The diversity of

Hippocampus abdominalis in New Zealand

A thesis

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Abstract:

This study investigates the diversity and population differentiation of the New Zealand Pot-belly seahorse *Hippocampus abdominalis* through the utilization of morphological and genetic data. Four microsatellite loci – Habd3, Habd6, Habd7 and Habd9 – and three mitochondrial DNA markers – cytochrome b (814 bp), cytochrome oxidase 1 (624 bp) and control region (404 bp) – in conjunction with quantified morphological features revealed a very high diversity but low population differentiation within New Zealand, suggesting very high levels of gene flow. Some sexual dimorphism was detected, in the terms of shorter snout length and trunk length, and a higher incidence of fronds and spotting in males. A sample size of 166 yielded 31-46 microsatellite alleles and no common multilocus genotypes, and 36-40 new sequences were generated for each mitochondrial DNA marker exposing 14-16 haplotypes, with a maximum of 0.7-2.2% sequence divergence. *H. abdominalis* were found to be widely dispersed mainly in low density populations. As this species is likely to be facing increased threats from exploitation and habitat degradation in the future it is hoped that this information contributes to the knowledge about *H. abdominalis* so that future conservation management would be easier to implement.

Acknowledgements:

From the beginning I was so grateful to work in the molecular biology and genetics laboratory, it has phenomenally intelligent Academics and incredibly helpful technicians and very welcoming students. My supervisor Ray Cursons gave me a very thorough overview of methods and was always willing to go along with whatever ideas I came up with. It was a very relaxed and enjoyable year, and I really appreciated your commitment to my 'out-there' project. I also appreciated the comments on analysis and formatting I received from my secondary supervisor Chrissen Gemmill as this assisted greatly in the professional layout of this Thesis. The people I never felt like I thanked enough were the SCUBA divers who went into the cold water with me (most often for me actually) and exercised their seahorse spotting skills. I believe anyone who hunts for seahorses would agree that I have been incredibly blessed to have achieved the sample sizes that feature in this study. An extension of this is that I am appreciative for the welcoming nature of all Aquariums managers that allowed me to come in and sample their display stock. A very honourable mention goes out to the Environment Waikato Environmental Initiatives Fund Council for believing that my project has merit to receive funding, and the same goes out to Mike Percy and the Western Underwater Research Team which also provided a lot of start up information for me, as did Chris Woods and Peter Smith - a big thank you to you for giving me the initial idea for this project. Last but not least come all the people who put up with hearing about the project day in and day out, my flatmates (which changed over the year) Esther, Lucy, Julie, Sehai and Paul. You let me do my work but were there for me in between – what more could I want! and Nala, I appreciate your company every day. I wouldn't even be here (in more ways than one) without my parents Petra and Reinhard – you are my rock, you may not really know what I have been learning and doing all year but have taught me all the most important things I will ever know, and I love you more than I say. The same goes for my incredibly wonderful partner Mark, who gives unwavering support and motivation to everything I do. It was a good experience, and even though I have gone on to cancer research for now, I won't lose my discovered passion for seahorses anytime soon.

Jennifer ☺

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References for Introduction

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1. Introduction

1.1. Distribution and Morphology

Hippocampus abdominalis is the only species of seahorse found in New Zealand but its range extends all the way to southeast Australia (Figure 1). Sightings have been recorded throughout the entire range of New Zealand from the Three Kings Islands in the north, to the Snares Islands in the south and as far east as the Chatham Islands. In Australia, they have been observed near Newcastle in New South Wales, through Victoria and Tasmania, all the way towards the Great Australian Bight in South Australia (Lourie *et al.*, 2004; Woods *et al.*, 2008). *H. abdominalis* is the largest seahorse species in the world, with juveniles at least 16 millimeters (mm) in size upon birth, and adults can reportedly reach a height up to 350 mm (Francis, 1996). It has been conjectured that adult *H. abdominalis* in New Zealand are slightly larger than those found in Australia (Francis, 1988).

Figure 1: Map of the world, depicting the location of Australia and New Zealand (within the bold square) and the geographical distribution of *H. abdominalis* (shaded in grey) between these two countries. Source: "The guide to the Identification of Seahorses" by (Lourie *et al.*, 2004).

Due to its markedly protruding abdomen, *H. abdominalis* has earned the non-scientific name of 'Big-belly' or 'Pot-belly' seahorse (Figure 2). Seahorses in general differ greatly in morphology from other bony fish. They have no pelvic or caudal fins, only a highly reduced anal fin, a curved trunk and a prehensile tail, and they carry their head at a 90° angle to the rest of their body (Foster, Vincent, 2004). A highly specialized vertebral system allows them to swim both vertically and horizontally (Storero, González, 2008), using the dorsal fin for propulsion and the pectoral fins on their head for steering and stabilization. Relative to most other fish they are slow moving and also do not have scales, rather their skin is stretched over bony plates that cause ridges which can look like rings around the trunk and tail (Foster, Vincent, 2004). It has been proposed that this acts as armour and also assists in axial bending of the tail (Hale, 1996). *H. abdominalis* in particular is a highly morphologically variable species mainly in regards to snout length, tail length and colouration (Woods, 2000).

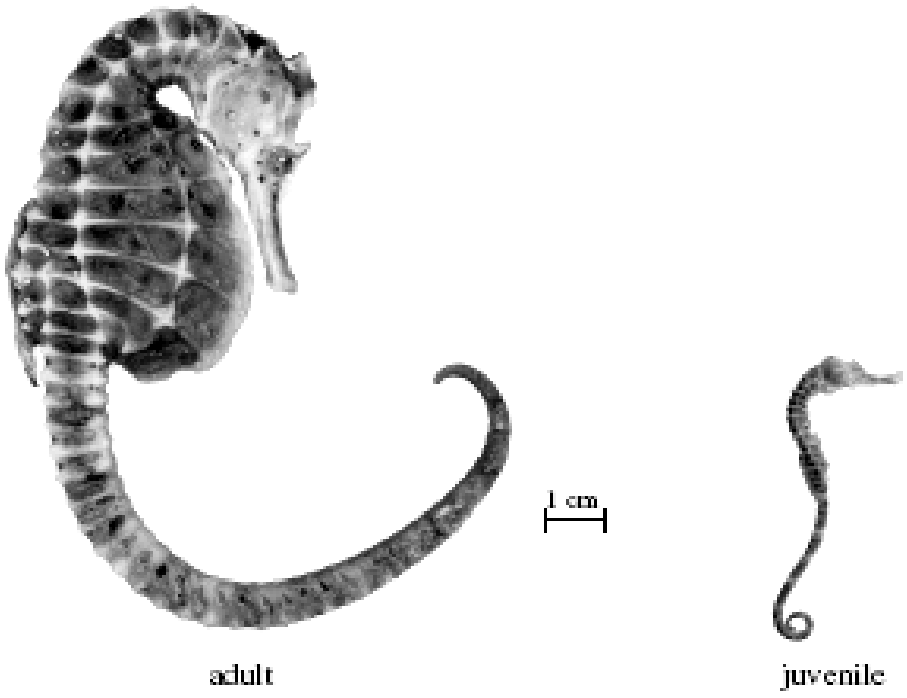


Figure 2: Illustrative example of a *Hippocampus abdominalis* adult (female) and juvenile, with a one centimeter scale for reference. Source: "The Guide to the Identification of Seahorses" by (Lourie *et al.*, 2004).

Seahorses are sexually dimorphic creature. The males receive the eggs from the females and then carry the embryos until birth, and so the most distinguishing sexual characteristic is their brood pouch. This emerges in *H. abdominalis* after approximately four months of age and it is usually lightly coloured or white with a yellow 'slash' above it (Woods, 2000). Apart from this *H. abdominalis* males are also described to be heavier marked with spotting, have a relatively longer tail and shorter snouts (Foster, Vincent, 2004; Lourie *et al.*, 2004) (Figure 3). Like many other seahorse species, *H. abdominalis* sometimes displays fronds (dermal filamentous growths from the head and trunk region), a characteristic that has not yet been linked with any determining factor(s) (Lourie et al., 2004). This species has been observed in a range of colours including black, yellow, grey, white, red, orange and various shades of brown (Mass, 2007; Woods, 2007), usually with dark spotting on the dorsal fin, head and trunk, and alternate light and dark banding on the tail (Lourie et al., 2004). It has been proven that *H. abdominalis* are able to alter their body colour to match their environment using the pigment cells in their skin (Mass, 2007), but there is very little published data describing the causes or mechanisms of this (Wardley, 2001). This ability is most likely for camouflage or communication as in some other fish species (Foster, Vincent, 2004).

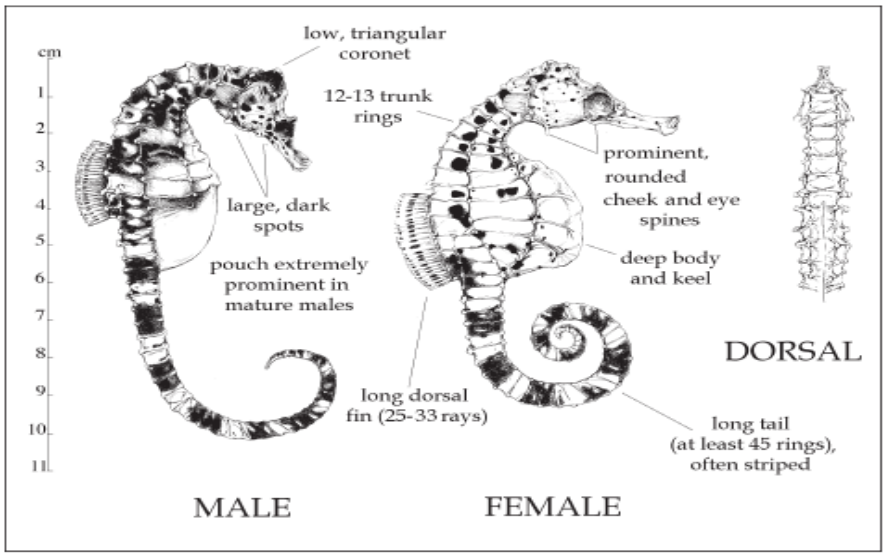


Figure 3: Illustration of a male and female *H. abdominalis* adult, displaying some of the sexually dimorphic traits such as the presence of a light coloured brood pouch, heavy spotting and shorter snout in males. Source: "The Guide to the Identification of Seahorses" by (Lourie *et al.*, 2004).

