGAGGCGCA-AAGTTTGGAGGCTGC
        menziesii_PC1   --------------GTTATTGTCCCGTTTTCTTGTTGAGGCGCA-AAGTTTGGAGGCTGC
        menziesii_PC2   --------------GTTATTGTCCCGTTTTCTTGTTGAGGCGCA-AAGTTTGGAGGCTGC
        menziesii_PC3   --------------GTTATTGTCCCGTTTTCTTGTTGAGGCGCA-AAGTTTGGAGGCTGC
```

```
menziesii_PC4          --------------GTTATTGTCCCGTTTTCTTGTTGAGGCGCA-AAGTTTGGAGGCTGC
aucklandica_consensus  ----------------------------TCTTGTTGAGGCGCAAAAGATTAGAGGCGGC
onekaka_consensus      ------------------------------------------------GGAGGCTGC

menziesii_1            ------------------------------------------------------------
menziesii_2            ------------------------------------------------------------
menziesii_3            ------------------------------------------------------------
menziesii_4            ------------------------------------------------------------
menziesii_5            ------------------------------------------------------------
menziesii_6            TTGGACAGTAG--TTCCTGGGCTGTTATTATTGGTTTTAGCTATCCCCTCTGTGCGGTTG
menziesii_7            TTGGACAGTAG--TTCCTGGGCTGTTATTATTGGTTTTAGCTATCCCCTCTGTGCGGTTG
menziesii_8            TTGGACAGTAG--TTCCTGGGCTGTTATTATTGGTTTTAGCTATCCCCTCTGTGCGGTTG
menziesii_M4           TTGGACAGTGG--TTCCTGGGCTGTTATTATTGGTTTTAGCTATTCCCTCTGTGCGGTTG
menziesii_M5           TTGGACAGTAG--TTCCTGGGCTGTTATTATTGGTTTTAGCTATCCCCTCTGTGCGGTTG
menziesii_M14          TTGGACAGTAG--TTCCTGGGCTGTTATTATTGGTTTTAGCTATCCCCTCTGTGCGGTTG
menziesii_PC1          TTGGACAGTAG--TTCCTGGGCTGTTATTATTGGTTTTAGCTATCCCCTCTGTGCGGTTG
menziesii_PC2          TTGGACAGTAG--TTCCTGGGCTGTTATTATTGGTTTTAGCTATCCCCTCTGTGCGGTTG
menziesii_PC3          TTGGACAGTAG--TTCCTGGGCTGTTATTATTGGTTTTAGCTATCCCCTCTGTGCGGTTG
menziesii_PC4          TTGGACAGTAG--TTCCTGGGCTGTTATTATTGGTTTTAGCTATCCCCTCTGTGCGGTTG
aucklandica_consensus  TTGGACAATAG--TTCCTGGGTTGTTATTATTAGTATTGGCTATTCCTTCTGTACGGTTG
onekaka_consensus      TTGAAACGATGGTTTCCTGGGTTGTTGTTATTGGTTTTGGCCATTCCTTCCGTACGATTA

menziesii_1            ------------------------------------------------------------
menziesii_2            ------------------------------------------------------------
menziesii_3            ------------------------------------------------------------
menziesii_4            ------------------------------------------------------------
menziesii_5            ------------------------------------------------------------
menziesii_6            TTGTATTTGTTGGATGAAATTGGTGGTCCTGTTGTGAGGATGAAGGCTATTGGGCATCAA
menziesii_7            TTGTATTTGTTGGATGAAATTGGTGGTCCTGTTGTGAGGATGAAGGCTATTGGGCATCAA
menziesii_8            TTGTATTTGTTGGATGAAATTGGTGGTCCTGTTGTGAGGATGAAGGCTATTGGGCATCAG
```

```
          menziesii_M4   TTGTATTTGTTGGATGAAATTGGTGGTCCTGTTGTGAGGATGAAGGCTATTGGGCATCAA
          menziesii_M5   TTGTATTTGTTGGATGAAATTGGTGGTCCTGTTGTGAGGATGAAGGCTATTGGGCATCAA
         menziesii_M14   TTGTATTTGTTGGATGAAATTGGTGGTCCTGTTGTGAGGATGAAG-CTATTGGGCATCAA
          menziesii_PC1   TTGTATTTGTTGGATGAAATTGGTGGTCCTGTTGTGAGGATGAAGGCTATTGGGCATCAA
          menziesii_PC2   TTGTATTTGTTGGATGAAATTGGTGGTCCTGTTGTGAGGATGAAGGCTATTGGGCATCAA
          menziesii_PC3   TTGTATTTGTTGGATGAAATTGGTGGTCCTGTTGTGAGGATGAAGGCTATTGGGCATCAA
          menziesii_PC4   TTGTATTTGTTGGATGAAATTGGTGGTCCTGTTGTGAGGATGAAGGCTATTGGGCATCAA
  aucklandica_consensus   TTGTATTTGTTAGATGAGGTTGGGAGCCCTGTTGTGAGGATAAAGGCTATTGGGCATCAG
      onekaka_consensus   TTATATTTGTTGGATGAGATTGGTGGTCCTGTCGTAAGGATGAAGGCTATTGGGCATCAG

