Assessing the use of molecular techniques to determine terrestrial invertebrate diversity in New Zealand mangroves and detection of blue ducks in New Zealand streams

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Abstract

Biodiversity monitoring requires reliable methods for species detection and identification. Here, I examine the use of molecular approaches, including mitochondrial DNA sequencing (barcoding) and environmental DNA (eDNA) analyses in two New Zealand-based case studies. In the first study, I used DNA barcoding to assess the diversity of arthropods collected from the mangroves within RAMSAR protected wetland area in the Firth of Thames, New Zealand. Over one year, a total of 9829 individuals were collected and of these 251 were sequenced. COI sequences were largely congruent with morphogroup designations. The sequences formed 101 putative species, 39% of which contained specimens from outside of New Zealand. 44% of the putative species found at the Thames sites had not been previously found in an inland habitat. I conclude that the terrestrial arthropod community of the mangrove forest in the Firth of Thames is distinctly different from other New Zealand habitats, and may include species not found elsewhere. In the second study, I examined the use of environmental DNA (eDNA) as a tool for detecting the presence of New Zealand's endemic blue duck, or whio, (*Hymenolaimus malacorhynchos*) in rivers, through water sampling. Species specific primers were designed to target a section of the mitochondrial control region. Water samples were collected from running water within artificial blue duck habitats using an in situ filtration system, eDNA was then extracted from the filter. Any blue duck DNA present in the sample was selectively amplified using the species specific primers. This allowed for blue ducks to be detected through sequencing of the PCR product. In 66% of these cases, it was possible to detect blue duck DNA from the filtered water

samples. On this basis, I conclude that there is sufficient "proof of concept" to warrant further investigation into the methods developed here, specifically, the testing of samples from natural environments, in which the presence of *H. malacorhynchos* has been confirmed by traditional methods.

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Chapter I

Thesis Introduction

Biodiversity monitoring often involves determining species composition and detecting changes in community composition or species' abundance (Buckland et al., 2005). However, establishing species' presence can pose a significant challenge when relying on traditional morphological methods of detection and identification (Oliver & Beattie, 1996; Cardoso et al., 2008). Fortunately, some of these challenges may be addressed through the use of molecular approaches, such as DNA barcoding (*sensu* Hebert et al., 2003) and the analysis of environmental DNA (eDNA) (Valiere & Taberlet, 2000). This thesis examines the use of both DNA barcoding for arthropod species identification (Chapter II) and the use of eDNA to detect the presence of New Zealand blue duck (Chapter III).

In Chapter II, I examine the use of mtDNA sequences and morphological analyses for assessing the diversity of terrestrial arthropods found in the mangrove forests of the Firth of Thames, New Zealand. The DNA barcoding approach was used in conjunction with the morphological approach to assess the accuracy of specimen identification.

Mitochondrial DNA (mtDNA) sequences are a common target for phylogenetic studies and taxon identifications among the metazoans (Zhang & Hewitt, 1996; Hebert et al., 2003) due to a highly conserved gene content (Boore, 1999), and lack of introns and recombination found in nuclear DNA (Hebert et al., 2003). The cytochrome *c* oxidase subunit I gene (COI), one of 37 mtDNA genes (Boore, 1999; Liu et al. 2013) has gained widespread acceptance for use in taxon identification. In particular, a 658 nucleotide "barcode" region of the gene has been recommended for species identification (Hebert et al., 2003; Collins &

Cruickshank, 2013), with effective primers now having been developed for most animal taxa (Folmer et al., 1994; Hebert et al, 2003). The Barcode of Life Data-systems (BOLD) database stores COI sequences, and can be used for DNA-based identification. The system has minimum information and quality requirements that must be met before sequences are included in the public database, which aims to reduce the risk of misidentifications. While researchers provide taxonomic identifications, to phylum or lower, at the time sequences are uploaded, this information is easily modified and updated. DNA "barcode" sequences are also attributed a Barcode Index Number (BIN) by BOLD. The BINs use a clustering algorithm to identify highly similar sequences across the database, with clusters having high concordance with species (http://www.boldsystems.org/).

In Chapter II, assessing of the diversity of the arthropod community found in the mangrove forests of the Firth of Thames, New Zealand, DNA barcoding will be used to supplement the morphological approach to arthropod specimen identification.

The phylum Arthropoda is the most diverse animal taxon and a major contributor to the biodiversity of most ecosystems (Kremen et al., 1993; Buddle et al., 2005; Losey & Vaughan, 2006; Rohr et al., 2007; Spafford & Lortie, 2013). Arthropods have critical roles in ecosystem function (Meades et al, 2002; Buddle et al., 2005; Losey & Vaughan, 2006; Spafford & Lortie, 2013) as they serve several roles, including pollination and nutrient cycling, in addition to being part of the food web (Losey & Vaughan, 2006). This high diversity and key ecosystem functions

makes it essential for arthropods to be considered in conservation efforts (Losey & Vaughan, 2006; Lovell et al., 2010). Arthropod community assemblages are also potential indicators of ecosystem health (Spafford & Lortie, 2013) which can be used to evaluate and monitor ecosystems, and habitat reconstruction efforts (Williams, 1993). Because of these applications, there is a need for quick and accurate methods for assessing the diversity of arthropod communities (Buffington & Redak, 1998; Cardoso et al., 2008). However, the effectiveness of invertebrate inventories in monitoring programs is complicated by the lack of detailed data available on arthropod diversity in general (Olvier & Beattie, 1996; Cardoso et al., 2008), with previously unknown insect species continuing to be found (Crisp et al., 1998).

Morphological keys traditionally used for specimen identification are typically based on individuals of specific life stages, such as adult forms, or on only one gender, making identification of individuals difficult when they differ from the life stage and gender of the one described by the dichotomous key (Hebert et al., 2003). The use of morphological keys also often requires expertise (Hebert et al., 2003), limiting their usefulness to non-specialists. This difficulty, combined with the large proportion of arthropods species that have yet to be formally identified, makes the use of morphogroups an attractive option in multi taxa studies, such as community surveys. The identification of morphogroups uses easily observed external characteristics, such as shape and colour, to group unknown specimens of morphological similarity (Derraik et al., 2010). Oliver and Beatie (1996), found non-specialist sorting of specimens into morphogroups resulted in splitting and lumping of specimen groups, where individuals of the same species are separated into different morphogroups, and multiple species are grouped into a single

morphogroup, respectively. However, these two sources of error were found to occur at similar rates, having little impact on the resulting estimates of species richness. The accuracy of separation of specimens into morphogroups varies between arthropod orders (Derraik et al., 2010), making specialist verification of morphogroups of particular importance when working with groups which are more difficult for non-specialists to separate (Oliver & Beattie, 1996), such as those which show sexual dimorphism (Derraik et al., 2010). The incorporation of specialist verification not only serves to identify errors, but reduces the error rate in subsequent studies (Oliver & Beattie, 1996). However, specialists may be unavailable for some taxa (e.g. Derraik et al., 2010), and may also make errors where there is phenotypic plasticity or otherwise morphologically cryptic taxa (Hebert et al., 2003).

Some of the limitations of morphology based identification methods may be addressed by including molecular markers in the identification process, as this allows for morphological identifications and groupings to be checked against genetic data assembled in databases or reference libraries (Schindel & Miller, 2005; Collins et al., 2012; Collins & Cruickshank, 2013). One such approach that is widely accepted uses short sequences of the cytochrome *c* oxidase subunit I (COI) gene. This 'DNA barcoding' approach utilizes the variations, such as point mutations, found within short regions of the COI gene as a tool for taxon identification and phylogenetic analyses (Wilson, 1995; Hebert et al., 2003). For the purposes of specimen identification, a specific region of an unknown specimen's DNA sequence is compared to an existing reference library of sequences obtained from specimens identified by morphological characteristics in

order to find a known specimen with a matching DNA barcode (Collins et al., 2012; Collins & Cruickshank, 2013).

DNA barcoding does not entirely remove the element of human error, in regard to the creation of these reference libraries (Becker et al., 2011). Where there are multiple laboratories working on the same taxa, specimens with identical sequences, but that have been identified using different morphological characteristics, can appear in a database library under different taxonomic names (Collins & Cruickshank, 2013). In order to prevent, or limit, misidentifications, detailed records and voucher specimens which can be checked and updated are required (Ratnasingham & Hebert, 2007), allowing users to use both the genetic information and the specimen information to potentially resolve problems, and prevent mis-identifications in future projects (Collins & Cruickshank, 2013). Some of the issues with the use of DNA barcoding will diminish with its use; as more researchers add to existing databases, the data available will become more robust. As the cost of sequencing decreases, the availability of barcoding for community assessment will improve, and the incorporation of barcode information is likely to become increasingly common practice in taxonomy (Wilson, 2003).

In Chapter III, the potential for eDNA analysis to be developed as a tool for the detection of the New Zealand blue duck (*Hymenolaimus malacorhynchos*) will be explored. DNA released into the environment (eDNA) through faeces, feathers, and other shed material (Taberlet et al., 1999) can be collected without directly handling or observing the organism. Because the animal does not have to be

directly observed, eDNA can be used as a tool for inferring the presence, or absence, of a species within a given area (Valiere & Taberlet, 2000; Ficetola et al., 2015; Giguet-Covex et al., 2014), and may be of particular use in circumstances where traditional field surveys require high investment of labour (Ficetola et al., 2008; Takahara et al., 2015), such as when working with small population sizes or rare species (Kohn & Wayne, 1997).

The mitochondrial control region, or D-loop, has a role in the initiation of transcription and replication of mtDNA molecules and is another region of the mitochondrial genome which has been used to explore taxonomic questions (Liu et al. 2013). While there is no database dedicated to accumulating control region sequences, as BOLD does with COI, the control region may have some advantages over COI in some studies. The control region has the greatest evolutionary rate of the mtDNA (Liu et al. 2013), and as such can be used to examine both inter and intra-specific relationships (Boore et al., 1999; Liu et al. 2013; Robertson et al. 2007).

Environmental DNA-based monitoring has been shown to have potential applications in bio-surveillance and the management of both threatened and invasive species in aquatic environments (Jerde et al., 2011) and wetlands, where eDNA isolated from water samples can be used to detect the presence of a target species (Ficetola et al., 2008; Lodge et al., 2012; Takahara et al., 2015). Because eDNA is often highly degraded, short DNA markers, of less than 300bp (Taberlet et al., 1999; Panasci et al., 2011; Broquet et al., 2007), which contain point

mutations which vary sufficiently to allow taxon identification, are targeted for PCR amplification and analyses (Wilson, 1995; Hebert et al., 2003).