1.2. Taxonomy

Seahorses are a genus (*Hippocampus*) belonging to the family Syngnathidae, of the order Gasterosteiformes which is included in the class Teleost (bony fish), which comprises over 24,000 known species (Nelson, 1994). *Hippocampus abdominalis* is one of 33 species of seahorse (Lourie *et al.*, 2004), although this number has risen to 36 with three recently discovered species (Lourie, Kuiter, 2008). The family Syngnathidae also includes pipehorses and seadragons (Solegnathinae), pipefishes (Syngnathinae and Doryhampinae), and pygmy pipehorses (Hippocampinae) which are most closely aligned to seahorses as they also have a prehensile tail, the absence of a caudal fin and a fully enclosed brood pouch in males (Kuiter, 2000). The Syngnathidae are very distinguished evolutionary, having adapted away from the basic morphological features of all other fish. Wilson *et al.* (2001) believes a major factor in Syngnathid radiation was the matter of specialized male parental care. The oldest Syngnathid fossils date back to the Eocene (Patterson, 1993) and a pre-Tethyan origin (i.e. 20 million years ago) has been hypothesized for seahorses specifically, due to their present high diversity in south-east Asia (Fritzsche, 1980).

The taxonomy of *Hippocampus* has in the past been largely based on morphology. At the beginning it was a challenge to define a standard method for measuring such a strangely shaped fish, further complicated by the fact that within the genus there is little differentiation (Lourie *et al.*, 1999). Leeson (1827) was the first to classify the *H. abdominalis* species. Fowler (1908) gave them the synonym names of *H. agnesæ* and *H. bleekeri*, followed by McCulloch (1911) naming it *H. graciliformis.* Relatively recently, Kuiter (2001) raised speculations that *H. abdominalis* is comprised of multiple species, naming the South Australian and Victorian populations *H. bleekeri* and the Tasmanian and New Zealand populations *H. abdominalis*, with the claim that there may be more even still. This confusion occurred due to the highly morphologically variable nature of the species and has since been refuted with other meristic or genetic studies (Armstrong, 2001; Lourie *et al.*, 2004). The guide by Lourie *et al.* (2004) is now the accepted version of

Hippocampus taxonomy based on morphology. Taxonomic agreement is vital so the species can be researched and communicated about in the scientific community, government, industry and conservation initiative groups such as the United Nations Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and the International Union for Conservation of Nature and Natural Resources (IUCN) (Woods *et al.*, 2008).

1.3. Behaviour

H. abdominalis live in low exposure coastal habitats (i.e. bays, harbours and estuaries) and spend much of their time anchored to holdfasts such as macroalgae or artificial structures (Kuiter, 2001). Artificial structures include concrete and wooden pilons such as under wharves, crayfish pots (Woods, 2007), anti-predator cages (Marshall, 2004) or netting (Clynick, 2007), and mussel farms (Carbines, 1993; Francis, 1988; Morrisey *et al.*, 2006). They are usually seen within 40 meters of the surface (Francis, 1988) but are also sometimes found as bycatch by various types of offshore trawlers (F. Lichtwark, personal communication). *H. abdominalis* adults exhibit some site fidelity *in situ* (Van Dijken, 2001) but are also known to be capable of travelling hundreds of meters a day (Martin-Smith, Vincent, 2005). They are usually seen as single individuals at low density but occasionally are found exhibiting group social structure (Martin-Smith, Vincent, 2005; Woods, 2000). Irrespective of density, promiscuous social behavior has been observed *in situ* (Martin-Smith, Vincent, 2005) and *ex situ* (Woods, 2000), but genetic analysis confirms reproductive monogamy at least over one brooding cycle (Wilson, Martin-Smith, 2007). Foster and Vincent (2004) speculate that the higher mobility of this species facilitates more encounters between individuals, and could be a cause of the more promiscuous lifestyle relative to other seahorse species.

It takes approximately thirty days for *H. abdominalis* males to brood their offspring, after which several hundred fully independent juveniles are released and start their two to four week pelagic phase (Wilson, Martin-Smith, 2007; Woods, 2000). During this stage it is assumed that the juveniles spend the entire time attached to floating macroalgae which acts for protection and prey aggregation (Holmquist, 1994) as this has been observed in entirety *ex situ* (Woods, 2000) and sporadically *in situ* (Gomon, Neira, 1998; Hickford, 2000; Kingsford, Choat, 1985). This pelagic phase is assumed to be the main dispersal mechanism for seahorses (Foster, Vincent, 2004). The degree to which pelagic young are at the mercy of ocean currents before

settlement depends on their sensory and locomotor capabilities and behaviour (Montgomery *et al.*, 2006), very little of which is known about in regards to seahorses. It is very likely that the pelagic phase is important for juvenile survival as there are many reports of adults consuming their young *ex situ* (e.g. Woods, 2007) and even *in situ* (e.g. Storero, González, 2008).

1.4. Prey and predators

Seahorses are highly inconspicuous visual ambush predators, sometimes keeping their bodies completely stationary apart from their independently swiveling eyes which track prey that, once within range, is sucked up their tubular snout. *H. abdominalis* have been observed to also be capable of dismantling their prey (Woods, 2002) and suppressing the release of kairomones to remain chemically inconspicuous (Cohen, Ritz, 2003). *H. abdominalis* feeds upon a wide range of epifaunal and planktonic prey, such as invertibrates, fish fry and particularly crustaceans such as copepods, amphipods, isopods, carideans, and also euphausid and mysid shrimps, as per availability (Woods, 2002).

Bony plates do not seem to deter many predators as *H. abdominalis* have been found in the stomachs of crustaceans (e.g. rock lobster), larger fish (e.g. skates, red cod, trumpeter, blue cod, ling, sea perch (Graham, 1974) and banded wrasse (Denny, Schiel, 2001)), and birds (Kuiter, 2000). Natural predation and pathogens (e.g. Alcaide *et al.*, 2001) of seahorses is a largely uninvestigated subject, and the effects of it in combination with exploitation remain unknown (Woods *et al.*, 2008).

1.5. Threats and conservation

Seahorses worldwide are listed as either endangered, vulnerable, or data deficient. Their feature on the IUCN red list of threatened species and CITES Appendix II suggests the urgency and scale of their neglected situation (Koldewey, 2005; Sanders *et al.*, 2008). The continuing decline in wild seahorse numbers is mainly due to the huge trade in dried seahorses for traditional medicine, but also the aquarium and curiosity trades. The traditional medicine trade was found to involve 32 countries and more than 16 million seahorses annually before 1996 (Vincent, 1996), a reassessment in 2002 estimated these figures to have risen to 77 countries and 24 million animals respectively. Additionally to this, habitat degradation and pollution of coastal areas pose further threat (Koldewey, 2005). Unfortunately for the *H. abdominalis* species, they have the capability to possess all the traits that are most highly valued in the traditional medicine trade: large size, pale colouring and smooth skin. Historically, they have not featured in the trade on a large scale but aquaculture efforts have been high since the 1990's to supply demand and lift pressure on other species. So far nearly all have failed due to problems with poor breeding and high juvenile mortality, with no resolution as of yet within New Zealand (Wright et al., 2007).

Foster and Vincent (2004) claim that wild seahorse populations are faring so poorly because some of their life history traits render them vulnerable; such as their low mobility (i.e. site fidelity), low distribution (i.e. difficulty in finding new mates) and monogamous mating patterns. However, Curtis and Vincent (2006) point out that seahorses also have some life history traits that should confer resilience, such as rapid growth and maturation. *H. abdominalis* are not as rigidly monogamous as other seahorse species, and they can breed all year round with brood sizes that can reach over a thousand juveniles, thus they display some resilience (Woods, 2000).

A nationwide survey of seahorse sightings was carried out voluntarily between the years 2000 and 2008 by an Auckland SCUBA dive club named the 'Western Underwater Research Team' between 2000 and 2008. This 'Survey Seahorse 2000' project is the only documented effort for monitoring seahorse abundance in New Zealand, and the organizers report a decline in sightings both by the public and recreational divers over this time period (M. Percy, personal communication). Martin-Smith and Vincent (2005) monitored three sites in the Derwent estuary of Tasmania between 2001 and 2004, and observed a 79-98% decline in abundance. This was assumed to be caused by either an invasive species or disease as there were no observations of direct exploitation or change in physiochemical conditions.

Both Australia and New Zealand are CITES parties, and in Australia Syngnathids have been protected under the Environmental Protection and Biodiversity Conservation Act since 2001 (Pogonoski *et al.*, 2002), but in New Zealand *H. abdominalis* is losing ground, having recently been rejected from entry into the quota management system and thereby were also removed from Schedule 4C of the Fisheries Act of 1996 (New Zealand Ministry of Fisheries, 2008). Both countries have minimal reporting and it is unknown how common bycatch or other forms of unregulated take are; this is likely their largest threat (Woods *et al.*, 2008).

1.6. Molecular markers and genetic diversity

Historically, morphology has had the largest influence on taxonomic classification of seahorses, but genetic analysis provides the opportunity of neutral markers to provide another perspective on evolutionary divergence (Wilson, 2006). A wide variety of molecular markers (e.g. mitochondrial DNA, nuclear microsatellites, nuclear genes, intergenic spacers, allozymes) are available to investigating a variety of levels of genetic diversity. The different types of markers have different constraints and modes of evolution, and therefore different (often changeable) rates of mutation and levels of polymorphism (Hellberg, 2007). Markers with low levels of polymorphism (e.g. nuclear genes) usually have high sequence conservation and can reveal evolutionary relationships, but are not good for recent or lower level analyses, whilst markers with higher levels of polymorphism (e.g. microsatellites) can provide information mainly on population history and even relatedness and gene flow (Anne, 2006; Hellberg, 2007; Wan *et al.*, 2004). All markers are based on the genetic sequence but the techniques used can provide a varying quantity of information. Studying sequence divergence by DNA sequencing is the most expensive yet direct method, but there are many other indirect methods that can provide meaningful data also (e.g. SSCP, RFLP, allozymes, etcetera) (DeWoody, 2005). Choosing which molecular marker to use is highly dependent on the biological question to be answered but inherently involves three steps: (1) choosing the level of variability required; (2) choosing the DNA region that provides the appropriate nature of information (e.g. considering the neutrality, mode of inheritance, recombination rate, ploidy); and lastly (3) deciding on the technique that is best employed based on the availability of resources and researcher experience (Anne, 2006). When trying to estimate true historical events, statistical power comes from the number of markers utilized (i.e. more is better) and their degree of genetic variability (Dewoody, 2005).