          menziesii_1    ------------------------------------------------------------
          menziesii_2    ------------------------------------------------------------
          menziesii_3    ------------------------------------------------------------
          menziesii_4    ------------------------------------------------------------
          menziesii_5    ------------------------------------------------------------
          menziesii_6    TGGTATTGGAGTTATGAGTATAGAGACGTGGGGCAGGTTGAGTATGATTCTTATATGGTT
          menziesii_7    TGGTATTGGAGTTATGAGTATAGAGACGTGGGGCAGGTTGAGTATGATTCTTATATGGTT
          menziesii_8    TGGTATTGGAGTTATGAGTATAGAGACGTGGGGCAGGTTGAGTATGATTCTTATATGGTT
          menziesii_M4   TGGTATTGGAGTTATGAGTATAGAGACGTGGGGCAGGTTGAGTATGATTCTTATATGGTT
          menziesii_M5   TGGTATTGGAGTTATGAGTATAGAGACGTGGGGCAGGTTGAGTATGATTCTTATATGGTT
         menziesii_M14   TGGTATTGGAGTTATGAGTATAGAGACGTGGGGCAGGTTGAGTATGATTCTTATATGGTT
          menziesii_PC1   TGGTATTGGAGTTATGAGTATAGAGACGTGGGGCAGGTTGAGTATGATTCTTATATGGTT
          menziesii_PC2   TGGTATTGGAGTTATGAGTATAGAGACGTGGGGCAGGTTGAGTATGATTCTTATATGGTT
          menziesii_PC3   TGGTATTGGAGTTATGAGTATAGAGACGTGGGGCAGGTTGAGTATGATTCTTATATGGTT
          menziesii_PC4   TGGTATTGGAGTTATGAGTATAGAGACGTGGGGCAGGTTGAGTATGATTCTTATATGGTT
  aucklandica_consensus   TGATATTGGAGTTATGAGTATAGGGATGTGGAGCAAGTTGAGTATGATTCTTATATAGTG
      onekaka_consensus   TGGTACTGGAGTTATGAATATAGGGATGTGGAGCAAGTTGAGTATGATTCTTATATGGTT

          menziesii_1    ------------------------------------------------------------
          menziesii_2    ------------------------------------------------------------
```

```
          menziesii_3   ------------------------------------------------------------
          menziesii_4   ------------------------------------------------------------
          menziesii_5   ------------------------------------------------------------
          menziesii_6   GGCGTTCCAGATATGGTGGATGGGGGTTATCGGTTGCTTGAGGTTGACAATCGTTGCGTG
          menziesii_7   GGCGTTCCAGATATGGTGGATGGGGGCTATCGGTTGCTTGAGGTTGATAATCGTTGCGTG
          menziesii_8   GGCGTTCCAGATATGGTGGATGGGGGCTATCGGTTGCTTGAGGTTGATAATCGTTGCGTG
         menziesii_M4   GGTGTTCCAGATATGGTGGATGGGGGCTATCGGTTGCTTGAGGTTGACAATCGTTGCGTG
         menziesii_M5   GGTGTTCCAGATATGGTGGATGGGGGCTATCGGTTGCTTGAGGTTGACAATCGTTGCGTG
        menziesii_M14   GGTGTTCCAGATATGGTGGATGGGGGCTATCGGTTGCTTGAGGTTGACAATCGTTGCGTG
         menziesii_PC1   GGCGTTCCAGATATGGTGGATGGGGGCTATCGGTTGCTTGAGGTTGATAATCGTTGCGTG
         menziesii_PC2   GGCGTTCCAGATATGGTGGATGGGGGCTATCGGTTGCTTGAGGTTGATAATCGTTGCGTG
         menziesii_PC3   GGCGTTCCAGATATGGTGGATGGGGGCTATCGGTTGCTTGAGGTTGATAATCGTTGCGTG
         menziesii_PC4   GGCGTTCCAGATATGGTGGATGGGGGCTATCGGTTGCTTGAGGTTGATAATCGTTGCGTG
aucklandica_consensus   GGTGCGTCGGATATGGTGGGTGGTGGTTACCGATTGCTTGAGGTCGATAATCGTTGTGTG
    onekaka_consensus   GGTGTTTCAGATATGGTGGATGGGGGTTATCGGTTGCTTGAGGTTGATAATCGTTGTGTG

          menziesii_1   ------------------------------------------------------------
          menziesii_2   ------------------------------------------------------------
          menziesii_3   ------------------------------------------------------------
          menziesii_4   ------------------------------------------------------------
          menziesii_5   ------------------------------------------------------------
          menziesii_6   TTGCCTTTTGGGGTGGATAGTCGGGTGCTTGTTAGGTCAATGGATGTAGTTCATGCTTGG
          menziesii_7   TTGCCTTTTGGGGTGGATAGTCGGGTGCTTGTTAGGTCAATGGATGTAGTTCATGCTTGG
          menziesii_8   TTGCCTTTNGGGGTGGATAGTCGGGTGCTTGTTAGGTCAATGGATGTAGTTCATGCTTGG
         menziesii_M4   TTGCCTTTTGGGGTGGATAGTCGGGTGCTTGTTAGGTCAATGGATGTAGTTCATGCTTGG
         menziesii_M5   TTGCCTTTTGGGGTGGATAGTCGGGTGCTTGTTAGGTCAATGGATGTAGTTCATGCTTGG
        menziesii_M14   TTGCCTTTTGGGGTGGATAGTCGGGTGCTTGTTAGGTCAATGGATGTAGTTCATGCTTGG
         menziesii_PC1   TTGCCTTTTGGGGTGGATAGTCGGGTGCTTGTTAGGTCAATGGATGTAGTTCATGCTTGG
         menziesii_PC2   TTGCCTTTTGGGGTGGATAGTCGGGTGCTTGTTAGGTCAATGGATGTAGTTCATGCTTGG
         menziesii_PC3   TTGCCTTTTGGGGTGGATAGTCGGGTGCTTGTTAGGTCAATGGATGTAGTTCATGCTTGG
```