Enviornmental samples often contain low quantities of DNA (Broquet et al., 2007; Panasci et al., 2011), fortunately, PCR is a highly sensitive method, capable of amplifying samples with DNA concentrations in the nanogram to microgram range (Navidi et al., 1992), allowing for the detection of eDNA at low concentrations (Ficetola et al., 2008), as might be expected where the target species has a low population density (Darling & Mahon, 2011). However, as eDNA contains non-target DNA (Broquet et al., 2007; Panasci et al., 2011), careful primer design is needed to reduce the risk of cross-reactivity leading to false positives, in which the DNA of a non-target species is amplified (Raut et al., 2007; Darling & Mahon, 2011). The possibility of false positives can be controlled for through the inclusion of both positive and negative control samples in DNA extractions and PCR reactions (Willis et al., 2011), and by sequencing the PCR product and comparing the sample to a reference sequence (Darling & Mahon, 2011).

In addition to false positives, false negatives, in which the target species is present but goes undetected, may also occur (Darling & Mahon 2011; Ficetola et al., 2015; Takahara et al., 2015). False negatives may be the result of a failure to collect the target species' DNA, and are expected to increase in frequency where samples sizes are small (Takahara et al., 2015), however this may also occur when the ratio of target DNA to contaminating non-target DNA impacts the effectiveness of the PCR amplification (Navidi et al., 1992). Low numbers of

replicate samples also contribute to the occurrence of false negatives (Ficetola et al., 2015). Increasing sample sizes and the number of replicates collected may reduce the impact of false negatives, however this also increases running costs (Ficetola et al., 2015; Takahara et al., 2015). Modifications made to survey methods in an effort to reduce this source of error need to be site and species specific, and be made with some knowledge of the sensitivity of the methods used (Hayes et al., 2005; Darling & Mahon, 2011; Willis et al., 2011).

The final chapter of this thesis (Chapter IV) summarises the findings and suggests avenues for future research related to my two projects.

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Chapter II

Assessing the diversity of terrestrial invertebrates in mangrove forests at Thames, North Island, New Zealand*

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Introduction

The grey mangrove or manawa (*Avicennia marina* ssp. *austalasica*) is the only mangrove species in New Zealand (de Lange & de Lange, 1994). Mangroves have been present in New Zealand for more than 9,800 years (Brownell, 2004). These trees form forests in the intertidal zone, a high stress environment where growth is limited by pressures such as high soil salinity, which increases the cost of obtaining freshwater (Lugo, 1980). These stresses may influence their growth, with different conditions resulting in either tall trees of up to 9 metres, or a stunted shrub form of around 2 metres in height (Burns & Ogden, 1985; de Lange & de Lange, 1994). In addition to the immediate challenges of growing in the intertidal zone, mangroves are subject to frequent disturbance events, such as storms, tidal movements and the impacts of human activities (Farnsworth, 1998; Farnsworth & Ellison, 1998), all of which affect these systems on both temporal and spatial scales (Farnsworth, 1998). Mangroves trap sediments and slow water movements, protecting coastlines. However, over time this accumulation of sediments may lead to a transition from mangrove forest to terrestrial habitats or freshwater wetlands (Green et al., 2003).

In many parts of the world mangrove forests are diminishing as the result of anthropogenic pressures (Farnsworth & Ellison, 1997; Schwarz, 2003). In contrast, the New Zealand mangrove forests have expanded in recent decades, as a result of increased sedimentation within estuaries, due to the deforestation which has taken place over the past 150 years, with accumulated sediments in tidal areas forming areas suitable for mangrove colonisation (Swales et al., 2008). This

expansion can impinge on human activities and aesthetic values as areas which were once open water are now mangrove forest (Green et al., 2003).

New Zealand's mangrove forests provide habitat for marine species, such as yellow eyed mullet (*Aldrichetta forsteri*) and smelt (*Retropinna retropinna*) (Green et al., 2003). However, little literature is currently available on the terrestrial arthropod component of New Zealand's mangroves. This is unfortunate as these areas may provide habitat for a range of native species. Here, we addressed this gap by using a combined morphological and molecular approach (DNA barcoding) to assess the diversity of terrestrial invertebrates in the mangrove forests in the Firth of Thames, New Zealand. Specifically, we focused on three sites of varying growth patterns, to determine the diversity of terrestrial arthropods. Specifically, we tested the hypotheses that: 1) different sites house different arthropod species; and 2) terrestrial arthropod species found in mangrove forests are unique to these habitats, by comparison with other available molecular data.

Materials and Methods

Sampling sites

The Firth of Thames, Coromandel, North Island, New Zealand, is a wetland of international importance, recognised under the RAMSAR convention. The Firth is a shallow embayment with a maximum depth of 35 metres, and a width of 11 to 14 nautical miles. The embayment merges with the Hauraki Gulf in the north, with the Firth receiving freshwater from the Waihou, Piako, Kauaeranga and Waitakaruru Rivers, with a total catchment area of roughly 3600km² (Brownell, 2004). Mangroves are frost-sensitive trees which are restricted to the North Island, at latitudes above 38º (Swales et al., 2008), placing the Firth of Thames within approximately 100km of the southern limit of mangrove forests (Cromarty & Scott, 1995). Three sites within the Firth of Thames were selected for this study (Figure 1). Site 1 is separated from the sea by tidal mudflats, with Sites 2 and 3 both being positioned on the banks of rivers that flow into the firth. The three sampling sites in this study experience varying levels of tidal water inundation, which appears to also influence the diversity of vegetation present at each site (Table 1).

Mangroves present a challenge to invertebrate sampling as they are regularly inundated with water, risking both damage to traps set up at the site and a loss of samples. Two of the three sites were also positioned close to roads and public access points, making any highly visible traps, such as light traps, vulnerable to interference. Due to these constraints, sweep netting and branch beating were used as the primary sampling methods.

Owing to the presence of surface water, pitfall traps were not included in the original survey design. However, during the August 2014 collection large numbers of Collembola were observed on the ground at Site 3. A pit fall trap was set up to collect these taxa, and left in place while the planned sampling was carried out.

Sample collection

Fourteen monthly collections were taken at each of the three sites between November 2013 and November 2014. Whenever possible, sampling was carried out beginning at low tide, with low tide occurring in the morning, on days without rain having been forecast by the Meteorological Service. To correspond with higher arthropod abundances during summer, two collections were carried out in January 2014. At each site and for each collection period, sweep netting and branch beating were carried for a minimum of 45 minutes each, with the exception of the June 2014 sampling at sites 2 and 3, which coincided with the duck hunting season. As active shooting was taking place, the sampling was truncated and consisted of only sweep netting. Sampling was carried out following the same path through each of the sites, in order to prevent any unidentified microclimates within the sites from influencing the data (Figure 2).

A net with a 30cm opening was used for sweep netting, and was emptied after every few sweeps in order to prevent damage to the specimens. To reduce sampling error associated with the individual collector's skill and reach (Buffington & Redak, 1998; Roulston et al., 2007) sweep netting was carried out by the same collector throughout the study, with the exception of the first

collection, in which training took place. Sweep netting was used to collect specimens from all heights of vegetation, from ground cover, to the maximum height that could be reached by the net. Branch beating was used on vegetation ranging from approximately knee height to shoulder height. In addition, specimens found clinging to foliage, and spiders resting on their webs, were collected by hand as they were observed. All specimens were placed in 100% ethanol on site, with collection information. The specimens were kept cool and stored at 4ºC on return to the laboratory.

Specimen Identification

Morphology

Individual specimens from each collection were sorted into groups based on morphological similarity, using easily observed morphological characterises, including wing shape and pattern, body size, leg modifications, and distinctive colour or markings. A representative specimen from each morphogroup was photographed. Where possible, morphogroups were identified to the genus or species level using field guides and dichotomous keys (a complete list of reference materials use for morphological identifications is provided in Appendix 2). Those morphogroups which could not be identified were named by the lowest level of identification that could be made with confidence, and then assigned an identification number (e.g. *Chironomus* M1). All specimen data (site, date, taxonomic identification or morphogroup number) was recorded. Where possible, sex, life stage and species authority were noted. The number of individual specimens of each taxon and the number of additional specimens removed for sequencing purposes were recorded (a complete list of species and morphogroups is provided in Appendix 3).

Molecular Analyses

A total of 285 specimens, representing 109 morphogroups, was selected for sequencing from the November 2013 to February 2014 collections. One to five specimens from each of the selected morphogroups were sequenced, depending on the number of specimens available. Specimens were photographed and tissue samples (right, rare leg) taken. Tissue samples were sent to the Canadian Centre of DNA Barcoding (CCDB) for DNA isolation and sequencing. Specimen information, photographs, and trace files were uploaded to the Barcode of Life Data system (BOLD), under the project code NZMAN.

Specimens were identified using the COI sequences by searching the full BOLD data base for similar sequences, with the BOLD full database search tool returning the 99 most similar sequence records in the database. All published records within the search results were considered. However, records listed as either Private or Early Release were disregarded from the identification process, as these records could not be accessed or verified. Unpublished sequence data were used where we had full access to these records. Taxonomic trees, constructed using the BOLD "Taxon ID Tree" tool, were used to visualise the similarity between all sequences from each search. These trees, and the percentage similarity with other records in the database, were then used to identify the unknown sequences of arthropods from our samples. Where percentage similarity was high (e.g. >98%) between the sequence of the unknown specimen and sequences from the database, the lowest common level of taxonomic identification was used. Details from each search, including the taxonomic identification of the most similar sequence from the

database and the percentage of similarity, were recorded for each sequence for the ease of cross checking and future examination.

Neighbour Joining (NJ) trees were constructed using the sequences within the NZMAN project, with separate trees being made for the Arachnida and each insect order. These trees were used to visualise variation within each of the groups, and to reveal any relationships among sites and genetic variation within a given species, which could indicate restricted dispersal between the sampling sites.

Specimens belonging to the same morphogroup of sequenced specimens that were identified using COI were assumed to be the same species as the COI identified specimen. The method of identification used was recorded for each specimen, allowing for those which were identified by sequence data, and those which were morphological matches to these specimens to be differentiated.

Statistical Analyses

Multidimensional scaling analyses were used to examine the relationship between the arthropod communities at each of the sampling sites, and the relationship between the arthropod community and seasonal changes. For these analyses, rare species, defined here as those morphogroups for which the highest count was less than four throughout the study or which were found in less than 9.5% of the collections, were removed from the species abundance data that were used. The

remaining data were log transformed before use in the analysis. Statistical data analysis was carried out using Primer 6 software.

Table 1. Summary of environmental characteristics. *Maximum height based on height measurements taken from 20 *A. marina australasica* trees, greater than 40cm in height, within the sampling area. The maximum height able to be measured accurately was 5m. **Means are based on measurements made below the first branching point of 20 *A. marina australasica* trees, over 40cm in height, within the sampling area.

	Site 1: Miranda	Site 2: Piako River	Site 3: Waihou River
Latitude	-37.1826	-37.2019	-37.1907
Longitude	175.32	175.5	175.56
Number of vascular plant species	2	9	15
Maximum tree height*	3m	>5m	>5m
Mean trunk circumference**	N/A	24.9	38.4
Growth pattern of mangroves	multi stemmed shrub	single trunk tree	single trunk tree
Prevalence of juveniles <40cm	limited	abundant	absent
Surrounding land use	Agriculture & conservation	Agriculture	Agriculture
Protection	QEII covenant	None	None

Figure 1. Map of the Firth of Thames and sampling sites (modified from www.google.co.nz/maps).