1.6.1. Mitochondrial DNA markers

In fish, the mitochondrial DNA (mtDNA) is additional and separate to the chromosomal DNA. It contains 36 genes as well as non-coding sequence, the largest of which is the control region which regulates the replication and transcription of the mtDNA molecule. This region has three identified domains, the central domain which is highly conserved, and hyper-variable region one and two. These have a high mutation fixation rate and evolve 5-10 times faster than single-copy nuclear DNA (scnDNA) (Lee et al., 1995). For seahorses, the only theorized estimate of mtDNA evolution is 1-4% sequence changes per million years (e.g. Casey *et al.*, 2004; Teske *et al.*, 2004). Mitochondrial DNA is well known for its haploid mode of maternal inheritance (i.e. no recombination), and therefore all mtDNA markers reveal a female biased story of evolutionary history. It is rare, but problems can arise due to the presence of nuclear mtDNA pseudogenes. However, since it is a very easy marker to work with, it is very commonly used (White et al., 2008).

MtDNA is a useful auxiliary marker to nuclear DNA. An example of the utilization of multiple markers was demonstrated by Armstrong's (2001) work in delineating the *H. abdominalis* species. Even though the number of samples and outgroups were small, he utilized six functional protein allozyme loci to expose a high genetic identity within the species (0.994-1.000), as opposed to between species (0.000-0.667), and 357 base pairs of the mitochondrial cytochrome b gene to reveal low divergence within the species (0.28-2.24%), as opposed to between species (8.68-22.13%)). It could be concluded that *H. abdominalis* varied more within than across the proposed species lines and that there is only one species of *H. abdominalis* within the geographic range currently described. Cytochrome b has been the most popular marker for investigating the genetic diversity and evolutionary history of *Hippocampus* species. For example: Lourie *et al.* (1999) were able to delineate *Hippocampus* species in the Vietnam region; Lourie and Vincent (2004) discovered that two distinct lineages (2.9% divergence) exist in seahorse species of the southeast Asia region; Casey *et al.* (2004) revealed intraspecific distances of up to 5.9% and

interspecific distances of 23.2% between 22 species; and Teske *et al.* (2004) used cytochrome b sequences in combination with nuclear genes for a similar purpose. Both the studies by Casey *et al.* (2004) and Teske *et al.* (2004) revealed that the *H. abdominalis* is sister to *H. breviceps* and part of an ancient lineage away from all other seahorse species. Although it was Jones *et al.* (2003) who discovered that these two species most likely split due to sympatric speciation via size assortative mating (Vincent, Sadler, 1995). It seems pertinent to mention that in the future, cytochrome b is likely be replaced by cytochrome oxidase 1 due to the International DNA barcoding initiative which is proposed to be a universal taxon identification technique (Hebert *et al.*, 2003; Ratnasingham, Hebert, 2007). Cytochrome b and cytochrome oxidase 1 both code for protein products which are involved within the mitochondria converting the chemical energy ATP and give a similar phylogenetic signal. The DNA barcoding idea shows merit for fishes. Ward *et al.* (2005) "barcoded" 207 species of Australian fishes using 655 base pairs of cytochrome oxidase 1 and was able to successfully differentiate between all of them. It is projected that representative cytochrome oxidase 1 sequences for all fish species will be available by 2012 (Ratnasingham, Hebert, 2007). Meanwhile, the most popular marker for studying population level genetics has been the mitochondrial control region. Mainly the rapidly evolving right domain has been utilized. For example: Teske *et al*. (2003) used it to detect high diversity and population structure between three populations of *H. capensis*; and Teske *et al.* (2005) used the same region for evaluating the level of migration and speciation in six populations of the global *H. kuda* complex.

1.6.2. Nuclear Genes

Due to low phylogenetic signal at the species level, nuclear genes have only rarely been used at the species level of *Hippocampus*. Teske *et al*.'s (2004) study utilized 640 base pairs of the first intron of the S7 ribosomal protein one gene, as well as 188 base pairs of the partial intron-exon region of the Aldolase gene but found only very little variation between species, and mtDNA data was heavily relied upon to yield a

species tree. Non-coding or neutral nuclear DNA such as intergenic spacers or microsatellites would seem like a more viable option in this regard.

1.6.3. Microsatellite markers

Microsatellite loci are diploid nuclear DNA sequences containing short (1-6 base) repetitive elements where the copy number varies among alleles (5-100 times per locus). They are considered to be codominant and evolving neutrally, unless they are tightly linked to a functional gene, and therefore display a high rate of mutation (100-1000 times faster than scnDNA). The mutational mechanism is still disputed, but likely to be 'slippage': where the polymerase enzyme accidentally adds or deletes a random number of repeats to the new DNA strand during replication. It is proven that longer alleles bring this event about more often and therefore mutate more rapidly (see Petit *et al.*, 2005). Two models are commonly considered to be representative of microsatellite evolution: the infinite allele model (IAM) by Kimura and Crow (1964) which proposes a mutation can involve any number of tandem repeats but always results in an allelic state not previously encountered, and the stepwise mutation model (SMM) by Kimura and Ohta (1978) which proposes the loss or gain of a single tandem repeat to have equal probability (if the model is symmetrical). The SMM is considered to be more likely to be representative of true microsatellite evolution (Pritchard, Feldman, 1996). These models are used to extrapolate observed data into population parameters, but it seems that true mutational patters often deviate significantly from both models (see Di Rienzo *et al.*, 1994; Estoup *et al.*, 2002).

It is important to mention that microsatellite scoring needs to be rigorously assessed for quality, the biggest problem is incorrect scoring which can occur either due to the technique or human error. There are many ways for errors to originate from the process. For example, slippage or inconsistent additions of Adenine nucleotides to the 3' end during PCR can give varying allele reads, or the nature of the primers can cause preferential amplification of an allele (i.e. allele drop out) or inhibition of

amplification (i.e. null allele) usually due to mutations in the binding sequences. Both bring about the illusion of excess homozygotes. Conversely, if contamination is present (i.e. misprinting occurs) then an illusion of multiple alleles arises (Wan *et al.*, 2004).

In *Hippocampus*, microsatellite markers have most commonly been utilized in the study of parentage and breeding systems. Jones *et al.* (1998) used four loci from 15 pregnant *H. angustus* males to show that their offspring resulted from monogamous pairing, and Jones *et al.* (2003) also investigated parentage but also provided information on the genetic diversity of *H. subelongatus* (i.e. three loci yielded a range of 24-42 alleles from approximately 100 adults). Pardo *et al.* (2007) isolated 12 microsatellite loci from *H. guttulatus* and 2-15 alleles were revealed from a sample size of 32 individuals. Galbusera *et al.* (2007) detected 15 loci and compared the diversity of a captive bred and wild collected group of *H. capensis*, in this case 2-8 alleles were found in the captive group which had a sample size of 25 and 3-18 alleles were found in the wild group which was comprised of 45 individuals. These markers were found to amplify in *H. abdominalis* also. Wilson and Martin-Smith (2007) managed to isolate four microsatellite loci for Australian populations of *H. abdominalis* specifically and discovered a high degree of polymorphism (25-35 alleles) in a sample size of 112. So far, their study is the only one that has employed microsatellites for *H. abdominalis* specifically.

1.7. New Zealand as a Habitat

The country of New Zealand consists of a geographically isolated archipelago of over 700 islands, spanning a large latitudinal range (29° south to 52° south). Many diverse features exist along its extensive coastline (18200 km) creating a variety of habitats for coastal flora and fauna. In combination with well-characterized oceanography, this environment becomes a valuable system for marine biogeographical research (Waters, Roy, 2004). There are two major islands (the North and South Island) which intersect the general west to east Tasman current flow. It is the East-Australian Current which brings warm, nutrient rich Tasman water and most of this is distributed over the northeast of New Zealand, with a small offshoot making up the West Auckland Current flowing down the west coast of the upper North Island. The Westland Current flows along the west coast of the South Island, the course depending on wind direction. The Southland Current takes the cold fresh Subantarctic water from under the South Island up the east coast. Once on the east side the water flow is directed towards the centre of New Zealand before being expelled to the east (Figure 4). This system creates rough coastal conditions along the entire west coast and forms permanent eddies on the more sheltered north-east coast. All currents are influenced by the continental shelf topology and vary with wave, tide and wind direction and strength, creating a very chaotic and complex system (De Lange *et al.*, 2003; Harris, 1990; Laing, Chiswell, 2003)

The capability of these currents to disperse pelagic young of marine organisms always depends on the intensity of the conditions and the duration of ocean swell exposure (Lester, Ruttenberg, 2005; Montgomery *et al.*, 2006). New Zealand weather is highly variable throughout all seasons and since *H. abdominalis* can breed all year round the juveniles must have the resilience to withstand this.