```
menziesii_PC4          TTGCCTTTTGGGGTGGATAGTCGGGTGCTTGTTAGGTCAATGGATGTAGTTCATGCTTGG
aucklandica_consensus  TTGCCTTATGGCGTGGATAGTCGAGTGCTAGTTAGATCTTTAGATGTGATTCATGCTTGG
onekaka_consensus      TTACCTTTTGGGGTGGATAGTCGGGTGCTTGTTAGGTCATTAGATGTAGTTCATGCTTGG

menziesii_1            ------------------------------------------------------------
menziesii_2            ------------------------------------------------------------
menziesii_3            ------------------------------------------------------------
menziesii_4            ------------------------------------------------------------
menziesii_5            ------------------------------------------------------------
menziesii_6            GCGGTTCCTTCCGTTGGGGTTAAAGCTGATGCTATTCCAGGGCGTATTAACCAGTTGGCT
menziesii_7            GCGGTTCCTTCCGTTGGGGTTAAAGCTGATGCTATTCCAGGGCGTATTAACCAGTTGGCT
menziesii_8            GCGGTTCCTTCCGTTGGGGTTAAAGCTGATGCTATTCCAGGGCGTATTAACCAGTTGGCT
menziesii_M4           GCGGTTCCTTCCGTGGGGGTTAAAGCTGATGCTATTCCAGGGCGTATTAACCAGTTGGCT
menziesii_M5           GCGGTTCCTTCCGTGGGGGTTAAAGCTGATGCTATTCCAGGGCGTATTAACCAGTTGGCT
menziesii_M14          GCGGTTCCTTCCGTGGGGGTTAAAGCTGATGCTATTCCAGGGCGTATTAACCAGTTGGCT
menziesii_PC1          GCGGTTCCTTCCGTGGGGGTTAAAGCTGATGCTATTCCAGGGCGTATTAACCAGTTGGCT
menziesii_PC2          GCGGTTCCTTCCGTGGGGGTTAAAGCTGATGCTATTCCAGGGCGTATTAACCAGTTGGCT
menziesii_PC3          GCGGTTCCTTCCGTGGGGGTTAAAGCTGATGCTATTCCAGGGCGTATTAACCAGTTGGCT
menziesii_PC4          GCGGTTCCTTCCGTGGGGGTTAAAGCTGATGCTATTCCAGGGCGTATTAACCAGTTGGCT
aucklandica_consensus  GCTGTTCCTTCGGTGGGGGTTAAAGCTGATGCTATTCCGGGGCGAATTAACCAGTTGGCT
onekaka_consensus      GCGGTTCCTTCGATTGGGGTTAAAGCGGATGCTATTCCTGGGCGTATTAATCAGTTGGCT

menziesii_1            ------------------------------------------------------------
menziesii_2            ------------------------------------------------------------
menziesii_3            ------------------------------------------------------------
menziesii_4            ------------------------------------------------------------
menziesii_5            ------------------------------------------------------------
menziesii_6            GTTCGTTTGATTGGGTCTGGGGTGATATATGGTCAGTGTAGTGAAATTTGTGGTGTTAAT
menziesii_7            GTTCATTTGATTGGGTCTGGGGTGATATATGGTCAGTGTAGTGAAATTTGTGGTGTTAAT
menziesii_8            GTTCATTTGATTGGGTCTGGGGTGATATATGGTCAGTGTAGTGAAATTTGTGGTGTTAAT
```

```
          menziesii_M4   GTTCATTTGATCGGGTCTGGGGTGATATATGGTCAGTGTAGTGAAATTTGTGGTGTTAAT
          menziesii_M5   GTTCATTTGATCGGGTCTGGGGTGATATATGGTCAGTGTAGTGAAATTTGTGGTGTTAAT
         menziesii_M14   GTTCATTTGATCGGGTCTGGGGTGATATATGGTCAGTGTAGTGAAATTTGTGGTGTTAAT
          menziesii_PC1   GTTCATTTGATTGGGTCTGGGGTGATATATGGTCAGTGTAGTGAAATTTGTGGTGTTAAT
          menziesii_PC2   GTTCATTTGATTGGGTCTGGGGTGATATATGGTCAGTGTAGTGAAATTTGTGGTGTTAAT
          menziesii_PC3   GTTCATTTGATTGGGTCTGGGGTGATATATGGTCAGTGTAGTGAAATTTGTGGTGTTAAT
          menziesii_PC4   GTTCATTTGATTGGGTCTGGGGTGATATATGGTCAGTGTAGTGAAATTTGTGGTGTTAAT
  aucklandica_consensus   ATTCATTTGGTTGGGCCTAGGGTTATGTATGGTCAGTGTAGTGAGATTTGTGGTGTTAAT
      onekaka_consensus   ATTCATTTGATTGGGTCGGGGGTGATATACGGTCAGTGTAGTGAGATTTGTGGGGTCAAT