Figure 2. Aerial images showing sampling area for each of the sites visited (http://www/google.co.nz/).

Results

Fourteen sampling visits were made to each of the three sites over thirteen months, for a total of 42 collections, and 9829 individual arthropod specimens. No other taxa were collected. The specimens were split into 505 morphogroups, from four taxonomic classes, based on a combination of morphological characteristics and sequence data. Of these, 397 were Insecta, 88 Arachnida, 15 Collembola (Entognatha), and 4 were Isopoda (Malacostraca). Of the 505 morphogroups 40 were identified to species level, 41 to genus, and the remainder to family level or higher.

A total of 285 specimens were sequenced, of which 251 were successful and produced sequences with an average sequence length of 655bp. BOLD attributed the sequences to 102 Barcode Index Numbers (BINs), four which were flagged by BOLD awaiting meta data compliance and were excluded from the analyses. Of the 98 BINs that were examined, 64 contained specimens previously collected from New Zealand sites outside of the study area (Figure 3). Sixty-two BINs were unique to New Zealand, 46 of which were found only within the Firth of Thames in the current research. Of these 46 BINs, 22 were singletons (i.e. single sequences fitting in to a BIN). One of these singletons was morphologically identified as a spotted lax beetle (*Parispalpus nigronataus*) which, while known to be common near mangroves, is not restricted to this habitat.

Juvenile and larval forms of 10 morphogroups were included among the specimens that were sequenced, and linked to their adult forms, where adult

arthropod specimens were found which shared 100% sequence similarity with the juvenile specimen's sequence.

The MDS plot of community comparison showed some overlap between the arthropod communities at different sites, those from Site 1 grouped together more strongly than those from Sites 2 and 3 (Figure 4). Pairwise testing of these relationships showed there were significant differences ($p < 0.002$ in all cases) between the arthropod communities at each of the sites.

The MDS plot of seasonal variation clearly showed groupings of collections by season, with the summer and winter samples being well separated from each other. In contrast, those groupings from spring and autumn, had data points overlapping with other seasons (Figure 5). Pairwise testing showed significant differences between each season, with p-values of 0.001 for all relationships, with the exception of summer/winter and summer/autumn, which returned p-values of 0.004 and 0.015 respectively. The June 2014 collections for Sites 2 and 3, for which no branch beating was carried out, grouped closely with the remaining collections for that season (winter).

Cicadas (*Cicadidae)* and bumble bees *(Bombus* sp.) where sighted at Sites 1, 2, and 3, and at Site 1, respectively, but were not captured at any stage. Similarly, white butterflies (*Pieris rapae*) were observed and black field crickets (*Teleogryllus commodus*) heard, at each of the sampling sites. These two species were observed near the fringe of sampling areas at Sites 1 and 2, and throughout

Site 3, which had ground cover absent from the other sites. Damselflies and dragonflies (Odonata), and honeybees (*Apis mellifera*) were frequently observed at Sites 1 and 2. However, capture rates for theses taxa were lower than expected given their occasionally high numbers within the sampling areas, with a total of only two Odonata, and ten *A. mellifera* specimens having been collected throughout the study.

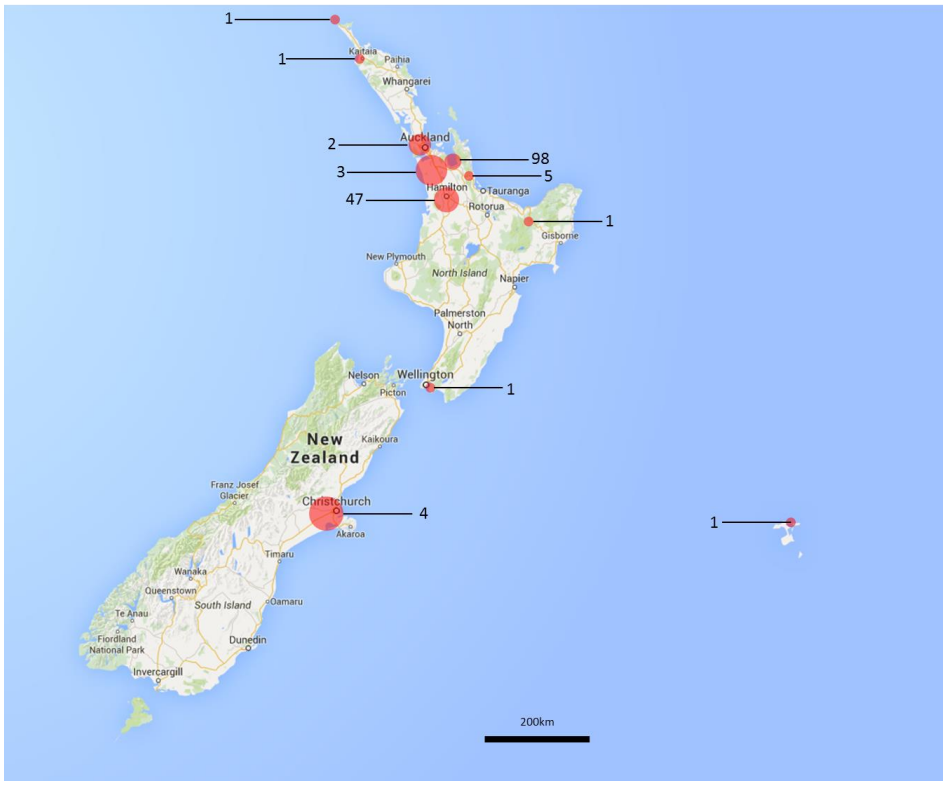

Figure 3. Map of New Zealand, showing the 11 areas from which specimens falling into 98 BINs were collected, and the number of BINs represented in each area.

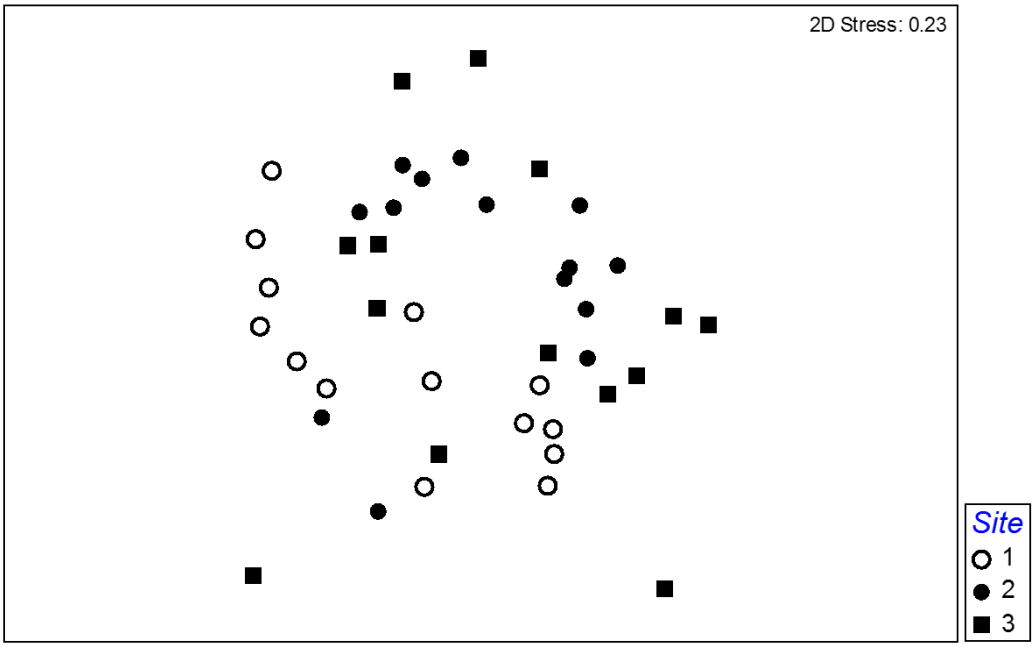

Figure 4. MDS of arthropod community composition at the three sampling sites over time.

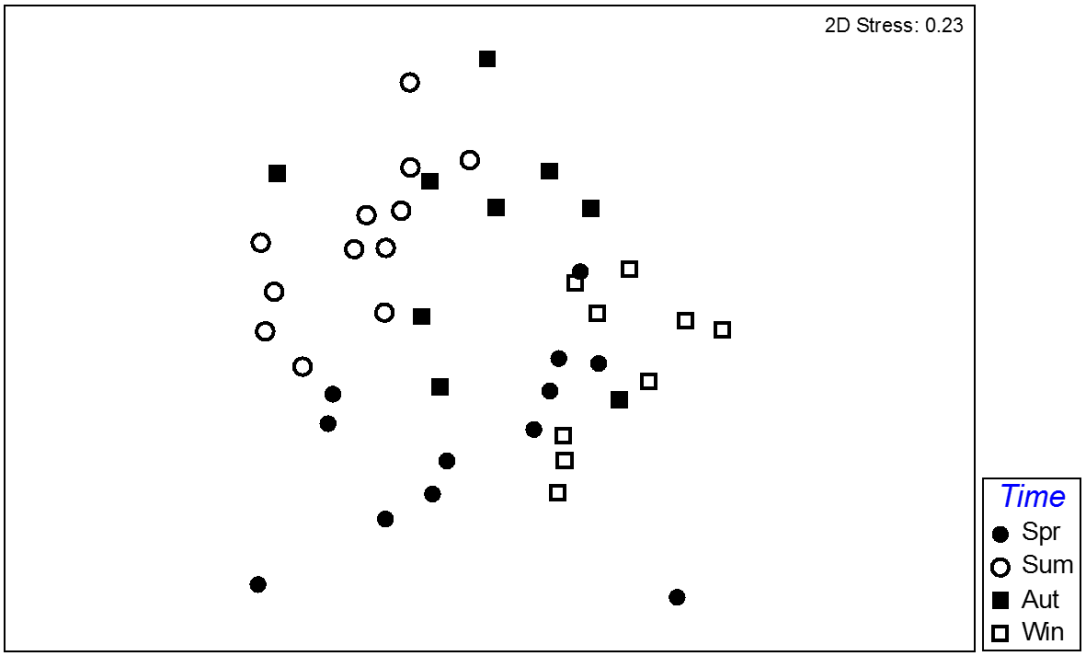

Figure 5. MDS plot of seasonal variation in the composition of the arthropod community in the Firth of Thames mangrove forests.

Discussion

Using a combination of morphological characteristics and COI sequence data, the 9829 individual arthropod specimens in this study were sorted into 505 morphogroups. Only 40 of the morphogroups could be identified to species, and 41 to genus. The remaining morphogroups were identified to family level or higher. Meades et al. (2002), identified 250 terrestrial arthropod morphogroups in mangrove forests in New South Wales, Australia, from only two sampling visits, and suggested that mangrove stands may host a greater diversity of terrestrial arthropods than they had been thought to. Our findings support this suggestion.