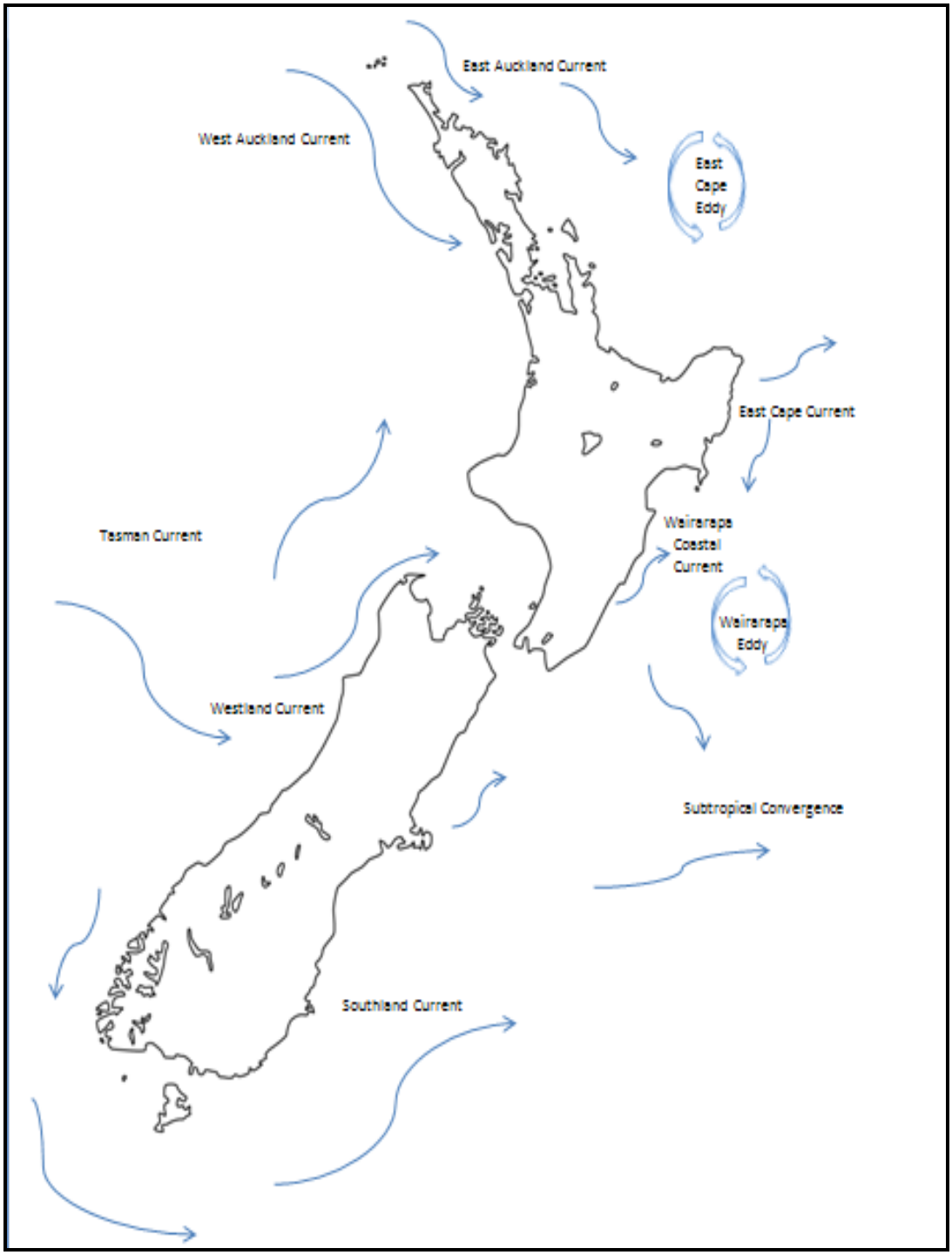


Figure 4: Map of New Zealand and the major ocean currents that surround it in a general west to east flow. Source: based on the information provided in (De Lange *et al.*, 2003).

1.8. Phylogeography in New Zealand marine organisms

Diversity, population structure and dispersal potential has never been studied in the New Zealand seahorse, but this has been done for other New Zealand marine organisms that undergo pelagic phases before settling as adults. Population structure in fishes is usually very weak due to high population numbers and high gene flow which is a product of the unlimited nature of the ocean (DeWoody, Avise, 2000). However, some phylogenetic structure has been inferred from the analysis of the cytochrome b gene in limpets (Goldstien *et al.*, 2006) which have a 3-11 day pelagic phase, the NADH IV region (also in the mtDNA) of the greenlip mussel (Apte, Gardener, 2002) which has a 3-5 week pelagic phase, and microsatellite markers in the snakeskin chiton (Veale, 2007) which have a 2-3 week pelagic phase. All of these studies have found a disjunction at the top of the South Island, perhaps due to upwelling, or alternatively in the Cook Strait, perhaps due to the turbulent conditions that the topology induces (Apte *et al.*, 2003; Goldstien *et al.*, 2006; Veale, 2007; Waters, Roy, 2004). The geographical scale and length of the pelagic phase have to be considered when comparing studies and these organisms do exhibit varying lifestyles so the comparative power is limited. At two to four weeks the *H. abdominalis* pelagic phase is relatively long. This would increase the degree of gene flow among populations and make it surprising to find any population structuring (P. Ritchie, personal communication). Ross *et al.* (in press) recommends for the selection of appropriate markers for testing specific hypotheses and that collaborations with physical oceanographers and modelers will aid in understanding of marine ecosystem structuring. Apte and Gardener (2002) calculated the theoretical travel distance of mussel spat to be approximately 926 kilometers in total (or 45 kilometers a day) over a three week pelagic phase but cautioned this may be an overestimation due to the changeability of currents; Stephens *et al.* (2006) used computational modeling to infer about the displacement of abalone larvae, which have a 4-8 day pelagic phase, and found that in calm/normal tidal currents this should result in distances of 4-50 km in total, depending on conditions.

1.9. Objectives

H. abdominalis is a unique species as it is the only species of seahorse found in New Zealand waters, and because it is the largest species (size-wise) in the world. Its taxonomy has taken a long time to delineate due to a high degree of morphological variability, and only very little is known about their dispersal capabilities. This study aims to address mainly the diversity of the species in New Zealand as a whole, but also includes some investigation into population differentiation and dispersal potential. This will require the collection of as many samples as possible, preferably from a wide range of locations and predominantly *in situ*. Two main tools will be used to pursue this aim: morphology and genetics. To describe the morphological diversity, a variety of continuous and discrete variables will be measured and summarized as baseline data for the species in New Zealand. This will also feature a component on sexual dimorphism. The genetic aspect will be elucidated via two types of markers which will hopefully provide information at the population and gene-flow level. Three sequences from the mitochondrial DNA (i.e. cytochrome b, cytochrome oxidase 1 and the control region) and four microsatellite loci will be utilized to describe the degree of diversity and nature of population structure in *H. abdominalis* within New Zealand. It is hoped that this data will also reconfirm that there really is only one species within this area and that this will contribute towards lifting *H. abdominalis* off the data deficient status so that conservation management can be applied when necessary in the future.

2. Materials and methods

2.1. Sample collection

Locations of seahorse habitation were identified mainly via extensive enquiries into previous sightings by commercial and recreational SCUBA divers, Ministry of Fisheries and Department of Conservation staff, through the literature review and the results of the 'Survey Seahorse 2000' project run by the Western Underwater Research Team. Ten *in situ* sampling sites and six Aquariums yielded a total of 169 individual *H. abdominalis* representations (Table 1) from around New Zealand. The ten *in situ* sample sites were explored (one was revisited due to easy accessibility) via SCUBA diving, over the course of the 2008 year 96 seahorses were caught individually by hand on these expeditions and subsequently released after sampling. A detailed map and description of each site is available in Appendix A. One site (Raglan) was revisited due to easy access, but samples from the second expedition, as well as the sample from the Titahi Bay area, were not included in pair-wise population comparisons as their sample sizes varied too far from those of the other populations. Aquarium specimens were still deemed acceptable for inclusion as all originated from New Zealand waters and any juveniles produced at these establishments are usually released into local waters, thereby still contributing to the overall gene pool. Three Aquariums – the Southern Encounter Aquarium in Christchurch, the Westpac Aquarium in Portobello, and the Stewart Island Aquarium – had previously obtained wild *H. abdominalis* specimens from their respective local waters for the purpose of display and kindly allowed their specimens (total n = 27) to participate in this study. Southern Encounter also provided one frozen specimen as an unexpected death occurred. The Westpac Aquarium also held six other seahorses with uncertain origins (putatively Dunedin or the Marlborough Sounds). These, in combination with the specimens from three additional Aquariums – the National Aquarium in Napier, Seahorse World in Picton, and Kelly Tarltons Underwater

World™ in Auckland – which also had no confirmed origins (being a mixture of locally caught and purchased individuals from the recently closed Seahorse Farm in Napier (which sourced all breeding stock from the Hawke's Bay), were sampled also (total n = 46) to add to the diversity aspect of the study.

The *in situ* sampling was permitted under Section 6(b) of the Ministry of Fisheries Special Permit (386) held by the University of Waikato. This permit is pursuant to Section 97(1)(a)(i), (ii) and (iii) of the Fisheries Act 1996. The handling of all seahorses was authorized by the University of Waikato Animal Ethics Committee (application protocol number 705). Sampling consisted of obtaining a photograph (out of water with a centimeter ruler for reference), and the non-destructive taking of a tissue sample (i.e. a 1-2 mm^2 fin clipping with surgical scissors) from the lower edge of the dorsal fin. This method has been used previously to obtain tissue samples from seahorses for genetic analysis (e.g. Galbusera *et al.*, 2007; Jones *et al.*, 2003; Lourie *et al.*, 2005; Teske *et al.*, 2003; Wilson, Martin-Smith, 2007), also see (Lourie, 2003a). Each sample was given a code upon collection in the form of two letters (e.g. 'AA', 'AB', 'AC') for identification and coupling the photo to the DNA sample. It was impossible to monitor the wellbeing of the *in situ* sampled specimens, but all specimens were handled similarly and no subsequent adverse effects were reported by any of the Aquarium managers. The geographical origins of the ten wild collected (*in situ* and *ex situ*) populations which will be used for the pairwise-population comparisons can be seen in Figure 5.

Table 1: 169 samples (comprised of 90 males and 79 females) were acquired from three types of circumstances*: in situ* (wild collected), *ex situ* (wild collected) and *ex situ* (uncertain origins). Reported for each group/population are the geographical origins (if available), the number of samples (n), the person/establishment in charge of the collections and the date the samples were taken along with a guide to show whether those samples were included in the pair-wise population comparisons.