          menziesii_1    ------------------------------------------------------------
          menziesii_2    ------------------------------------------------------------
          menziesii_3    ------------------------------------------------------------
          menziesii_4    ------------------------------------------------------------
          menziesii_5    ------------------------------------------------------------
          menziesii_6    CATTCGTTTATGCCTATTGGCTTGGAGAGGGTTTCTCCTAAGGTTTTTTTCCATTGGTTA
          menziesii_7    CATTCGTTTATGCCTATTGGCTTGGAGAGGGTTTCTCCTAAGGTTTTTTTCCATTGGTTA
          menziesii_8    CATTCGTTTATGCCTATTGGCTTGGAGAGGGTTTCTCCTAAGGTTTTTTTCCATTGGTTA
          menziesii_M4   CATTCGTTTATGCCTATTGGCTTGGAGAGGGTTTCTCCTAAGGTTTTTTTTCATTGGTTA
          menziesii_M5   CATTCGTTTATGCCTATTGGCTTGGAGAGGGTTTCTCCTAAGGTTTTTTTTCATTGGTTA
         menziesii_M14   CATTCGTTTATGCCTATTGGCTTGGAGAGGGTTTCTCCTAAGGTTTTTTTTCATTGGTTA
          menziesii_PC1   CATTCGTTTATGCCTATTGGCTTGGAGAGGGTTTCTCCTAAGGTTTTTTTCCATTGGTTA
          menziesii_PC2   CATTCGTTTATGCCTATTGGCTTGGAGAGGGTTTCTCCTAAGGTTTTTTTCCATTGGTTA
          menziesii_PC3   CATTCGTTTATGCCTATTGGCTTGGAGAGGGTTTCTCCTAAGGTTTTTTTCCATTGGTTA
          menziesii_PC4   CATTCGTTTATGCCTATTGGCTTGGAGAGGGTTTCTCCTAAGGTTTTTTTCCATTGGTTA
  aucklandica_consensus   CATTCGTTTATACCTATCGGTTTAGAGAGGGTTTCTCCTAAGGTTTTTTTTCATTGATTG
      onekaka_consensus   CACTCATTTATGCCTATTGGCTTGGAGAGGGTTTCTCCTAAGGTTTTTTTTCATTGGTTG

          menziesii_1    ------------------------------------------------------------
          menziesii_2    ------------------------------------------------------------
```

```
          menziesii_3   ------------------------------------------------------------
          menziesii_4   ------------------------------------------------------------
          menziesii_5   ------------------------------------------------------------
          menziesii_6   GTTGGAGTTTAAGT-GTG-GTAACTGT-GATATAGGAAGTGTGGGGCTAATAT-----GT
          menziesii_7   GTTGGAGTTTAAGT-GTG-GTAATTGT-GATATAGGAAGTGTGGGGCTAATAT-----GT
          menziesii_8   GTTGGAGTTTAAGT-GTG-GTAATTGT-GATATAGGAAGTGTGGGGCTAATAT-----GT
         menziesii_M4   GTTGGAGTTTAAGT-GTG-GTAATTGT-GGTATAGGAAGTGTGGGGCTAATAT-----GT
         menziesii_M5   GTTGGAGTTTAAGT-GTG-GTAATTGT-GGTATAGGAAGTGTGGGGCTAATAT-----GT
        menziesii_M14   GTTGGAGTTTAAGT-GTG-GTAATTGT-GGTATAGGAAGTGTGGGGCTAATAT-----GT
         menziesii_PC1   GTTGGAGTTTAAGT-GTG-GTAATTGT-GGTATGGGAAGTGTGGAGTTAATAT-----GT
         menziesii_PC2   GTTGGAGTTTAAGT-GTG-GTAATTGT-GGTATGGGAAGTGTGGAGTTAATATG-----T
         menziesii_PC3   GTTGGAGTTTAAGT-GTG-GTAATTGT-GGTATGGGAAGTGTGGAGTTAATATG-----T
         menziesii_PC4   GTTGGAGTTTAAGT-GTG-GTAATTGT-GGTATGGGAAGTGTGGAGTTAATAT-----GT
aucklandica_consensus   GTGGGAGTCTAGTTAGAGTGTATTTGTAGGGGTCGTTTGGGTTGATTTATTATAGTAAGT
    onekaka_consensus   GTTGG-GTTTTAGTATAA--TAACTGT-GATA-GGGAAGTGTGAGGTCAGTCT-----GT

          menziesii_1   ------------------------------------------------------------
          menziesii_2   ------------------------------------------------------------
          menziesii_3   ------------------------------------------------------------
          menziesii_4   ------------------------------------------------------------
          menziesii_5   ------------------------------------------------------------
          menziesii_6   GCTTGTGGGTATTTAGGTTGGTGGTTTTGACTTGCGGTGGCTATGTTCTACTAATCATAA
          menziesii_7   GCTTGTGGGCATTTAGGTTGGTGGTTTTGACTTGCGGTGGCTATGTTCTACTAATCATAA
          menziesii_8   GCTTGTGGGCATTTAGGTTGGTGGTTTTGACTTGCGGTGGCTATGTTCTACTAATCATAA
         menziesii_M4   GCTTGTGGGTATTTAGGTTGGTGGTTTTGACTTGCGGTGGCTATGTTCTACTAATCATAA
         menziesii_M5   GCTTGTGGGTATTTAGGTTGGTGGTTTTGACTTGCGGTGGCTATGTTCTACTAATCATAA
        menziesii_M14   GCTTGTGGGTATTTAGGTTGGTGGTTTTGACTTGCGGTGGCTATGTTCTACTAATCATAA
         menziesii_PC1   GCTTGTGGGTATTTAGGTTGGTGGTTTTGACTTGCGGTGGCTATGTTCTACTAATCATAA
         menziesii_PC2   GCTTGTGGGTATTTAGGTTGGTGGTTTTGACTTGCGGTGGCTATGTTCTACTAATCATAA
         menziesii_PC3   GCTTGTGGGTATTTAGGTTGGTGGTTTTGACTTGCGGTGGCTATGTTCTACTAATCATAA
```