The 251 sequences obtained in this study represent only 2.55% of the 9829 specimens that were collected. However, these sequences were largely congruent with the morphogroup analyses, with the 109 morphogroups that were sequenced being grouped into 102 BINs by BOLD. This rate of error is lower than reported by previous authors comparing non-expert morphology based grouping and expert identification (e.g. Derraik et al., 2010). This suggests that the morphogroups were a good surrogate for species diversity. Twenty-three BINs showed significant morphological similarity to those of another BIN, making consistent separations of specimens into their correct groups difficult without the aid of sequence data. Conversely, specimens in three of the BINs showed sufficient morphological variation that they were originally placed into six separate morphogroups, but have been re-grouped into three BINs based on their COI sequences. By re-assessing morphogroup designations in light of the sequence data, small morphological differences that had been previously over looked were identified. This allowed for the majority of these morphogroups to be redefined

and for morphologically similar specimens for which there was no sequence data to be placed into these re-assessed groups with reasonable confidence.

Ninety-seven of the 102 BINs are metadata compliant, and have been considered in the analyses. Of these metadata compliant BINs, 46 appeared to be unique to the Firth of Thames when they were compared to the full BOLD database. One of the BINs which was identified morphologically as a spotted lax beetle (*Parisopalpus nigronataus*), which is known to be common near mangroves. Some of the remaining 42 BINs may represent species which have not been added to the BOLD database. However, it is also possible that some represent species that are unique to either the Thames mangrove forest, or New Zealand mangrove forest habitats in general.

The majority of the 51 BINs which also contained New Zealand sequences from outside of the Firth of Thames were within approximately 100km of the study area. The approximate north-south orientation of New Zealand's landmass creates a climatic gradient, which restricts the dispersal of some species, which may explain some of the apparent restricted distribution of some BINs.

The pressures associated with the coastal marine environment make mangroves dynamic systems (Farnsworth, 1998), with a simplified vegetative community (Lugo, 1997), and reduced biodiversity compared with tropical forests (Farnsworth, 1998). However, if the disturbance events and environmental pressures ease for a prolonged period, terrestrial systems may replace mangroves

forests (Lugo, 1980). This shift towards becoming a terrestrial system may be occurring at Site 3, which has limited tidal inundation and the greatest diversity of plant species of the three sites. The resulting increase in plant diversity would be expected to create a more diverse habitat, and therefore the diversity of the arthropod community would also be expected to increase (Crisp et al, 1998). The differences in the vegetation at the sampling sites may explain the variation found between the arthropod communities. The arthropod community at Sites 2 and 3 were more similar to each other than either was to Site 1, which also had a less diverse plant community compared to the other two sites.

The finding that community composition at each site changed with the seasons was expected, as the detectability of species may vary over time (Buckland et al., 2005). Seasonal variation in the environment would be expected to influence the abundance, behaviour, life histories and metabolic activity of arthropods, which would in turn affect changes in the susceptibility of some taxa to being collected (DeLong, 1932). This, combined with the sensitivity of the sampling methods to wind and rain (DeLong, 1932), may explain some of the difference seen between the winter and summer collections; some taxa may have been undetected during winter, rather than absent if they were less abundant or relatively inactive. Weather conditions may also have an impact on sampler effort (Buckland et al., 2005). The overlap seen between the autumn and spring collections was also expected, as these seasons are transitional in nature.

Sweep netting has been found to be ineffective at collecting small arthropods which may be dispersed by movement of the net itself (Buffington & Redak,

1998). While numerous specimens with body length of 1mm were collected during this study, these specimens may not represent the full diversity of small bodied taxa present at the sampling sites. However, the taxa which were observed but not captured, or which were collected in low numbers, *Cicadidae*, *Bombus* sp., Odonata and *A. mellifera*, were relatively large animals. This would seem to contradict Cooper & Whitmore (1990), who suggested that heavier and more active arthropods may be more likely to be captured by sweep netting.

In summary, the combined morphological and molecular approach used here has demonstrated the diversity found in three areas within the Thames mangrove forest. The morphogroup concept was tested using molecular (DNA barcoding) data and found it to be a reasonable surrogate for actual species diversity. Future work examining a range of mangrove forests around the upper North Island of New Zealand will be needed to fully assess the diversity found within these habitats.

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Chapter III

Detecting New Zealand blue ducks (*Hymenolaimus malacorhynchos*) using environmental DNA (eDNA)*

*To be published under the same title as: Doyle, E. J., Hogg, I., Banks, J., & Gemmill, C. E. C.

Introduction

New Zealand's endemic blue duck, or whio, (*Hymenolaimus malacorhynchos* Gmelin 1789), is morphologically similar to other riverine Anseriformes. However analysis of the mitochondrial genome has shown that this monotypic genus has no close relationship with any other genera within the family Anatidae (Robertson & Goldstien, 2012). This river specialist (Robertson et al., 2007a; King et al., 2000; Robertson & Goldstien, 2012), was widespread prior to the arrival of humans to New Zealand, but has since gone into decline and is now restricted to forested, fast-flowing rivers, often in remote areas (Figure 1) (Williams, 1991; Collier et al., 1993; King et al., 2000). This reduction in population size and distribution has occurred due to the loss of suitable habitat, with the modification of waterways and the removal of riparian vegetation, coupled with the introduction of mammalian predators (Adams et al., 1997; Glaser et al., 2010). Stoats (*Mustela erminea*) have been identified as the main threat to *H. malacorhynchos* in some areas (Glaser et al., 2010).

Under the New Zealand Threat Classification System (NZTCS), *H. malacorhynchos* is currently classified at Nationally Vulnerable, with the population in decline, and in need of active management to prevent extinction (Glaser et al., 2010). The IUCN has also recognised this species as being under threat of extinction, listing *H. malacorhynchos* as Endangered on their Red List, due to their small and highly fragmented population and continuing decline (http://www.iucnredlist.org/).

Locating individuals in the wild is an important step in protecting a threatened species. However, in the case of *H. malacorhynchos*, this can require a considerable effort, as these birds have slate-blue plumage, which provides effective camouflage in their rocky habitat (Glaser et al., 2010), making them difficult to spot. *H. malacorhynchos* also tend to be crepuscular feeders, reducing the chances of sighting them on the water in the middle of the day (Glaser et al., 2010). An additional difficulty is their small population density as they form enduring, territory holding pairs versus forming large flocks (Williams, 1991; Collier et al., 1993).

The goal of the current research was to develop a technique for using environmental DNA (eDNA) as a tool for locating *H. malacorhynchos* through water sampling. Such a technique would allow for the rapid genetic screening of numerous waterbodies for the presence of *H. malacorhynchos* in a relatively short time period, which would then allow conservation efforts to be focused on areas which have been inferred as having these birds present.

Figure 1. Distribution of *H. malacorhynchos* wild populations (Robertson et al., 2007b).

Materials and Methods

Primer design, specificity and sensitivity

Twelve 894 nucleotide long *H. malacorhynchos* DNA sequences, representing each of the twelve mtDNA control region haplotypes identified by Robertson et al. (2007a) were downloaded from GenBank (GenBank accession no. EF395946-EF395957). These sequences were aligned using Geneious (version 7), and a 149bp region that contained comparatively few SNPs was identified as a potential marker region. The marker sequence was entered in GenBank's BLASTn (Basic Local Alignment Search Tool nucleotide) (Altschul et al. 1990) algorithm, to examine its similarity to other avian species within the database. The marker region was found to have 100% query cover, and 91-100% identical sequence to the 12 *H. malacorhynchos* haplotypes. BLASTn also identified *T. tadorna* (common shelduck), as having a similar sequence, however query cover was 20-57% and sequence similarity was 79-93%, making this species clearly distinguishable from *H. malacorhynchos* using this marker region (Figure 2).

The first 20 nucleotides at the 5´ end and the last 19 nucleotides at the 3´ end of the marker region were separately searched for in GenBank using the BLASTn algorithm to examine their specificity as potential primer binding sites. The sequences were found to have 100% query cover, and 100% identical sequence to *H. malacorhynchos,* as expected. Both sequences were also found to have some similarity to non-target species from a diverse range of taxa that would not be expected to be found in *H. malacorhynchos* habitat. The query cover for the majority of this non-target species was below 85%. The 5´ end sequence was found to have 85% query cover and 100% identical sequence to the non-target

species, *Apteryx austalis* (southern brown kiwi), and the 3´ end sequence was found to have 100% query cover and 100% identical sequence to *Ovis canadensis* (bighorn sheep), two species not likely to be found in the fast flowing rivers that *H. malacorhynchos* inhabits. These were the most similar non-target sequences found for the two sequences being tested. However, PCR primers based on these sequences were unlikely to amplify these non-target species when used together. Based on these results, PCR primers were designed to amplify the identified marker region; Control Region Forward (CRF: 5´-CCCATTACGCATGGACTAAA-3´) and Control Region Reverse (CRR: 5´-CGTGGGACCTTGTTTGTGG-3´).

DNA was extracted from blood samples of *H. malacorhynchos* and chicken (*Gallus gallus domesticus*) and from muscle tissue of seven non-target species found in New Zealand (little blue penguin (*Eudyptula minor*), common pheasant (*Phasianus colchicus*), black bird (*Turdus merula*), thrush (*Turdus philomelos*), prion (Procellariidae), sacred kingfisher (*Todiramphus sanctus*) and koi carp (*Cyprinus carpio*)) using the Qiagen Blood and Tissue Kit, following the protocols recommended for purification of total DNA from nucleated blood and muscle tissues by spin column, as appropriate. The DNA samples were PCR amplified in 15 µL reactions containing 7.5 µL *i*-Taq (iNtRON), 3.9 MQ water, 1 µL of the forward primer (10 µM), 1 µL of the reverse primer (10 µM), and 2 µL of the template DNA. A negative control, in which 2 µL of MQ water was substituted for the template DNA was included in each PCR. Thermal cycling was conducted in an Eppendorf Cycler (Vapo. Protect ™). DNA was denatured for 2 minutes at 94ºC, followed by 30 cycles of denaturation at 94ºC for 15 seconds, a primer annealing period of 30 seconds at 50ºC, and elongation period of 30

seconds at 72ºC, followed by an extension period of 60 seconds at 72ºC. The PCR products were run out on a 1% agarose 0.5X TBE gel, with 1 µL Redsafe™, and visualized in an Alpha Imager.

To check the primer sensitivity to DNA concentration, a dilution series was made of *H. malacorhynchos* DNA samples, with two replicates, each reducing in concentration by 50% at each step, from 100% down to 0.005%. The nucleic acid concentration of each sample was measured using a NanoDrop® (Thermo Scientific) spectrophotometer, with readings ranging from 16ng/µg to below the minimum detectable amount. To determine the approximate concentration of DNA needed to obtain the single 150bp band expected, these dilutions were PCR amplified using the *H. malacorhynchos* CRF and CRR primers.