Geographical origins	**Code**	**n**	**Collector:**	**Sample date**	**Analysis guide:**
In situ, wild collected:					
Raglan	RAG	13	J E Nickel	4 Feb 2008	Included in population comparisons
New Plymouth	NPY	10	J E Nickel	25 Apr 2008	Included in population comparisons
Whangateau	WHA	5	J E Nickel	24 Mar 2008	Included in population comparisons
Tauranga	TAU	8	J E Nickel	28 Mar 2008	Included in population comparisons
Napier	NAP	8	J E Nickel	1 Aug 2008	Included in population comparisons
Wellington	WEL	10	J E Nickel	23 May 2008	Included in population comparisons
Jackson Bay	JAB	5	J E Nickel	30 Oct 2008	Included in population comparisons
Dunedin	DUN	4	J E Nickel	29 Oct 2008	Included in population comparisons
Raglan2	RAG2	32	J E Nickel	26 Oct 2008	Excluded from population comparisons due to (n>20)
Titahi Bay	TIB	1	J E Nickel	23 May 2008	Excluded from population comparisons due to (n<5)
Ex situ, wild collected:					
Stewart Island	STW	9	R Dennis	2 Nov 2008	Included in population comparisons
Christchurch	CHC	14	D Bradshaw	1 Sept 2008	Included in population comparisons
Dunedin	DUN	4	Portobello Aquarium	30 Oct 2008	Included in population comparisons
Ex situ, uncertain origins:					
N/a	MPQ	6	Portobello Aquarium	30 Oct 2008	For diversity analyses only due to uncertain origins
N/a	MSQ	8	Seahorse World	2 Nov 2008	For diversity analyses only due to uncertain origins
N/a	NAQ	18	National Aquarium	3 Apr 2008	For diversity analyses only due to uncertain origins
N/a	KTQ	14	Kelly Tarltons	16 Apr 2008	For diversity analyses only due to uncertain origins
TOTAL	169 (90 males and 79 females)				

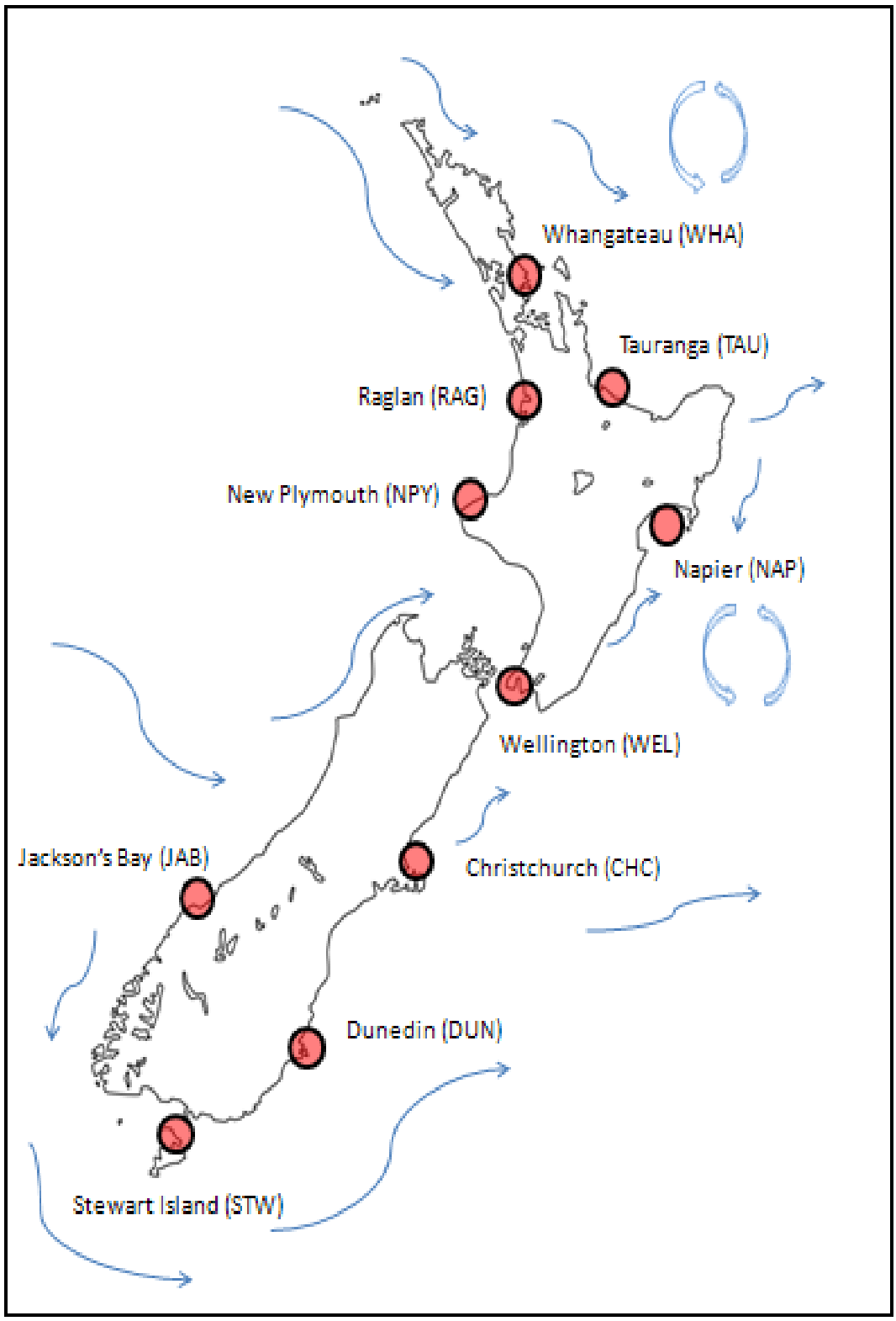


Figure 5: Map of New Zealand showing the origins and codes of the ten wild collected *H. abdominalis* populations (red shaded circles) and directional indications of the major ocean currents (blue arrows).

2.2. DNA extraction

The fin clipped tissue samples were stored in labeled 1.7 ml clear Axygen microtubes containing 1 mL of 100% ethanol for transport from the field site to the laboratory. Genomic DNA was extracted individually from each tissue sample by the following method which is based on the protocol by Sambrook *et al.* (1989) and using the solutions as they are given in Appendix C.

1. Ethanol was removed by pipette, leaving only the tissue sample behind, which was air dried in the 37°C incubator for 5 minutes.
2. 350 µL of SDS-EDTA-Tris Lysis solution was added, along with 10 µL Proteinase K (60 ng/µL) and left to digest in the 37°C incubator overnight.
3. Once digested, 360 µL of 5M Lithium Chloride was added, followed by 720 µL of Chloroform (100%), following which samples were continually inverted for 30 minutes.
4. Once well mixed, the samples were spun for 10 minutes at 13200 rotations per minute (rpm) in a 5415D Centrifuge (Eppendorf AG), and the top layer extracted by pipette into a new tube with the corresponding label.
5. Then, 600 µL of Isopropanol (100%) was added to allow the DNA to precipitate out of solution for (at least) 1 hour.
6. Following this, the samples were spun for 10 minutes at 13200 rpm for the DNA to condense as a pellet at the base of the tube and then the supernatant was removed.
7. The DNA pellets were washed once by the addition of 1 ml of Ethanol (70%) and spun for 5 minutes at 13200 rpm.
8. Following this, the Ethanol was removed by pipette and the pellets air dried in the 37°C incubator for 5 minutes.
9. The pellets were subsequently resuspended in 50 µL of Tris-EDTA buffer (10mM:1mM) and stored at 4°C.

To assess the quality of the DNA extraction, 5 µL of each sample (along with 1 µL of gel loading dye) were run on an UltraPure™ agarose gel (1%) containing 0.5 µL ethidium bromide (10mg/ml), in Saline-Borate buffer, at 110 volts for a duration of 30 minute on an Owl Separation Systems B1A electrophoresis system using a Lighting volt™ OSP-250L power supply (Owl Scientific Inc.). A 100 base pair ladder (Invitrogen) was loaded also to enable sizing of the fragments for quality control. The outcome was visualized using ultra-violet light and captured using a COHU 4910 Series Monochrome CCD Camera (COHU Electronics Division, Inc.). To assess the concentration of each DNA extract, 1 µL was tested on the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies Inc.) to give a quantitative value. All samples were diluted to have a final working concentration of 20-50 µL/ng and stored at 4°C.

2.3. PCR and DNA Sequencing/Genotyping

2.3.1. Mitochondrial DNA Sequence Markers

Three mitochondrial DNA markers cytochrome b, cytochrome oxidase 1 and the mitochondrial control region were amplified via polymerase chain reaction (PCR) using the primers described in Table 2. Three to five samples were selected at random from each population for each marker as to maximize participation for the diversity analysis and have all individuals contribute in representing their population. A spreadsheet outlining which marker was sequenced for which samples is available in Appendix B. Reactions were in a total volume of 50 μL: each had a final concentration of 1x PCR buffer, 2 mM Mg^{2+}, 250 μM of each dNTP, 0.4 mM primer set (forward and reverse), 0.5U Hot-start iSTAR *Taq* (iNtRON Biotechnology Inc.) and 20-50 ng of DNA template.

Table 2: Descriptions of the forward (F) and reverse (R) primers utilized for the amplification of the three mitochondrial markers: cytochrome b (CYTB), cytochrome oxidase 1 (COX1) and mitochondrial control region (MtCR), and their sources.

MtDNA marker	**Primer pairs**	**Source**
CYTB	F: SHF 5'-CTACCTGCACCATCAAATATTTC-3' R: SHR2 5'-CGGAAGGTGAGTCCTCGTTG-3'	(Lourie, Vincent, 2004)
COX1	F: FishF1 5'-TCAACCAACCACAAAGACATTGGCAC-3' R: FishR1 5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'	(Ward et al., 2005)
MtCR	F: HCAL2: 5'-CACACTTTCATCGACGCTT-3' R: HCAH2: 5'-TCTTCAGTGTTATGCTTTA-3'	(Teske et al., 2003)

The PCR was performed in a Peltier Thermal Cycler PTC-200 (MJ Research Inc.): After an initial step of 95°C for 2 minutes (to activate the *Taq* polymerase), samples were subjected to 30 cycles of 95°C for 20 seconds, 50-60°C for 20 seconds (annealing temperature depended on the primer pair for each marker: 50°C was used for amplifying the mitochondrial control region, and 60°C was used for both cytochrome b and cytochrome oxidase 1), and 68°C for 30-60 seconds (extension time depended on the primer pair for each marker: 30 seconds was used for

amplifying the mitochondrial control region as the primer pair used for this marker is expected to yield a fragment under 500 DNA bases, and 60 seconds was used for both cytochrome b and cytochrome oxidase 1 as their primer pairs are expected to yield fragments of 500-1000 DNA bases). At the end of the 30 cycles, a final extension step of 68°C for 5 minutes finished the process.