```
        menziesii_PC4   GCTTGTGGGTATTTAGGTTGGTGGTTTTGACTTGCGGTGGCTATGTTCTACTAATCATAA
aucklandica_consensus   GGTGAGGGTTAGTGGATTAAGCGGTTTTGGTTTGCGGTGGTTGTGTTCTACTAATCATAA
    onekaka_consensus   GTTTAAGG-TGTCTGGGCTAGTCCTCTTGGTTTGCGGTGGTTATGTTCTACTAATCATAA

        menziesii_1   ------------------------------------------------------------
        menziesii_2   ------------------------------------------------------------
        menziesii_3   ------------------------------------------------------------
        menziesii_4   ------------------------------------------------------------
        menziesii_5   ------------------------------------------------------------
        menziesii_6   GGATATTGGGACTTTGTATTTACTCTTTGCCTTGTGGTCTGGGATGATTGGGCTTGCTTT
        menziesii_7   GGATATTGGGACTTTGTATTTACTCTTTGCCTTGTGGTCTGGGATGATTGGGCTTGCTTT
        menziesii_8   GGATATTGGGACTTTGTATTTACTCTTTGCCTTGTGGTCTGGGATGATTGGGCTTGCTTT
       menziesii_M4   GGATATTGGGACTTTGTATTTACTCTTTGCCTTGTGGTCTGGGATGATTGGGCTTGCTTT
       menziesii_M5   GGATATTGGGACTTTGTATTTACTCTTTGCCTTGTGGTCTGGGATGATTGGGCTTGCTTT
      menziesii_M14   GGATATTGGGACTTTGTATTTACTCTTTGCCTTGTGGTCTGGGATGATTGGGCTTGCTTT
       menziesii_PC1   GGATATTGGGACTTTGTATTTACTCTTTGCCTTGTGGTCTGGGATGATTGGGCTTGCTTT
       menziesii_PC2   GGATATTGGGACTTTGTATTTACTCTTTGCCTTGTGGTCTGGGATGATTGGGCTTGCTTT
       menziesii_PC3   GGATATTGGGACTTTGTATTTACTCTTTGCCTTGTGGTCTGGGATGATTGGGCTTGCTTT
       menziesii_PC4   GGATATTGGGACTTTGTATTTACTCTTTGCCTTGTGGTCTGGGATGATTGGGCTTGCTTT
aucklandica_consensus   GGATATTGGAACTCTGTATATTTTGTTTGCTTTGTGGTCTGGGATGATTGGGCTTGCTTT
    onekaka_consensus   GGATATTGGGACTTTGTATTTACTTTTTGCCTTGTGGTCTGGGATGATTGGGCTCGCTTT

        menziesii_1   ----------------------------CAGCCTGGGAGATTATTGGGTGATGACCAGTT
        menziesii_2   ----------------------------CAGCCTGGGAGATTATTGGGTGATGACCAGTT
        menziesii_3   ----------------------------CAGCCTGGGAGATTATTGGGTGATGACCAGTT
        menziesii_4   ----------------------------CAGCCTGGGAGATTATTGGGTGATGACCAGTT
        menziesii_5   ----------------------------CAGCCTGGGAGATTATTGGGTGATGACCAGTT
        menziesii_6   GAGGTTGTTGATTCGGGCTGAGTTGGGGCAGCCTGGGAGATTATTGGGTGATGACCAGTT
        menziesii_7   GAGGTTGTTGATTCGGGCTGAGTTGGGGCAGCCTGGGAGATTATTGGGTGATGACCAGTT
        menziesii_8   GAGGTTGTTGATTCGGGCTGAGTTGGGGCAGCCTGGGAGATTATTGGGTGATGACCAGTT
```

```
menziesii_M4            GAGGTTGTTGATTCGGGCTGAGTTGGGGCAGCCTGGGAGATTATTGGGTGATGACCAGCT
menziesii_M5            GAGGTTGTTGATTCGGGCTGAGTTGGGGCAGCCTGGGAGATTATTGGGTGATGACCAGCT
menziesii_M14           GAGGTTGTTGATTCGGGCTGAGTTGGGGCAGCCTGGGAGATTATTGGGTGATGACCAGCT
menziesii_PC1           GAGGTTGTTGATTCGGGCTGAGTTGGGGCAGCCTGGGAGATTATTGGGTGATGACCAGTT
menziesii_PC2           GAGGTTGTTGATTCGGGCTGAGTTGGGGCAGCCTGGGAGATTATTGGGTGATGACCAGTT
menziesii_PC3           GAGGTTGTTGATTCGGGCTGAGTTGGGGCAGCCTGGGAGATTATTGGGTGATGACCAGTT
menziesii_PC4           GAGGTTGTTGATTCGGGCTGAGTTGGGGCAGCCTGGGAGATTATTGGGTGATGACCAGTT
aucklandica_consensus   GAGATTGCTAATTCGAGCCGAGTTGGGGCAGCCTGGAAGATTGCTAGGTGATGATCAGTT
onekaka_consensus       GAGGCTGTTAATTCGGGCTGAGTTGGGGCAGCCTGGTAGGTTATTGGGTGATGATCAATT