Field sampling

Water samples were collected from artificial habitats, where a known number of *H. malacorhynchos* were present (Table 1). The sites chosen for this were the free flight aviary at the Hamilton Zoo, Hamilton, the blue duck enclosure at the Otorohanga Kiwi House, Otorohanga, and one of the two aviaries at the National Trout Centre, Turangi. At each of these positive control sites, artificial streams ran though the enclosures. At the Hamilton Zoo and the Otorohanga Kiwi House, the streams pass through multiple bird enclosures before entering the *H. malacorhynchos* enclosures. However, water for the artificial stream in the National Trout Centre's *H. malacorhynchos* enclosures is sourced directly from a spring in a well forested area, and not exposed to non-target avian species before entering the enclosure. Sampling was carried out twice at each site, with samples

collected at 1-3 points within the artificial streams, depending on the accessibility allowed by the surrounding plantings and structures. With the exception of the Hamilton Zoo site, samples were collected from the inlet outlet points. Negative control samples were collected from three sites where *H. malacorhynchos* were known to be absent.

Water samples were collected using a battery powered pump, which drew water through a filter, allowing eDNA to be collected and concentrated *in situ*. Two filter methods were tested: 1) a larger pore synthetic wool filter; and 2) a finer GF/C Millipore® filter. The synthetic wool was a porous, plastic material widely used in the filter systems of aquariums, and available in most pet supply stores. This material is designed to allow the rapid movement of water through the filter system, while removing biological material from the water. Circular filters (40mm diameter) of 4mm thickness were used. Filters were UV sterilised for 20 minutes per side. As the filter pads were porous, it was assumed that the UV light would be sufficient to sterilize the filter's internal surfaces in addition to the immediate surface. The sterilized filter pads were immediately transferred into individual UV sterilized sealable plastic bags.

The use of a filter with large pore size allowed for large volume of water to be filtered in a short time span, with fine suspended sediments passing through the filter, rather than clogging it, as occurs with finer Millipore® filters. The aim was to capture organic material released into the environment, such as mucus or faeces. We assumed that organic material would take time to break up into fragments small enough to pass through the filter, and that the filter will collect

large eukaryotic cells, rather than small prokaryotic cells or naked DNA molecules. This addressed the need for smaller pore sizes (0.22-0.45µm), which are recommended by most protocols (e.g. MoBio® Powerwater® kit protocol). This was also intended to reduce the impact of false positives, in which the DNA of the target species is detected after the organism is no longer in the area, as the larger pore size will be less likely to collect particulate DNA which may have persisted in the environment.

The GF/C Millipore® filter was used to collect eDNA from the National Trout Centre *H. malacorhynchos* aviary, with sample sizes of 20, 40 and 60L (Table 2). GF/C filters were used directly from the package without further treatment. These were expected to collect more material, but also become clogged more quickly.

The number of samples that could be collected at any one site was subject to logistical constraints (e.g. time to filter each sample) as well as minimising the disturbance to animals. Where the volume of water filtered per sample was increased, the number of samples that could be collected in the time available was reduced.

Isolation of eDNA

Environmental DNA was extracted from the filters on the same day of collection in order to reduce the risk of bacterial proliferation within the filters, and the degradation of DNA by nucleases within the sample. eDNA was isolated from the filters using the MoBio® PowerWater® DNA isolation kit, following the

manufacturer's instructions, with the following modifications: At Step 4, the filters were cut in half, and inserted into separate bead beating tubes, forming two subsamples per filter. The initial bead beating step was carried out for 10 minutes, and the centrifuging step that followed was extended to six minutes. At Step 16 the two subsamples were combined, with both subsamples being passed through a single spin filter column. The quantity of eDNA in each sample was evaluated using a NanoDrop® spectrophotometer, with a subset of the samples further measured using a Qubit™ fluorimeter.

Hymenolaimus malacorhynchos DNA within the eDNA samples was amplified by PCR, using the primers CRF and CRR, and visualised after electrophoresis in a 1% agarose 0.5X TBE gel. The samples were considered positive only when a single discrete PCR product of 150bp, matching that of the *H. malacorhynchos* DNA positive control, was amplified. To confirm that the amplified DNA was *H. malacorhynchos*, these samples that had a single PCR product of the appropriate size were cleaned up using ExoSAP (Exoneclease I- Shrimp Alkaline Phosphate, GE Health Care) and sequenced bi-directionally using the same primers as per the PCR on an ABI model 3130xl Genetic Analyser at the University of Waikato sequencing facility.

Resulting sequences were edited by eye using Geneious, then queried using GenBank's BLASTn search algorithm. eDNA sequences with high similarity to one or more of the 12 sequences on the GenBank database were recorded as being positive for blue duck DNA. However, as eDNA is expected to be degraded (Broquet et al., 2007; Panasci et al., 2011), 100% similarity was not expected.

Low concentrations of DNA and/or the presence of contaminating non-target DNA was expected in the eDNA samples. Therefore, further optimization of the method was required to improve the results. The eDNA samples were PCR amplified in 15 µL reactions containing 7.5 µL *i*-Taq (iNtRON), 2.9 µL MQ water, 0.8 µL of the control region forward (CRF) primer, 0.8 µL of the control region reverse (CRR) primer, and 3 µL of the template DNA. A negative control without DNA, in which 3 µL of MQ water was substituted for the template DNA, and a second negative control with 3 µL of *C. carpio* DNA and a positive control using 3 µL of *H. malacorhynchos* were included in each run. The touchdown cycling began with the DNA being denatured for 5 minutes at 94ºC, followed by 30 cycles of denaturation at 94ºC for 30 seconds, a primer annealing period of 30 seconds at 60ºC, and elongation period of 60 seconds at 72ºC. This was done for a single cycle, with the annealing temperature being reduced by 0.5ºC in each of the following 10 cycles, until the annealing temperature of 55ºC was reached at the eleventh cycle, which was repeated for a total of 35 cycles. This was followed by an extension period of 10 minutes at 72ºC. The PCR products were run out on a 1% agarose 0.5X TBE gel, with 1 µL Redsafe™ (iNtRON), and visualized using an Alpha Imager. A subset of the samples which amplified, producing a single 150bp band by this method were sequenced. Sequences were queried in GenBAnk using the BLASTn search algorithm.

Table 1. Environmental DNA sampling sites with number of samples collected using the synthetic wool filter.

Site	Number of blue ducks	Non-target species present	Sun exposure	Sediment type	Total samples collected (20 L)	Total samples collected (40 L)	Total samples collected (60 L)
Hamilton Zoo free flight aviary	2	Multiple	Well shaded	Hard bottom	19	0	0
Otorohanga Kiwi House	2	Multiple	Half shaded	Soft sediment	17	0	0
National Trout Centre aviary	6+	None	Full exposure	Hard bottom	14	3	1
Waikato River, Hamilton City District	0	Multiple	Full exposure	Soft sediment	6	0	0
National Trout Centre artificial stream point source	0	None	Full shade	Mixed	1	0	0
Knighton Lake, University of Waikato campus	0	Multiple	Full exposure	Soft sediment	6	0	0

Table 2. Environmental samples collected from the National Trout Centre aviary using the GF/C Millipore® filter.

	20 L	40 L	60 L
Number of replicate samples	3	4	2

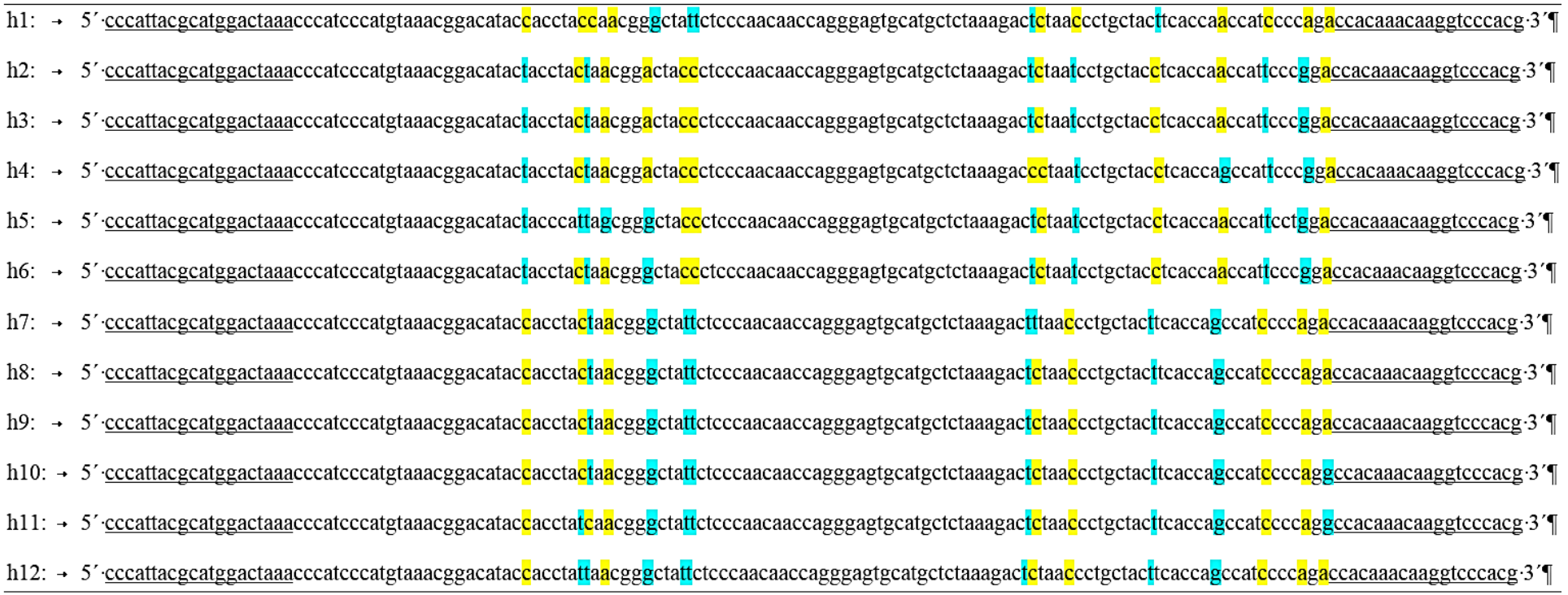

Figure 2. Blue duck mtDNA marker region variations within each of the twelve haplotypes identified by Robertson et al. (2007a), (GenBank accession no. EF395946-EF395957).

Results

Primer design and sensitivity

The CRF and CRR PCR primers reliably amplified an approximately 150bp DNA fragment using samples of *H. malacorhynchos* DNA derived from blood from 11 individual birds. The sequences obtained using these samples typically showed greater sequence length in the reverse direction. When the 11 *H. malacorhynchos* sequences were entered into the GenBank's BLASTn algorithm, they each returned a match of 92-100% similarity to three or more of the *H. malacorhynchos* control region sequences in the database. There were an additional six matches made to *T. tadorna* with 93% similarity.

Six of the seven non-target samples (*E. minor*, *P. colchicus*, *T. merula*, *T. philmelos*, *T. sanctus*, and *C. carpio*) failed to amplify. While the Procellariidae DNA sample did amplify this was not of the expected 150bp size (Figure 3).