To determine if the amplifications were successful, 10 µL of each of the PCR products (along with 1 µL gel loading dye) were run in the same manner as described at the end of section 2.2. The remaining 40 µL were processed, via the following polyethylene glycol-sodium chloride precipitation method based on the protocol by (Sambrook et al., 1989) and using solutions as described in Appendix C.

1. Add an equal volume (40 µL) of polyethylene glycol-sodium chloride solution to the PCR product solution and allow the DNA to precipitate for (at least) 10 minutes.
2. Then spin at 13200 rotations per minute (rpm) for 10 minutes on a 5415D Centrifuge (Eppendorf AG) to condense the precipitated DNA as pellets in the tubes.
3. Remove the supernatant by pipette, and add 200 µL of Ethanol (100%) and spin at 13200 rpm for 5 minutes.
4. Remove the supernatant again by pipette, and add 200 µL of Ethanol (70%) and spin at 13200 rpm for a further 2 minutes.
5. Following these washes, remove all the Ethanol with a pipette and air dry in the 37°C incubator for 2 minutes.
6. Subsequently resuspend the pellets in 15 µL Tris-EDTA buffer and store at 4°C.

The following steps were carried out at the University of Waikato DNA Sequencing Facility: DNA templates were prepared using Big Dye v3.1 terminator chemistry (Applied Biosystems) and subsequently sequences were resolved using a 3130xl

Genetic Analyzer System fitted with 50 cm capillary arrays (Applied Biosystems) loaded with POP-7 polyacrylamide matrix (Applied Biosystems).

2.3.2. Nuclear DNA Microsatellite Markers

Four nuclear microsatellite loci (Habd3, Habd6, Habd9 and Habd7) were amplified by PCR, using the primers designed by Wilson and Martin-Smith (2007). In each set only the forward primer was fluorescently labeled (Table 3). An attempt was made to use different probes for each locus but as it was unlikely Habd3 and Habd9 would overlap, these were given the same label. All four loci were amplified separately for each sample, and then multiplexed via dilution before genotyping. Reactions were in a total volume of 20 μL: each had a final concentration of 1x PCR buffer, 2 mM Mg^{2+}, 250 μM of each dNTP, 0.4 mM primer set (forward and reverse), 0.5U Hot-start iSTAR *Taq* (iNtRON Biotechnology Inc.) and 20-50 ng of DNA template.

Table 3: Descriptions of the forward (F) and reverse (R) primers utilized for the amplification of the four microsatellite loci (Habd3, Habd6, Habd9, Habd7) designed by Wilson and Martin-Smith (2007).

Microsatellite Marker	**Primer pairs**
Habd 3	F: 5' – 6-FAM-CAGGTTATTAGTGCTGGGACAA – 3' R: 5' – GGGACAAGGTGGAAAGTGAA – 3'
Habd 6	F: 5' – HEX-GCGTGTGTCTTCAACGTGTC – 3' R: 5' – GCTCCATGCTTCCTGTGACT – 3'
Habd 9	F: 5' – 6-FAM-GCTAATTGCGGATACCCAGA – 3' R: 5' – TAGTCCCTCACCTCCCAAAA – 3'
Habd 7	F: 5' – TAMRA-GGCGTTTGTGTACCGCATGTC – 3' R: 5' – TCCGTCAGGCGACACAC – 3'

The PCR was performed in a Peltier Thermal Cycler PTC-200 (MJ Research Inc). After an initial step of 95°C for 2 minutes (to activate the *Taq* polymerase enzyme), samples were subjected to 40 cycles of 95°C for 20 seconds, 55-60°C for 20 seconds (annealing temperature depended on the marker and its primer pair: 55°C was used for Habd9, and 60°C was used for Habd3, Habd6 and Habd7), and 68°C for 30 seconds (as all amplified fragments were under 500 DNA bases in size). It is unusual to use so many cycles for microsatellite marker amplification but due to the multiplex dilution it was found that 40 cycles were required to give a consistently strong signal. A final extension step of 68°C for 5 minutes finished the process.

To determine if the amplifications were successful, 10 μL of each PCR product (along with 1 μL gel loading dye) were run in the same manner as described at the end of section 2.2. The remainder did not require further purification due to multiplexing, which involved adding 1 μL of each amplified product to 6 μL of MQ water, giving a total volume of 10 μL.

Genotyping was carried out at the University of Waikato DNA Sequencing Facility: 2 μL of each multiplexed pooled stock was added to a plate well containing 0.25 μL of GENESCAN - 1200 LIZ size standard and 7.75 μL of HiDi Formamide (Applied Biosystems). Genotypes were then resolved using a 3130xl Genetic Analyzer System fitted with 50 cm capillary arrays (Applied Biosystems) loaded with POP-7 polyacrylamide matrix (Applied Biosystems) using the GS1200LIZ version 2.0 run module.

2.4. Editing and quality control

2.4.1. Morphology

The photographs were taken with a variety of digital cameras as subject to availability: the Kodak Easyshare CX7300 (3.2 Megapixels); the Panasonic Lumix DMC-FX9 6.2 Megapixels); and the Canon EOS 400D (10.7 Megapixels). 155 photographs of *H. abdominalis*, comprising 80 males and 75 females, were of sufficient quality to be assessed for the morphological traits as described in Table 4 and some of which are illustrated in Figure 6. This includes the continuous length measurements (all in millimeters) of snout depth (SnD), snout length (SnL), head length (HL), head depth (HD), trunk length (TrL), tail length (TaL) and two subsequently calculated measurements named standard sength (StdL) and the head length to snout length ratio (HL:SnL). The photos were individually calibrated, using the refernce ruler in each photo, and the traits measured using ImagePro plus version 5.1.2.59 (Media Cybernetics Inc.). Each measurement was taken three times independently and averaged to give a final representative score, in the aim of minimizing human measurement error. Eight discrete morphological traits were also assessed from the photos, including the presence or absence of fronds, spines and tail banding, the degree of spotting (i.e. heavy, medium or low), and an estimation of dominant body colour and its overall base shading (i.e. dark or light). In the case of males this also extended to the incidence of a white brood pouch and a yellow 'slash'. These traits were assessed from the researcher's perspective following the guidelines in Table 4. Any features which could not be clearly resolved from the photos, due to low quality or ill-timed camera angle, were treated as missing data (i.e.'?'). Photographs were used instead of live measuring to dramatically decrease handling time and stress for the animal, and to allow the opportunity for future reassessment. The photographs and the raw measurement data are available on CD in Appendix D.

Table 4: Summary of all the continuous and discrete variables measured to assess the morphological variation of New Zealand *Hippocampus abdominalis.* Most of the assessment methods are based on descriptions in "Measuring Seahorses" by Lourie (2003b).

Morphological trait	**Assessment method**
Continuous variables (all samples)	
Snout depth (SnD)	The narrowest distance between the dorsal and ventral surfaces of the snout.
Snout length (SnL)	The distance from the tip of the snout to the eye spine.
Head length HL)	The distance from the tip of the snout to the mid-point of the cleithral ring.
Head depth (HD)	The distance from the lowest point of the depression immediately behind the coronet to immediately behind the cheek spine.
Trunk length (TrL)	The curved-line distance from the mid-point of the cleithral ring to the lateral mid-point of the last trunk ring.
Tail length (TaL)	The distance from the last trunk ring, along the midline, to the tip of the tail.
Calculated continuous variables (all samples)	
Standard length (StdL)	The sum of head length, trunk length (curved) and tail length.
Head length: snout length ratio (HL:SnL)	The number of times that snout length fits into the head length.
Discrete variables (all samples)	
Fronds	Presence or absence – filamentous growth from the head and/or trunk at any observable length.
Spines	Presence or absence – multiple raised and obviously protruding ridges from the upper and lower regions of the trunk.
Spotting	Heavy (dense spotting on head and trunk), medium (sparse spotting on head and trunk) or low (few spots on head or trunk).
Tail bands	Presence of absence – dark/light alternating variations in colour on the tail.
Body colour	Researchers perspective of dominant body colour.
Base shade	Dark or light impression of body colour.
Discrete variables (males only)	
White brood pouch	Presence or absence – lightening of the brood pouch relative to the body colour
Yellow slash	Presence or absence – any yellow colouration above the brood pouch.

Figure 6: An illustrative description of the continuous morphological variables measured from *H. abdominalis* from photographs such as this, showing the snout depth, snout length, head depth, head length, trunk length and tail length, as well as the ruler for reference and calibration.

2.4.2. Mitochondrial DNA sequence markers

The cytochrome b and cytochrome oxidase 1 sequences were verified by generating a forward and reverse read for each sample. The mitochondrial control region sequences were read twice in the reverse direction (often after reamplification) as the 5' portion of the light strand contains a thymine repeat which disrupts sequencing in the forward direction (see Teske *et al.*, 2003). In all cases, the duplicate sequences were identical to the original sequences.

To confirm that the markers amplified were authentic (i.e. *Hippocampus*), a sequence similarity search was carried out using the program BLAST (Altschul *et al.*, 1990). All chromatogram files were loaded into Geneious version 4.5.3 (Drummond *et al.*, 2008) and aligned as a single contig per sample, then checked for quality by eye. When there were differences in the forward and reverse sequences, the information from the better quality chromatogram was used. Contigs were subsequently aligned and truncated so haplotypes could be delineated. The original chromatogram files, contigs and alignments are available on CD in Appendix E. A printed alignment of haplotypes can be viewed in Appendix F.

2.4.3. Nuclear DNA microsatellite markers

Genotypes were automatically scored for each sample using Peak Scanner™ Software version 1.0 (Applied Biosystems) and also verified by eye. All four microsatellite markers (Habd3, Habd6, Habd9 and Habd7) were able to be amplified by PCR in all samples (n=169). However, three samples (ID: BC, BX, CC) had ambiguous results and were therefore excluded from all analyses. Genotypes were checked for normality independently within Microsatellite Analyser version (Dieringer, Schlötterer, 2003) and Micro-Checker version 2.2.3. (Van Oosterhout et al., 2004), no evidence of scoring errors was detected in the remaining 166 samples. The Peak Scanner™ files are available on CD in Appendix G. The allele scores that were read from these files and used for analysis are available in Appendix B.