menziesii_1             ATATAATGTTATTGTAACGGCGCATGCTTTTATAATGATTTTTTTTTTGGTGATGCCTAT
menziesii_2             ATATAATGTTATTGTAACGGCGCATGCTTTTATAATGATTTTTTTTTTGGTGATGCCTAT
menziesii_3             ATATAATGTTATTGTAACGGCGCATGCCTTTATAATGATTTTTTTTTTGGTGATGCCTAT
menziesii_4             ATATAATGTTATTGTAACGGCGCATGCCTTTATAATGATTTTTTTTTTGGTGATGCCTAT
menziesii_5             ATATAATGTTATTGTAACGGCGCATGCCTTTATAATGATTTTTTTTTTGGTGATGCCTAT
menziesii_6             ATATAATGTTATTGTAACGGCGCATGCCTTTATAATGATTTTTTTTTTGGTGATGCCTAT
menziesii_7             ATATAATGTTATTGTAACGGCGCATGCTTTTATAATGATTTTTTTTTTGGTGATGCCTAT
menziesii_8             ATATAATGTTATTGTAACGGCGCATGCTTTTATAATGATTTTTTTTTTGGTGATGCCTAT
menziesii_M4            ATATAATGTTATTGTAACGGCGCATGCTTTTATAATGATTTTTTTTTTGGTGATGCCTAT
menziesii_M5            ATATAATGTTATTGTAACGGCGCATGCTTTTATAATGATTTTTTTTTTGGTGATGCCTAT
menziesii_M14           ATATAATGTTATTGTAACGGCGCATGCTTTTATAATGATTTTTTTTTTGGTGATGCCTAT
menziesii_PC1           ATATAATGTTATTGTAACGGCGCATGCTTTTATAATGATTTTTTTTTTGGTGATGCCTAT
menziesii_PC2           ATATAATGTTATTGTAACGGCGCATGCTTTTATAATGATTTTTTTTTTGGTGATGCCTAT
menziesii_PC3           ATATAATGTTATTGTAACGGCGCATGCTTTTATAATGATTTTTTTTTTGGTGATGCCTAT
menziesii_PC4           ATATAATGTTATTGTAACGGCGCATGCTTTTATAATGATTTTTTTTTTGGTGATGCCTAT
aucklandica_consensus   ATATAATGTTATTGTAACAGCGCATGCTTTTATGATGATTTTTTTTCTAGTAATACCTAT
onekaka_consensus       ATATAATGTTATTGTAACGGCGCATGCTTTTATAATGATTTTTTTCTTGGTGATACCTAT

menziesii_1             AATGATTGGTGGTTTTGGGAACTGGTTGATTCCTTTGATGTTGGGGGCTCCTGATATGGC
menziesii_2             AATGATTGGTGGTTTTGGGAACTGGTTGATTCCTTTGATGTTGGGGGCTCCTGATATGGC
```

```
menziesii_3          AATGATTGGTGGTTTTGGGAACTGGTTGATTCCTTTGATGTTGGGGGCTCTTGATATGGC
menziesii_4          AATGATTGGTGGTTTTGGGAACTGGTTGATTCCTTTGATGTTGGGGGCTCCTGATATGGC
menziesii_5          AATGATTGGTGGTTTTGGGAACTGGTTGATTCCTTTGATGTTGGGGGCTCCTGATATGGC
menziesii_6          AATGATTGGTGGTTTTGGGAACTGGTTGATTCCTTTGATGTTGGGGGCTCCTGATATGGC
menziesii_7          AATGATTGGTGGTTTTGGGAACTGGTTGATTCCTTTGATGTTGGGGGCTCCTGATATGGC
menziesii_8          AATGATTGGTGGTTTTGGGAACTGGTTGATTCCTTTGATGTTGGGGGCTCCTGATATGGC
menziesii_M4         AATGATTGGTGGTTTTGGGAACTGGTTGATTCCTTTGATGTTGGGGGCTCCTGATATGGC
menziesii_M5         AATGATTGGTGGTTTTGGGAACTGGTTGATTCCTTTGATGTTGGGGGCTCCTGATATGGC
menziesii_M14        AATGATTGGTGGTTTTGGGAACTGGTTGATTCCTTTGATGTTGGGGGCTCCTGATATGGC
menziesii_PC1        AATGATTGGTGGTTTTGGGAACTGGTTGATTCCTTTGATGTTGGGGGCTCCTGATATGGC
menziesii_PC2        AATGATTGGTGGTTTTGGGAACTGGTTGATTCCTTTGATGTTGGGGGCTCCTGATATGGC
menziesii_PC3        AATGATTGGTGGTTTTGGGAACTGGTTGATTCCTTTGATGTTGGGGGCTCCTGATATGGC
menziesii_PC4        AATGATTGGTGGTTTTGGGAACTGGTTGATTCCTTTGATGTTGGGGGCTCCTGATATGGC
aucklandica_consensus AATGATTGGAGGGTTTGGTAATTGGTTGATTCCTTTGATGCTGGGGGCTCCTGATATAGC
onekaka_consensus    GATGATTGGGGGGTTTGGGAACTGATTGATTCCTTTGATGTTGGGTGCTCCGGATATGGC