Dilutions of the *H. malacorhynchos* DNA derived from blood, which were then PCR amplified showed some visually detectable banding on the gel electrophoresis down to the 0.05% dilution (Figure 4). However, the bands at these low concentrations were very faint, and would not generally be strong enough to be taken as a positive result. The bright bands, taken as positive results in test situations, were produced by the dilution of around 0.195% and higher. The NanoDrop® measurement of nucleotide concentration showed that the 0.195% DNA dilution had a concentration that was below the minimum dateable amount.

Environmental DNA Samples

The NanoDrop® measurements of nucleotide concentration showed some variability in the estimated concentration of eDNA between the first and second sampling visits to each site. However, 48 of the 70 samples had DNA concentrations below the minimum detectable threshold, making it difficult to establish a pattern. The measurements made using the Qubit™ were similarly low, with eighteen of the samples measured having nucleotide concentrations below 0.5µg/µL, and therefore too low even to be measured by the Qubit™. Twelve of the 17 samples from the National Trout Centre had concentrations below 0.5µg/µL.

The Qubit™ measurements of the GF/C samples were compared to those collected from the same site, on a previous sampling occasion, using the synthetic wool filter. Despite the finer pore size of the GF/C filter, the concentration of eDNA per sample was lower than those of the same volume collected using the synthetic wool filter. Of the nine eDNA samples collected using the GF/C filter, only one had a sufficiently high concentration to be measured using the Qubit™, with a eDNA concentration of 0.077µg/µL. Four of the eight eDNA samples collected using the synthetic wool filter were successfully measured using the Qubit™, with a mean concentration of 0.568 µg/µL.

Of the total 15 20L synthetic wool filter samples from the Hamilton Zoo which were amplified using the first PCR protocol, optimized using DNA from blood samples, six were successfully amplified. However, only four produced strong bands, and were subsequently sequenced (Figure 5). The four sequences were

found to be strong matches to the *H. malacorhynchos* control region sequences in GenBank, based on a BLASTn search with 99% similarity and 99% query cover. Amplification was not observed in any of the remaining eDNA samples from artificial *H. malacorhynchos* habitats (Table 3). Of the total 13 negative control site samples, collected from the three negative sites, no amplification occurred.

In contrast, when the touchdown PCR protocol was used, 43 of the 65 positive control eDNA samples produced a band of the desired size (Table 4). The positive controls were all found to have 95-100% sequence similarity to *H. malacorhynchos*, and 93% similarity to *T. tadorna*. Amplification did occur in *C. carpio* and several additional eDNA samples, producing fragments >150bp and/or smearing, which were not taken to be positive results. The *C. carpio* DNA amplified using the CRF and CRR primers. However, this amplification was seen as a smear in the electrophoresis gel and not a discrete band. Amplification was also observed in each of the negative control eDNA samples. However, only two of these produced a distinct band of 150bp that could be potentially be mistaken as being from *H. malacorhynchos*. The negative control samples, which had produced 150bp bands when PCR amplified, did not have any significantly similar matches to any records within GenBank, with BLASTn returning no query results.

Table 3. Summary of eDNA based presence/absence test using an unmodified PCR. Positive results were defined as the amplification of a 150bp band, and high sequence similarity to one or more of the *H. malacorhynchos* control region sequences in GenBank.

	Hamilton Zoo		National Trout Centre		Otorohanga Kiwi House	
Sample size	**Positive**	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
20L	4	15	0	14	0	14
40L	-	-	0	9	-	-
60L	-	-	0	3	-	-

Table 4. Summary of eDNA amplification success using incressed volumes of template DNA and a touchdown cycling profile.

	Hamilton Zoo		National Trout Centre		Otorohanga Kiwi House	
Filter used	**Positive**	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
Synthetic wool filter	14	5	14	1	10	7
GF/C	-	-	8	0	-	-

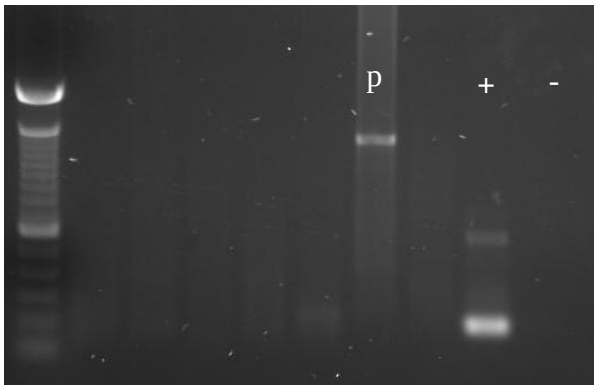

Figure 3. Electrophoreses of non-target DNA amplified using the CRF and CRR PCR primers. A positive control of *H. malacorhynchos* DNA (+) amplified as expected, while the only one non-target sample amplified. Procellariidae (p) produced a larger DNA fragment than the *H. malacorhynchos* control sample, making it easily distinguished from a positive result.

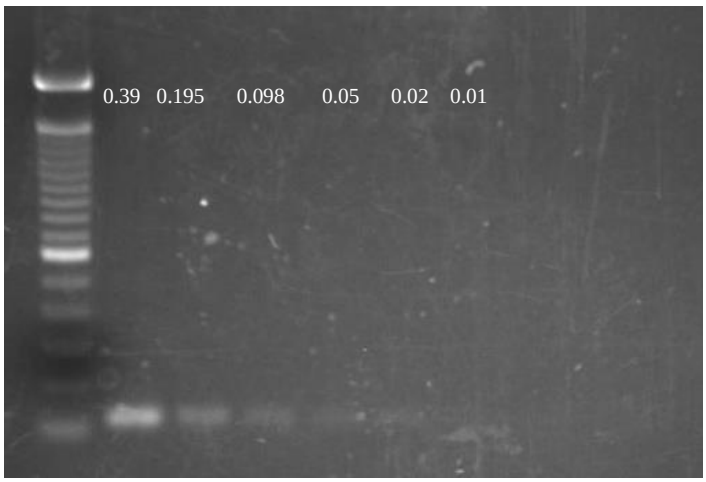

Figure 4. Electrophoresis of diluted *H. malacorhynchos* DNA, ranging from 0.39-0.01% DNA, amplified with the CRF and CRR PCR primers.

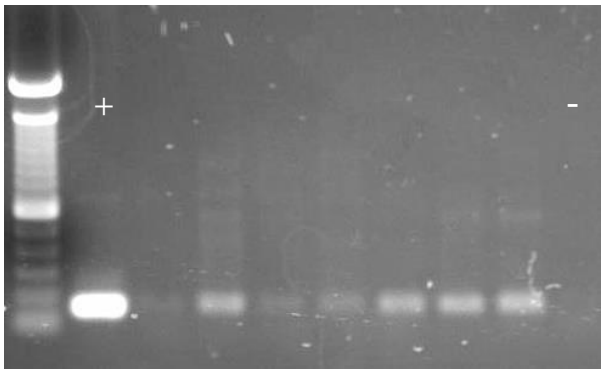

Figure 5. Electrophoresis of the first set of Hamilton Zoo samples. Of the seven eDNA samples, four produced strong bands of the expected 150bp size. The presence of pale bands in two additional samples is suggestive of a positive result, but samples with this weak a signal were not considerd worth sequencing.

Discussion

Here, we have developed a relatively simple protocol that could be used to detect *H. malacorhynchos* using eDNA derived from water samples. The original PCR protocol, which was based on using DNA from whole blood samples, appeared to be ineffective when working with eDNA samples, with only four of the eDNA samples amplified using the method (Figure 3). This was improved by modifying the laboratory protocol to accommodate the limitations of the eDNA samples. As suggested by Willis et al. (2011), this method modification may frequently be required when working with environmental samples from different sites, or collected from the same site at different times. Increasing the volume of template DNA in the PCR and the use of a touchdown temperature cycling profile significantly increased the number of eDNA samples that amplified, to 46 out of the total 70 eDNA samples. Of these 46 amplifications of a single 150bp fragment, 2 were from negative control sites (i.e. no *H. malacorhynchos* present). However, when sequenced, these samples were not found to have significant sequence similarity to any sequences in GenBank. This indicates that eDNA samples which are successfully amplified should be sequenced to confirm the presence of the target species. This may be particularly important when working with rare species, such as *H. malacorhynchos*, in order to reduce the risk of false positives (Darling & Mahon, 2011).

The variation between the sites, in terms of the concentration of eDNA in the samples, and the success rate of *H. malacorhynchos* DNA amplification, may have been due to environmental variations, such as flow rate, exposure to sunlight, and sediment type, which can affect the eDNA isolation and

amplification (Jerde et al., 2011). While environmental factors may also affect the rate of DNA degradation, and therefore its detection. However, how these factors interact is not fully understood, and cannot be fully controlled for (Lodge et al., 2012; Giguet-Covex et al. 2014).

When the *H. malacorhynchos* CRF and CRR primers designed here were tested on non-target DNA samples, the majority of the samples failed to amplify, and in the few cases where amplification did occur, the size of the DNA fragments differed from those of the *H. malacorhynchos* controls. This would suggest that these primers were species specific. This was also supported by the lack of similar sequences from other species in GenBank. However, we caution that the diversity of non-target DNA samples available to be tested, and sequences available in GenBank, may not be fully representative of all species present in *H. malacorhynchos* habitat. For example, all DNA from avian species present in the watershed may enter the system via runoff during rain events.

Both of the control region primers designed in the current research generated reliable, discrete and reproductive results. While the sequences produced using the reverse primer were of sufficient quality to be used, the forward sequences were frequently too short or of low quality. A redesign of the forward primer to produce longer sequences, of higher quality, would allow for alignment of the forward and reverse sequences.

The benefit of the synthetic wool filters over the GF/C filter was the relatively easy flow of water and small particles thought the filter, allowing for large volumes of water to be filtered without clogging. The GF/C filters were found to perform to a comparable standard; allowing for large volumes to be filtered, and with all eDNA collected using this filter amplifying successfully using the touchdown method. However, in the field, the GF/C filters were more difficult to use than the synthetic wool filters, as they were easily damaged when handled in wet conditions. These filters may also have been damaged by the pump that was used, as they were found to have small tears and indentations when removed from the filter system. Further sampling would be required to make a direct comparison between the two filter types tested here. However, results may vary with target species, and the nature of the target organic material being collected in the filter.

In summary, we have successfully designed PCR primers that can amplify a maker region of New Zealand's blue duck's mitochondrial control region. These primers were used in a touchdown cycling temperature profile to amplify a 150bp DNA fragment, which could be sequenced for confirmation of the presence *H. malacorhynchos* DNA in eDNA samples isolated from water. Having established a working method for inferring the presence of *H. malacorhynchos* from water samples in artificial habitats, the next phase of the project is to trial this method on eDNA samples from natural environments.

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Chapter IV

Thesis summary and Conclusions

In this thesis, I have used molecular methods to aid with the identification of arthropod specimens collected in the mangrove forests of the Firth of Thames over the course of a year (Chapter II), and to infer the presence of blue ducks (*H. malacorhynchos*) in artificial streams by the analysis of eDNA isolated from water samples.