2.5. Statistical Data Analysis

The statistical data analysis has two main parts: (1) species diversity is measured using all samples (irrespective of origin) that have sufficient sample quality to provide information for the New Zealand population as a whole, and (2), a pair-wise population differentiation assessment using only wild collected groups/populations, that have adequate sample size and sufficient sample quality, to investigate any differences there may be due to geographical origin.

2.5.1. Morphology

In regards to the continuous variable measurements, summary statistics were calculated in Microsoft® Office Excel (2007) and graphed as a box and whisker plot to show a distribution for the species. These variables were also separated by sex and tested for significant difference using a Mann Whitney Wilcoxon (MWW) test. This is a non-parametric test for assessing whether two 'samples' come from the same distribution (Hollander, Wolfe, 1999). Snout depth, snout length, head depth, head length, trunk length and tail length were graphed as a function of standard length to show their dependence on total size (see Appendix J). Therefore standard length was the only variable by which wild collected populations were compared (box and whisker plot). The frequency proportions of dominant body colour were displayed on a pie graph, as was the degree of spotting, and the presence/absence of frond for males and females. The proportions of all other discrete variables were reported for the species, and when appropriate also for the sexes and wild collected populations. For some traits the observational comments for the species were sufficient. All statistical analyses for morphology were computed in Statistica version 8 (StatSoft Inc.).

2.5.2. Mitochondrial DNA sequence markers

Haplotypes were defined manually for each marker and loaded into Arlequin version 3.1 (Excoffier et al., 2005) which gave output data on haplotype summary statistics, nucleotide diversity and composition, haplotype diversity (Nei, 1987), the mean number of pair-wise differences and population genetic estimations such as Tajima's D (Tajima, 1989), Fu's F_S (Fu, 1997), shared haplotypes and a mismatch distribution (100 bootstraps) with the corresponding Harpending's raggedness index (Harpending, 1992). The maximum pair-wise divergence was calculated manually from the inter-haplotype pair-wise difference matrix. Tajima's D and Fu's F_S measure the distribution of genetic variation based on neutral evolution and consequently allow inference about selective pressures (i.e. under the null hypothesis both would equal zero). Their significance is calculated under a model of population equilibrium. The observed mismatch distribution is generated via pair-wise comparisons of mtDNA sequences, and this is compared to neutral expectations under a unimodal model of recent population expansion (e.g. a population at equilibrium is expected to have a multimodal mismatch distribution), backed up by the Harpending's raggedness index which tests for departure from the expectations of the sudden expansion model (Rogers, Harpending, 1992). Due to the low number of samples representing each wild collected population, no breakdown is given for the molecular diversity or population genetic parameters, nor was pair-wise F_{ST} calculated. The proportions of haplotype occurrence were graphed on a map of New Zealand to show the distribution of shared haplotypes and phylogenetic relationships between these haplotypes and (Bilofsky, Burks, 1988) derived outgroups (Table 5) were visualized using neighbor joining, maximum parsimony and maximum likelihood methods within PAUP* (Swofford, 2002). The neighbor joining algorithm follows an agglomerative data clustering method and produces a tree that allows for variable rates of evolution across lineages (Saitou, Nei, 1987). Generally, at least one outgroup sequence is involved to root the tree. Distances based on the pair-wise differences were used to construct the initial neighbor joining tree for each marker and these were then further examined with 1000 non-parametric bootstrap replicates

to give a more robust consensus estimate. Bootstrapping is the statistical method of sampling with replacement, and the values are generally displayed above the branches, it is cautioned that these are not synonymous with statistical significance (Balding, 2006). The maximum parsimony algorithm is a non-parametric method which prioritizes taxa by the least evolutionary changes required to explain the observed data. Maximum parsimony trees were constructed via stepwise addition heuristic search and tree bisection reconnection branch swapping. Gaps were treated as missing data. This was followed by 1000 bootstraps (majority rule) to produce a consensus tree. Tree length is reported and tells the observer how many substitutions occur in the tree. The confidence index is also given as well as the retention index which measures the amount of synapomorphy on the tree (these values vary from zero to one). Both the neighbor joining and maximum parsimony tree was visualized using Figtree version 1.2 (Rambaut, 2008). The maximum likelihood estimation is considered to be superior to the other two methods as it assumes a model of evolution to explain the observed data. This model was selected within Modeltest version 3.7 (Posada, Crandall, 1998) and in combination with the data was then processed in the phyml program within the web-based service Mobyle (Neron et al., 2005) and then visualized in their 'drawgram' program.

Table 5: Details on the Genbank derived outgroups which were utilized in the phylogenetic analyses of cytochrome b (CYTB), cytochrome oxidase 1 (COX1) and the mitochondrial control region (MtCR), showing the identification 'name' that each is referred to within this study, their Genbank accession number, the species and locality the sequence represents and their source.

MtDNA Marker	**ID in this study**	**Genbank Accession #**	**Species**	**Locality**	**Author Source**
CYTB	H.abdNZL40.1	AF192640.1	*H. abdominalis*	New Zealand	(Casey et al., 2004)
	H.abdNZL41.1	AF192641.1	*H. abdominalis*	New Zealand	(Casey et al., 2004)
	H.abdAUS39.1	AF192639.1	*H. abdominalis*	Australia	(Casey et al., 2004)
	H.abdAUS38.1	AF192638.1	*H. abdominalis*	Australia	(Casey et al., 2004)
	H.breviceps	AF192647.1	*H. breviceps*	Australia	(Casey et al., 2004)
	H.erectus	AF192660.1	*H. erectus*	Australasia	(Casey et al., 2004)
	H.kuda	AP005985.1	*H. kuda*	Australasia	(Kawahara et al., 2008)
COX1	H.kuda	AP005985.1	*H. kuda*	Australasia	(Kawahara et al., 2008)
MtCR	H.kuda	AP005985.1	*H. kuda*	Australasia	(Kawahara et al., 2008)

2.5.3. Nuclear DNA microsatellite markers

As to acquire a further perspective of genetic diversity, four microsatellite loci were genotyped and the results reported, for each locus, at the whole New Zealand level and, as an average, for wild collected populations. The number of alleles, their frequencies, ranges and means, as well as the expected number of alleles under the stepwise mutation model and the infinite allele model were calculated in Microsatellite Analyzer version 4.05 (Dieringer, Schlötterer, 2003). Whereas the expected and observed heterozygosities, the fixation index F_{IS} (Weir, Cockerham, 1984), the Garza-Williamson index (Garza, Williamson, 2001), the deviation from Hardy-Weinberg equilibrium, linkage disequilibrium and pair-wise F_{ST} (Weir, Cockerham, 1984) values were calculated in Arlequin version 3.1 (Excoffier et al., 2005). The frequency of alleles at each locus were graphed for New Zealand (bar graph) and for wild collected populations (heatmap) through Microsoft® Office Excel 2007. Deviation from Hardy-Weinberg equilibrium was tested through a Marcov Chain Monte Carlo method involving 1,000,000 chains and 10,000 dememorization steps and linkage disequilibrium was assessed for each combination of microsatellite loci using the likelihood ratio test, with a permutation procedure of 10 initial conditions for expectation-minimization and 16,000 permutations, as this level has been shown to come within 1% of significance (Guo, Thompson, 1992). The Garza-Williamson index is a statistic that is calculated by dividing the number of alleles at a locus by the allelic range plus one. A low index arises when there are a lower number of alleles than the range would suggest, thus it is sensitive to recent population bottlenecks. There is no significance test for this statistic. To estimate phylogenetic and phylogeographic relationships, Nei's standard genetic distance (Nei, 1978) was calculated in Microsatellite Analyzer version 4.05 (Dieringer, Schlötterer, 2003) between individuals and populations and was then used to produce neighbor joining trees in the Neighbor program within the Phylip package version 3.68 (Felsenstein, 1993), and then subsequently visualized in FigTree (Rambaut, 2008). The total genotypic variation among wild collected populations was assessed with pair-wise F_{ST}. The F_{ST} measurement assumes an infinite alleles model of

mutation and is a more conservative measure of divergence and population structure than R_{ST} when the number of loci is low (in this case 4) and samples per population (in this case $5<n<14$) are small (Gaggiotti et al., 1999). F_{ST} values can range from zero, where all populations share the same allelic frequencies, to one, where all populations are fixed for different alleles (Weir, Cockerham, 1984). Significance was estimated via 1023 permutations.

3. Results

3.1. Morphology

3.1.1. Continuous variables

The standard length of New Zealand *Hippocampus abdominalis* (calculated from 155 samples) ranged from 105.1-313.2 millimeters (mean = 194.0 mm; standard deviation = 44.5). This was calculated through the addition of the head length (range = 14.1-48.3 mm; mean = 29.7 mm; s.d. = 6.9), trunk length (range = 24.3-84.6 mm; mean = 46.6 mm; s.d. = 11.2) and tail length (range = 60.4-191.1 mm; mean = 117.8 mm; s.d. = 28.6). The head length was also utilized in combination with the snout length (range = 4.7-23.0 mm; mean 11.3 mm; s.d. = 3.8) to calculate the head length to snout length ratio (range = 1.9-3.9 mm; mean = 2.7 mm; s.d. = 0.5). Besides these, the other continuous variables measured were the snout depth (range = 2.5-6.4 mm; mean = 4.1 mm; s.d. = 0.82) and the head depth (range = 6.4-20.4 mm; mean = 14.1 mm; s.d. 2.9). The distributions of these measurements are illustrated on a box and whisker plot (Figure 7) and the full summary statistics are available in Appendix H.