menziesii_1          TTTTCCTCGATTAAATAATTTGAGTTTTTGGCTACTTGTGCCTGCGTTGTTTT-TGTTGT
menziesii_2          TTTTCCTCGATTAAATAATTTGAGTTTTTGGCTACTTGTGCCTGCGTTGTTTT-TGTTGT
menziesii_3          TTTTCCTCGATTAAATAATTTGAGTTTTTGGCTACTTGTGCCTGCATTGTTTT-TGTTGT
menziesii_4          TTTTCCTCGATTAAATAATTTGAGTTTTTGGCTACTTGTGCCTGCATTGTTTT-TGTTGT
menziesii_5          TTTTCCTCGATTAAATAATTTGAGTTTTTGGCTACTTGTGCCTGCATTGTTTT-TGTTGT
menziesii_6          TTTTCCTCGATTAAATAATTTGAGTTTTTGGCTACTTGTGCCTGCATTGTTTT-TGTTGT
menziesii_7          TTTTCCTCGGTTAAATAATTTGAGTTTTTGGCTACTTGTGCCTGCATTGTTTT-TGTTGT
menziesii_8          TTTTCCTCGGTTAAATAATTTGAGTTTTTGGCTACTTGTGCCTGCATTGTTTT-TGTTGT
menziesii_M4         TTTTCCTCGATTAAATAATTTGAGTTTTTGGCTCCTTGTGCCTGCGTTGTTTT-TGTTGC
menziesii_M5         TTTTCCTCGATTAAATAATTTGAGTTTTTGGCTCCTTGTGCCTGCGTTGTTTT-TGT---
menziesii_M14        TTTTCCTCGATTAAATAATTTGAGTTTTTGGCTCCTTGTGCCTGCGT-GTTTT-TGT---
menziesii_PC1        TTTTCCTCGATTAAATAATTTGAGTTTTTGGCTACTTGTGCCTGCGTTGTTTG-TGT---
menziesii_PC2        TTTTCCTCGATTAAATAATTTGAGTTTTTGGCTACTTGTGCCTGCGTTGTTTTGTGT---
menziesii_PC3        TTTTCCTCGATTAAATAATTTGAGTTTTTGGCTACTTGTGCCTGCGTTGTTTTGTGT---
```

```
menziesii_PC4          TTTTCCTCGATTAAATAATTTGAGTTTTTGGCTACTTGTGCCTGCGTTGTTTT-TGT---
aucklandica_consensus  TTTTCCGCGATTAAATAACCTTAGGTTTTGGTTGCTCGTGCCAGCGTTATTCT-TGTT--
onekaka_consensus      TTTTCCTCGATTAAATAATTTGAGATTTTGGTTACTTGTGCCTGCGTTGTTTT-TGTTGT

menziesii_1            TAAGGTCGTCTTTGGTTGAGAGGGGTGTTGGGACTGGGTGGACTGTTTATCCCCCCTTAT
menziesii_2            TAAGGTCGTCTTTGGTTGAGAGGGGTGTTGGGACTGGGTGGACTGTTTATCCCCCCTTAT
menziesii_3            TAAGGTCGTCTTTGGTTGAGAGGGGTGTTGGGACTGGGTGGACTGTTTATCCCCCCTTGT
menziesii_4            TAAGGTCGTCTTTGGTTGAGAGGGGTGTTGGGACTGGGTGGACTGTTTATCCCCCCTTGT
menziesii_5            TAAGGTCGTCTTTGGTTGAGAGGGGTGTTGGGACTGGGTGGACTGTTTATCCCCCCTTGT
menziesii_6            TAAGGTCGTCTTTGGTTGAGAGGGGTGTTGGGACTGGGTGGACTGTTTATCCCCCCTTGT
menziesii_7            TAAGGTCGTCTTTGGTTGAGAGGGGTGTTGGGACTGGGTGGACTGTTTATCCCCCCTTGT
menziesii_8            TAAGGTCGTCTTTGGTTGAGAGGGGTGTTGGGACTGGGTGGACTGTTTATCCCCCCTTGT
menziesii_M4           TAAGGTCGTCTTTGGTTGAGAGGGGTGTTGGGACTGGGTGGACTGTTTATCCCCCCTTGT
menziesii_M5           ------------------------------------------------------------
menziesii_M14          ------------------------------------------------------------
menziesii_PC1          ------------------------------------------------------------
menziesii_PC2          ------------------------------------------------------------
menziesii_PC3          ------------------------------------------------------------
menziesii_PC4          ------------------------------------------------------------
aucklandica_consensus  ------------------------------------------------------------
onekaka_consensus      TGAGGTCGTCTTTGGTTGAGAGTGGAGTTGGGACTGGGTGGACTGTTTACCCTCCTTTGT

menziesii_1            CTGGGAATGTTGCTCATTCTGGTGCGTCTGTCGATTTGGCTATTTTTTCTTTGCATCTTG
menziesii_2            CTGGGAATGTTGCTCATTCTGGTGCGTCTGTCGATTTGGCTATTTTTTCTTTGCATCTTG
menziesii_3            CTGGGAATGTTGCTCATTCTGGTGCGTCTGTAGATTTGGCTATTTTTTCTTTGCATCTTG
menziesii_4            CTGGGAATGTTGCTCATTCTGGTGCGTCTGTAGATTTGGCTATTTTTTCTTTGCATCTTG
menziesii_5            CTGGGAATGTTGCTCATTCTGGTGCGTCTGTAGATTTGGCTATTTTTTCTTTGCATCTTG
menziesii_6            CTGGGAATGTTGCTCATTCTGGTGCGTCTGTAGATTTGGCTATTTTTTCTTTGCATCTTG
menziesii_7            CTGGGAATGTTGCTCATTCTGGTGCGTCTGTAGATTTGGCTATTTTTTCTTTGCATCTTG
menziesii_8            CTGGGAATGTTGCTCATTCTGGTGCGTCTGTAGATTTGGCTATTTTTTCTTTGCATCTTG
```