In Chapter II, I catalogued 9829 individual arthropod specimens, collected from three areas within the Firth of Thames mangrove forests, assigning them to 505 morphogroups. DNA barcoding of 251 of these specimens allowed for some morphogroups to be identified to the species level, or higher, by comparing these DNA sequences to those already available on the BOLD database (Ratnasingham & Hebert, 2007). The sequence data indicated that about half of the morphogroups which had been sequenced had not been previously found outside of the sampling area, based on an absence of highly similar sequences originating from other areas in the BOLD database. This suggests that the arthropod community in the Firth of Thames mangrove forests may be distinctly different from other New Zealand habitats. With the potential for lumping of genetically distinct species, shown to have occurred for at least some of the morphogroups here, the finding of 505 morphogroups may be an underestimation of the true diversity of species in the mangrove forests.

Moeed & Meads (1985), found that botanically diverse New Zealand forests had greater invertebrate diversity compared to forests with less diverse plant life. This effect on invertebrate diversity is likely due to the increased range of host plants present and range of habitats available (Crisp et al., 1998). By collecting samples

from sites with differing plant diversity, small differences in the arthropod community that may be being shaped by the diversity of plant life were observed (e.g. Moeed & Meads, 1985). Additionally, by collecting samples over the course of a year, changes in arthropod community composition that occurred with the passage of the seasons were also detected. Understanding these seasonal variations in community composition may be of use in the future to provide insight into how arthropod communities are changing over time, and with environmental modification or changes that occur in the interim. The information collected here provides a baseline for future research in New Zealand mangrove forests, and provides important information for management of the RAMSAR protected mangrove forests in the Firth of Thames (e.g. Brownell, 2004).

I conclude that the terrestrial arthropod community of the mangrove forest in the Firth of Thames is distinctly different from other New Zealand habitats, and may include species not found elsewhere.

In Chapter III, I developed PCR primers capable of amplifying *H. malacorhynchos* DNA from blood samples. I optimized a protocol for this purpose, through trialling varied PCR master mixes and temperature profiles. The primers were tested on DNA from non-target species, and found to either not amplify at all, or to amplify a DNA fragment of a different size. From this, it was determined that the primers were sufficiently specific to be used in environmental samples.

Water samples were collected from artificial habitats, in which *H. malacorhynchos* were present, and control sites where they were absent. Initial testing was largely unsuccessful. Various additional modifications to the laboratory testing of these samples were tested, until a modified PCR master mix and temperature profile were found that improved the amplification success of the samples. The modified protocol allowed for DNA to be amplified in by taking multiple samples per site, and using sequencing as a confirmation tool, I was able to infer the presence of *H. malacorhynchos* DNA in 66% of the eDNA samples isolated from water.

As each site has its own specific conditions and sources of contamination, the effectiveness of eDNA analysis of *H. malacorhynchos* in natural environments is uncertain. However, the success of the method in the artificial habitats containing blue duck is encouraging. I conclude that there is sufficient "proof of concept" to warrant further investigation into the methods developed here, specifically, the testing of samples from natural environments, in which the presence of *H. malacorhynchos* has been confirmed by traditional methods.

Future research

The Firth of Thames mangrove forest species list generated here contains a large proportion of morphogroups that remain unidentified. In order to determine how different these forests are from other habitats, identifying these specimens would be beneficial. As the terrestrial arthropods of New Zealand's mangrove forests are a relatively unexplored group, further efforts to identify these morphogroups and sequencing of a larger proportion of the specimens will not only give a more precise indication of the diversity in this area, but it may also reveal previously undescribed species.

For the purposes of ecological monitoring, future work should revisit the Firth of Thames sampling sites, to asses any changes that have occurred over time, or with modifications to the environment. Resources allowing, carrying out sampling in additional areas would provide additional context for the interpretation of these results.

The research done here, on exploring eDNA as a tool for detecting blue ducks, has been carried out in artificial habitats. Future work should take the methods developed here into the field, to test the effectives of the method in natural environments. This should also provide further information as to the rate of false negatives under natural conditions, which would allow for an effective field sampling protocol to be developed.

The range of New Zealand species for which there are mitochondrial control regions sequences presently in GenBank is limited. Adding sequences of the same region from non-target species to the database would make the use of eDNA for species detection more robust.

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Appendices

Appendix 1: Plant species lists for Firth of Thames sampling sites

Site 1	Site 2	Site 3
Avicennia marina subsp. *australasica*	*Avicennia marina* subsp. *australasica*	*Apium prostutum*
Sarcocornia quinqueflora	*Glycerea maxima*	*Avicennia marina* subsp. *australasica*
	Muenleaseckia complexa	*Heminthotheca echioides*
	Plagianthus divarrcatus	*Metrosideros excelsa*
	Sarcocornia quinqueflora	*Myoporum lactum*
		Plagianthus divaicatus
		Poa praensis
		Polypogon mospeliasis
		Sarcocornia quinqueflora

Appendix 2: References used for arthropod identifications

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Appendix 3: Thames mangroves species list

Species list for the Firth of Thames mangrove forest, and total number of individuals of each species found at each of the sites between November 2013 and November 2014.

Morphogroup/ Species List	Site 1	Site 2	Site 3	Total
Hydrellia triici	781	131	502	1414
Diptera M21	539	17	93	649
Opisthoncus polyphemus	265	71	99	435
Diptera M24	93	142	180	415
Ectopocus M1	286	49	36	371
Trichopsocidae M1	5	248	10	263
Chironomus M1	32	148	75	255
Collembola M9	25		226	251
Diptera M4	139	71	8	218
Halmus chalybeus	85	86	47	218
Diptera M69	57	57	99	213
Sitobion M1		4	206	210
Micromus tasmaniae	124	51	18	193
Diptera M15	6	16	139	161
Diptera M6	122	2	33	157
Diptera M128	104	9	41	154
Psocoptera M1	10	121	7	138
Technomymex M1	9	32	80	121
Diptera M91	64	17	31	112
Diptera M33	32	27	51	110
Dolomedes minor	75	20	1	96
Diptera M40	49	22	17	88
Diptera M71	83		2	85
Psocoptera M10	65	10	9	84
Chironomus M2	3	51	19	73
Latridiidae M2	39	12	22	73
Diptera M22	13	51	8	72
Sciaridae M1	19	14	36	69
Collembola M4	7	58	1	66
Diptera M16	4	59	1	64
Coleoptera M6	8	41	14	63
Diptera M25	25	26	11	62
Psocoptera M2	12	27	21	60
Eriophora pustulosa	19	29	11	59
Psocoptera M8	9	21	27	57
Diptera M102	55			55
Diptera M3	21	13	21	55
Diptera M92	17	13	25	55
Parisopalpus nigronotatus	36		17	53
Diptera M63	42	2	8	52
Ceratozetidae M1		45	4	49

Ochetellus glaber	1	21	25	47
Cixiidae M1		2	39	41
Collembola M3		30	11	41
Chloropidae M2	1	8	29	38
Theridiidae M1		1	37	38
Psocoptera M7	15	16	6	37
Rhopalosiphum padi	13	17	6	36
Tipulidae M1	19	5	10	34
Diptera M182			33	33
Chloropidae M1	4	17	11	32
Tetragnatha nitens	11	15	5	31
Cynipidae M1	5	12	12	29
Miridae M2	18	4	7	29
Araneidae M1	20	6	2	28
Clubonia M1	27	1		28
Diptera M44	22	4	2	28
Diptera M5			28	28
Diptera M50	3		24	27
Diptera M79	22	1	2	25
Diptera M99	1	6	18	25
Psocoptera M4	3	22		25
Psocoptera M5	16	2	7	25
Theridiidae M4	5	19	1	25
Diptera M115	19		4	23
Hymenoptera M9	18		4	22
Psocoptera M13	1	12	9	22
Coleoptera M2	1	19	1	21
Curculionidae M1	1	19	1	21
Erigone M1	4	14	3	21
Hemiptera M15	9		12	21
Araneae M1	2	15	3	20
Diptera M114	20			20
Psocoptera M6	8	6	6	20
Salticidae M8	13	1	6	20
Araneae M11	2	16	1	19
Coleoptera M4	8	4	7	19
Diptera M51	3		16	19
Nezara viridula	8		11	19
Phoridae M1	10	5	4	19
Diptera M56	3	10	5	18
Tenuphantes tenuis	2	9	7	18
Diptera M120	16	1		17
Scolypopa australis	1		16	17
Diptera M31		2	14	16
Merophyas M1	5	11		16
Coccinellidae M2	4	1	10	15
Diptera M142			15	15
Ceratozetidae M2		14		14

Diptera M149		14		14
Hemiptera M2	3	11		14
Diptera M20	10	2	1	13
Hymenoptera M24	10	1	2	13
Lepidoptera M6	8	1	4	13
Diptera M29	1	3	8	12
Diptera M98	1		11	12
Dolichopodidae M2	2	7	3	12
Psocoptera M21	11		1	12
Tipulidae M6	7	4	1	12
Coleoptera M16	10	1		11
Coleoptera M17	10		1	11
Diptera M110			11	11
Formicidae M4		11		11
Apis mellifera	10			10
Coccinellidae M1	1	4	5	10
Diptera M34	3	1	6	10
Diptera M8	9		1	10
Diptera M86	6	2	2	10
Hemiptera M13	2		8	10
Scatella M1	2	5	3	10
Diptera M122			9	9
Diptera M48	1	8		9
Diptera M85		5	4	9
Dolichopodidae M1		9		9
Hemiptera M1	1	4	4	9
Isopoda M2			9	9
Lepidoptera M9	4	5		9
Theridiidae M6		7	2	9
Arachnida M3		8		8
Curculionidae M5			8	8
Diptera M75	7	1		8
Diptera M89			8	8
Helpis minitabunda	3		5	8
Psocoptera M3	4	2	2	8
Araneae M23		6	1	7
Araneidae M10	7			7
Ceroplastes sinensis	3	3	1	7
Diptera M10	2	2	3	7
Diptera M23	3		4	7
Diptera M38	6		1	7
Diptera M54	7			7
Diptera M83	7			7
Lepidoptera M3	3	3	1	7
Araneae M2		4	1	5
Diptera M130			6	6
Diptera M138		3	3	6
Diptera M139		1	5	6