```
          menziesii_M4   CTGGGAATGTTGCTCATTCTGGTGCGTCTGTCGATTTGGCTATTTTTTCTTTGCATCTTG
          menziesii_M5   ------------------------------------------------------------
         menziesii_M14   ------------------------------------------------------------
          menziesii_PC1  ------------------------------------------------------------
          menziesii_PC2  ------------------------------------------------------------
          menziesii_PC3  ------------------------------------------------------------
          menziesii_PC4  ------------------------------------------------------------
  aucklandica_consensus  ------------------------------------------------------------
      onekaka_consensus  CTGGGAATATTGCTCATTCAGGTGCTTCTGTCGATTTAGCTATTTTTTCTTTACATCTTG

          menziesii_1    CCGGTGCTTCTTCTATTCTTGGGGCTATTAATTTTATTTCTACTGTTGGAAACATGCGGT
          menziesii_2    CCGGTGCTTCTTCTATTCTTGGGGCTATTAATTTTATTTCTACTGTTGGAAACATGCGGT
          menziesii_3    CTGGTGCTTCTTCTATTCTTGGGGCTATTAATTTTATTTCTACTGTTGGAAATATGCGGT
          menziesii_4    CCGGTGCTTCTTCTATTCTTGGGGCTATTAATTTTATTTCTACTGTTGGAAATATGCGGT
          menziesii_5    CTGGTGCTTCTTCTATTCTTGGGGCTATTAATTTTATTTCTACTGTTGGAAATATGCGGT
          menziesii_6    CCGGTGCTTCTTCTATTCTTGGGGCTATTAATTTTATTTCTACTGTTGGAAATATGCGGT
          menziesii_7    CCGGTGCTTCTTCTATTCTTGGGGCTATTAATTTTATTTCTACTGTTGGAAATATGCGGT
          menziesii_8    CCGGTGCTTCTTCTATTCTTGGGGCTATTAATTTTATTTCTACTGTTGGAAATATGCGGT
          menziesii_M4   CCGGTGCTTCTTCTATTCTTGGGGCTATTAATTTTATTTCTACTGTTGGAAACATGCGGT
          menziesii_M5   ------------------------------------------------------------
         menziesii_M14   ------------------------------------------------------------
          menziesii_PC1  ------------------------------------------------------------
          menziesii_PC2  ------------------------------------------------------------
          menziesii_PC3  ------------------------------------------------------------
          menziesii_PC4  ------------------------------------------------------------
  aucklandica_consensus  ------------------------------------------------------------
      onekaka_consensus  CCGGTGCTTCTTCTATTCTTGGGGCTATTAATTTTATTTCTACTGTCGGAAACATGCGGT

          menziesii_1    CTGTTGGGTTGGTCGCTGAGCGGATTCCTTTGTTTGTGTGGAGTGTTACGGTAACGGCCA
          menziesii_2    CTGTTGGGTTGGTCGCTGAGCGGATTCCTTTGTTTGTGTGGAGTGTTACGGTAACGGCCA
```

```
          menziesii_3   CTGTTGGGTTGGTCGCTGAGCGGATTCCTTTGTTTGTGTGGAGTGTTACGGTAACGGCCA
          menziesii_4   CTGTTGGGTTGGTCGCTGAGCGGATTCCTTTGTTTGTGTGGAGTGTTACGGTAACGGCCA
          menziesii_5   CTGTTGGGTTGGTCGCTGAGCGGATTCCTTTGTTTGTGTGGAGTGTTACGGTAACGGCCA
          menziesii_6   CTGTTGGGTTG-------------------------------------------------
          menziesii_7   CTGTTGGGTTGGTCGCT----------------------------------------------
          menziesii_8   CTGTTGGGTTGGTCGCT----------------------------------------------
         menziesii_M4   CTGTTGGGTTG-------------------------------------------------
         menziesii_M5   ---------------------------------------------------------------
        menziesii_M14   ---------------------------------------------------------------
        menziesii_PC1   ---------------------------------------------------------------
        menziesii_PC2   ---------------------------------------------------------------
        menziesii_PC3   ---------------------------------------------------------------
        menziesii_PC4   ---------------------------------------------------------------
aucklandica_consensus   ---------------------------------------------------------------
    onekaka_consensus   CCGTCGGGTTGGTGGCTGA-----------------------------------------

          menziesii_1   TTTTGTTGGTTGCCTCTTTGCCTG
          menziesii_2   TTTTGTTGGTTGCCTCTTTGCCTG
          menziesii_3   TTTTGTTGGTTGCCTCTTTGCCTG
          menziesii_4   TTTTGTTGGTTGCCTCTTTGCCTG
          menziesii_5   TTTTGTTGGTTGCCTCTTTGCCTG
          menziesii_6   ------------------------
          menziesii_7   ------------------------
          menziesii_8   ------------------------
         menziesii_M4   ------------------------
         menziesii_M5   ------------------------
        menziesii_M14   ------------------------
        menziesii_PC1   ------------------------
        menziesii_PC2   ------------------------
        menziesii_PC3   ------------------------
```

```
        menziesii_PC4   ------------------------
aucklandica_consensus   ------------------------
    onekaka_consensus   ------------------------
```