Diptera M2	4	2		6
Diptera M41			6	6
Diptera M72	2	3	1	6
Lepidoptera M13		5	1	6
Oxysarcodexia varia	3	3		6
Psocoptera M9	4	1	1	6
Tetragnatha M2	2	4		6
Tettigoniidae M1			6	6
Tipulidae M2	2	3	1	6
Agromyzidae M1	4	1		5
Aphidiidae M8	5			5
Araneae M13		5		5
Chironomus M6		5		5
Coleoptera M1	2	2	1	5
Collembola M7			5	5
Culex M1		5		5
Diptera M100	4	1		5
Diptera M104	5			5
Diptera M146		5		5
Diptera M152		5		5
Diptera M55	3	2		5
Diptera M70	2	1	2	5
Diptera M81			5	5
Diptera M96			5	5
Hemiptera M9	2	2	1	5
Insecta M12	1	4		5
Lepidoptera M10	1	2	2	5
Lepidoptera M17		5		5
Mymicinae M1		5		5
Pentatomidae M1	2		3	5
Psocoptera M12		5		5
Psocoptera M16	5			5
Scymnus loewii	4		1	5
Sidymella longipes			5	5
Sidymella M1			5	5
Sphragistius M1	3		2	5
Araneae M15		4		4
Araneae M3		4		4
Araneidae M2	4			4
Cavariella aegopodii	1	3		4
Chironomus M3	1	2	1	4
Coccinella undecimpunctata	3		1	4
Coleoptera M18		4		4
Diptera M105	4			4
Diptera M135	1	1	2	4
Diptera M136	3	1		4
Diptera M87			4	4
Hemiptera M3	2	2		4

Hymenoptera M12		1	3	4
Hymenoptera M16	3	1		4
Ichneumonidae M1	4			4
Salticidae M12			4	4
Smitta M1	1	3		4
Agromyzidae M2		3		3
Anthicidae M1			3	3
Aphidiidae M11			3	3
Aphidiidae M7		1	2	3
Arachnida M14		3		3
Arachnida M28		2	1	3
Arachnida M7	1	2		3
Araneae M17		1	2	3
Araneidae M12	3			3
Argyrodes antipodianus		3		3
Coleoptera M15	2		1	3
Coleoptera M19		3		3
Collembola M1			3	3
Curculionidae M2	1		2	3
Diptera M101	3			3
Diptera M108	3			3
Diptera M113	1	2		3
Diptera M127			3	3
Diptera M131		3		3
Diptera M67			3	3
Diptera M97			3	3
Hemiptera M12	1	1	1	3
Hemiptera M14	2	1		3
Hymenoptera M11		1	2	3
Hymenoptera M13	1	1	1	3
Hymenoptera M22			3	3
Hymenoptera M6	1	1	1	3
Insecta M13	2		1	3
Longitarsus M2	2	1		3
Metopolophium dirhodum		1	2	3
Opogona omoscopa			3	3
Psocoptera M20		3		3
Psocoptera M23		1	2	3
Sidymella M2			3	3
Sphaeroceridae M1		3		3
Tachinidae M1		1	2	3
Tetragnatha M1	2		1	3
Tetramorium M1	3			3
Theridiidae M2	2	1		3
Adalia bipunctata	1		1	2
Arachnida M1		2		2
Arachnida M29			2	2
Araneae M12		2		2

Araneae M14		2		2
Araneae M18		2		2
Araneae M21		2		2
Araneae M5	2			2
Araneae M8		1	1	2
Bathyphantes M1			2	2
Chironomidae M1		1	1	2
Coleoptera M10			2	2
Coleoptera M21	2			2
Collembola M13		2		2
Collembola M16		1	1	2
Collembola M8	2			2
Culex pipiens		2		2
Curculionidae M6	2			2
Curculionidae M7		1	1	2
Curculionidae M9	1		1	2
Dipriidae M1			2	2
Diptera M111		1	1	2
Diptera M12	1	1		2
Diptera M121			2	2
Diptera M124			2	2
Diptera M125			2	2
Diptera M126		1	1	2
Diptera M137		1	1	2
Diptera M141			2	2
Diptera M144	2			2
Diptera M151	1	1		2
Diptera M28	2			2
Diptera M59		1	1	2
Diptera M60		2		2
Diptera M68			2	2
Diptera M82	1		1	2
Diptera M9	2			2
Diptera M90	1		1	2
Diptera M93		1	1	2
Diptera M94	1		1	2
Diptera M95	1		1	2
Exapion M1	2			2
Formicidae M5			2	2
Geometridae M1			2	2
Glaucias amyoti	1		1	2
Hemiptera M18	2			2
Hemiptera M5		2		2
Hymenoptera M1	1		1	2
Hymenoptera M15		1	1	2
Hymenoptera M23		1	1	2
Hymenoptera M26		2		2
Hymenoptera M28	1	1		2

Hymenoptera M29		2		2
Hymenoptera M7	1		1	2
Insecta M10	1	1		2
Insecta M5		2		2
Isopoda M1		2		2
Isopoda M3			2	2
Latridiidae M3	2			2
Lepidoptera M11	2			2
Lepidoptera M14			2	2
Lepidoptera M15		2		2
Lepidoptera M8	1		1	2
Leucauge dromedaria		3		3
Longitarsus M1	2			2
Mermessus M1		1	1	2
Miridae M1			2	2
Mymicinae monomorium			2	2
Nyctemera amica	2			2
Orthoptera M1		2		2
Pisauridae M1	2			2
Polistes chinensis	1	1		2
Psocoptera M18	2			2
Psocoptera M24		2		2
Ptyomaxia M1	2			2
Salticidae M1			2	2
Salticidae M2		2		2
Scaptomyza M1			2	2
Spilogona M1		2		2
Tenebrionidae M1			2	2
Tipulidae M5	1	1		2
Acyrthosiphon M1		1		1
Aphidiidae M10			1	1
Aphidiidae M12		1		1
Aphidiidae M9	1			1
Arachnida M10	1			1
Arachnida M12			1	1
Arachnida M13		1		1
Arachnida M15		1		1
Arachnida M16		1		1
Arachnida M2	1			1
Arachnida M30			1	1
Arachnida M31		1		1
Arachnida M4		1		1
Arachnida M5		1		1
Arachnida M8			1	1
Araneae M16			1	1
Araneae M19			1	1
Araneae M20	1			1
Araneae M22			1	1

Araneae M24		1		1
Araneae M6	1			1
Araneae M7		1		1
Araneae M9		1		1
Araneidae M11			1	1
Araneidae M13	1			1
Araneidae M14		1		1
Araneidae M3		1		1
Araneidae M4	1			1
Araneidae M5			1	1
Araneidae M6	1			1
Araneidae M7		1		1
Araneidae M8	1			1
Araneidae M9	1			1
Artysona M1		1		1
Austrolestes colensonis		1		1
Backobourkia M1		1		1
Bethelium signiferum	1			1
Cavariella M1	1			1
Chironomus M4			1	1
Chironomus M5			1	1
Chloropidae M3			1	1
Chrysomelidae M1	1			1
Coleoptera M14		1		1
Coleoptera M20		1		1
Coleoptera M22	1			1
Coleoptera M23	1			1
Coleoptera M24			1	1
Coleoptera M25			1	1
Coleoptera M26	1			1
Coleoptera M27			1	1
Coleoptera M28			1	1
Coleoptera M29			1	1
Coleoptera M31		1		1
Coleoptera M9		1		1
Collembola M10			1	1
Collembola M11			1	1
Collembola M12			1	1
Collembola M14	1			1
Collembola M17			1	1
Collembola M2			1	1
Collembola M5		1		1
Collembola M6		1		1
Conocephalua M1			1	1
Cryptanea M1	1			1
Curculionidae M3			1	1
Curculionidae M4			1	1
Curculionidae M8			1	1

Dialectica M1	1			1
Diptera M1		1		1
Diptera M103	1			1
Diptera M106	1			1
Diptera M107	1			1
Diptera M11			1	1
Diptera M116	1			1
Diptera M117	1			1
Diptera M119	1			1
Diptera M123			1	1
Diptera M129			1	1
Diptera M132		1		1
Diptera M133			1	1
Diptera M134	1			1
Diptera M140			1	1
Diptera M145	1			1
Diptera M147	1			1
Diptera M148	1			1
Diptera M150	1			1
Diptera M156		1		1
Diptera M27		1		1
Diptera M32			1	1
Diptera M36	1			1
Diptera M37	1			1
Diptera M43	1			1
Diptera M45	1			1
Diptera M47	1			1
Diptera M49	1			1
Diptera M53	1			1
Diptera M57	1			1
Diptera M61			1	1
Diptera M66		1		1
Diptera M73	1			1
Diptera M74	1			1
Diptera M76	1			1
Diptera M78		1		1
Diptera M84		1		1
Diptera M88			1	1
Formicidae M1		1		1
Formicidae M3			1	1
Formicidae M6		1		1
Gerris M1			1	1
Haliplidae M1	1			1
Harmonia conformis		1		1
Hemianax papuensis	1			1
Hemiptera M10		1		1
Hemiptera M11			1	1
Hemiptera M16		1		1

Hemiptera M20		1		1
Hemiptera M21			1	1
Hemiptera M22			1	1
Hemiptera M23		1		1
Hemiptera M24			1	1
Hemiptera M25	1			1
Hemiptera M6	1			1
Hemiptera M7			1	1
Hemiptera M8		1		1
Hymenoptera M10	1			1
Hymenoptera M17			1	1
Hymenoptera M18	1			1
Hymenoptera M2	1			1
Hymenoptera M20	1			1
Hymenoptera M21			1	1
Hymenoptera M27	1			1
Hymenoptera M3			1	1
Hymenoptera M30		1		1
Hymenoptera M31			1	1
Hymenoptera M32			1	1
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Hymenoptera M34			1	1
Hymenoptera M35			1	1
Hymenoptera M36			1	1
Hymenoptera M4			1	1
Hymenoptera M5			1	1
Hymenoptera M8		1		1
Ichneumonidae M2	1			1
Illeis galbula			1	1
Insecta M1	1			1
Insecta M11		1		1
Insecta M14	1			1
Insecta M16		1		1
Insecta M17			1	1
Insecta M18		1		1
Insecta M19	1			1
Insecta M2	1			1
Insecta M3	1			1
Insecta M4		1		1
Insecta M8		1		1
Insecta M9			1	1
Isopoda M4			1	1
Latridiidae M1			1	1
Latridiidae M4		1		1
Lepidoptera M16			1	1
Lepidoptera M4	1			1
Lepidoptera M5			1	1
Lepidoptera M7			1	1

Mantodae M1		1		1
Miomantis caffra			1	1
Miridae M3	1			1
Miridae M4			1	1
Orchrocydus huttoni	1			1
Orthodera novaesealandiae			1	1
Phoridae M2	1			1
Psocoptera M11			1	1
Psocoptera M14	1			1
Psocoptera M15	1			1
Psocoptera M17	1			1
Psocoptera M19	1			1
Psocoptera M22	1			1
Psocoptera M25	1			1
Psocoptera M50		1		1
Psocoptera M51		1		1
Psocoptera M52		1		1
Psychodidae		1		1
Salticidae M10		1		1
Salticidae M11			1	1
Salticidae M13			1	1
Salticidae M5			1	1
Scymnodes lividigaster	1			1
Tenuiphantes M1			1	1
Tetragnathidae M1		1		1
Theridiidae M3		1		1
Theridiidae M5		1		1
Theridiidae M7		1		1
Thysanoptera M1	1			1
Tipulidae M3	1			1
Tipulidae M4			1	1
Tipulidae M7			1	1