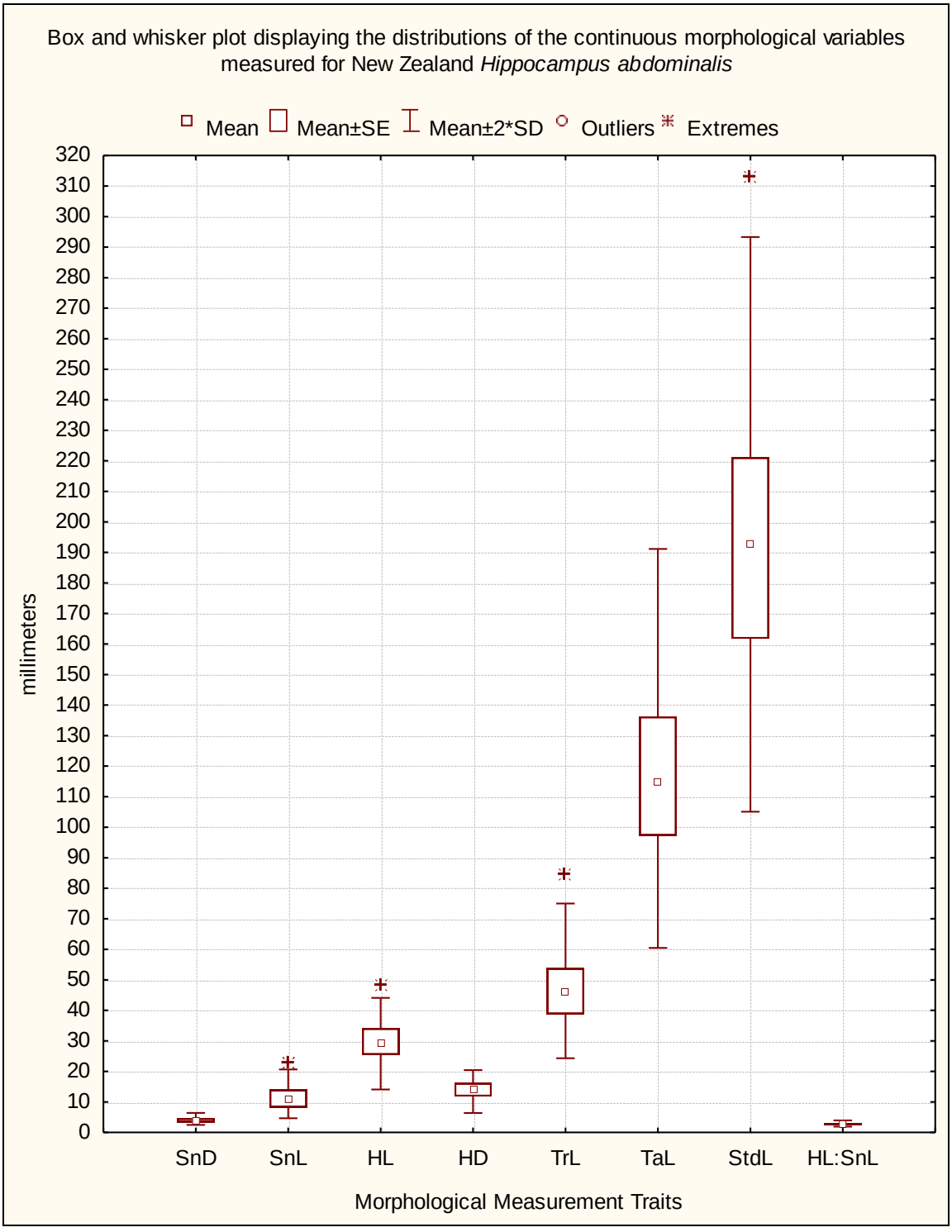


Figure 7: Box and whisker plot showing the distributions of the continuous variables measured for all samples (n=155) of New Zealand *H. abdominalis*. These include snout depth (SnD), snout length (SnL), head length (HL), head depth (HD), trunk length (TrL), tail length (TaL), standard length (StdL) and the head length to snout length (HL:SnL) and are shown on the x-axis. The y-axis represents size in millimeters.

Males have shorter snouts and trunk lengths than females, and a higher head length to snout length ratio (Table 6). The snout length (U statistic = 2213; p-value = 0.005), trunk length (U statistic = 2271; p-value = 0.009) and head length to snout length ratio (U statistic = 2135; p-value = 0.001) were found to be significantly different between males and females via the Mann Whitney Wilcoxon (MWW) test. Whilst the range of each measured trait is generally smaller in males, none distinguish the sex. Further details of the MWW test are available in Appendix I.

Table 6: Summary Statistics in the form of range and mean with standard deviation (SD) for the continuous morphological variables for male (n=80) and female (n=75) New Zealand *H. abdominalis*. Also shown is the p-value of the Mann Whitney Wilcoxon (MWW) test. All measurements are in millimeters. The significantly different traits ($p<0.05$) are highlighted in red.

Continuous variable	**Males**		**Females**		**MWW test P-value**
	Mean (+/- SD)	**Range**	**Mean (+/- SD)**	**Range**	
SnD	3.96 (0.73)	2.64 - 5.47	4.15 (0.90)	2.47 - 6.39	0.252627
SnL	10.48 (3.39)	5.41 - 20.64	12.21 (4.07)	4.67 - 22.95	0.004861
HL	28.73 (6.35)	16.85 - 43.69	30.63 (7.30)	14.06 - 48.29	0.056566
HD	14.05 (2.85)	7.78 - 19.42	14.14 (2.97)	6.35 - 20.42	0.834086
TrL	44.10 (9.22)	24.31 - 66.07	49.21 (12.57)	25.89 - 84.57	0.009096
TaL	119.73 (27.86)	65.68 - 186.21	115.76 (29.50)	60.44 - 191.11	0.403119
StdL	192.56 (41.84)	110.02 - 284.35	195.60 (47.32)	105.08 - 313.22	0.637751
HL:SnL	2.85 (0.46)	2.06 - 3.94	2.61 (0.42)	1.92 - 3.47	0.001966

The standard length of seahorses varied widely between the wild collected populations (Figure 8). The population in Whangateau (WHA), and to a lesser extent the population in Raglan (RAG), exhibited large ranges in standard length, whereas the other populations displayed much smaller ranges (i.e. range<110mm). The population in Tauranga (TAU) was unique by the way that it consisted of only small (standard length<160 mm) individuals, whilst those in all other populations were considerably larger on average. As the other continuous variables measured are dependent on the standard length (Appendix J), its high degree of variability between populations hinders their use for investigating significant population differentiation.

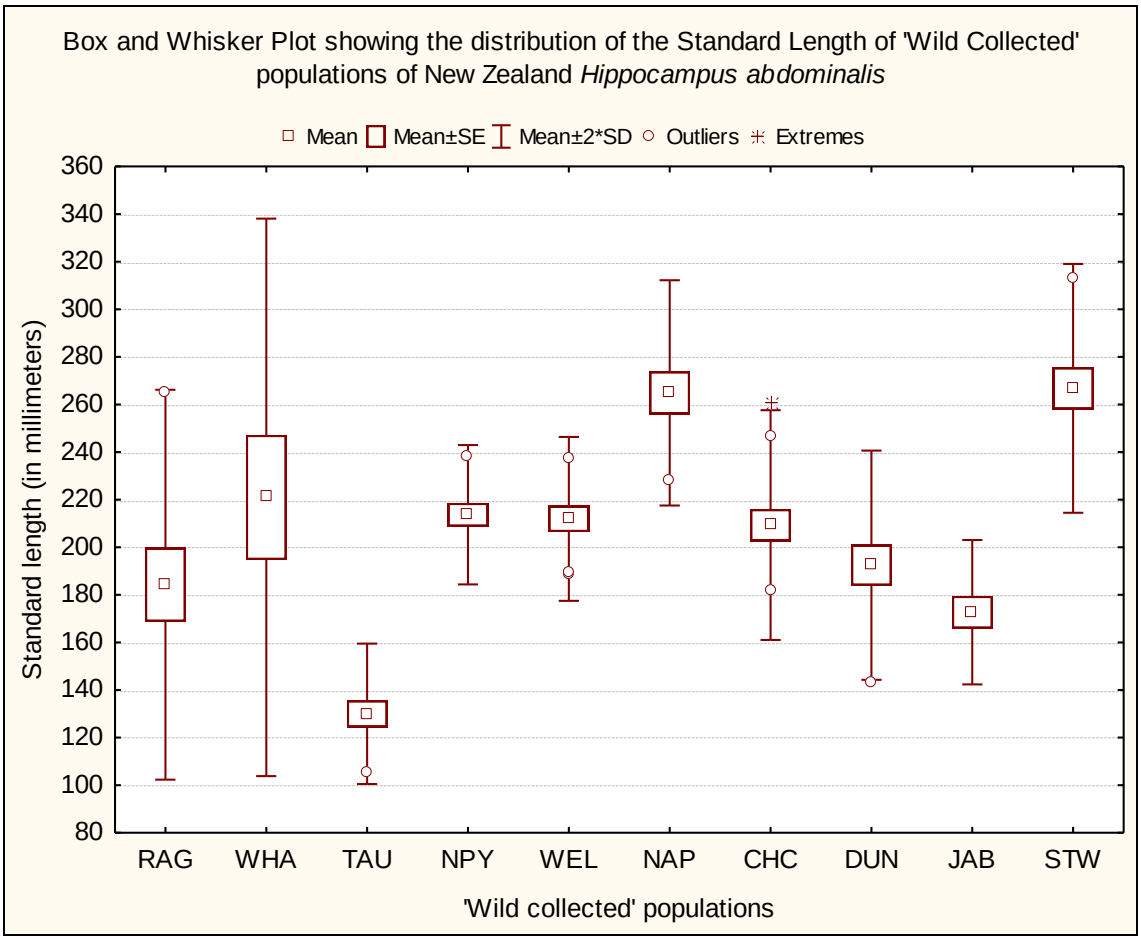


Figure 8: Box and whisker plot showing the distribution of standard length throughout wild collected populations of *H. abdominalis*. Populations are represented on the x-axis and the measurement is in millimeters on the y-axis.

3.1.2. Discrete variables

New Zealand *Hippocampus abdominalis* were found displaying ten dominant body colours (Table 7). The majority of the samples in this study originated directly from *in situ* (64%) and would not have been influenced by non-natural Aquarium conditions. A quarter of the sampled individuals had a brown-yellow base colouring, and almost three quarters were either a shade of brown or black (Figure 9). Only 6% could be classified as having a white dominant body colour, which is very similar to the 3% that were grey. Orange and red were the most striking but these were only observed at proportions of 3% and 2% respectively. Overall, only 18% could be described to be lightly coloured (i.e. white, grey or pale brown).

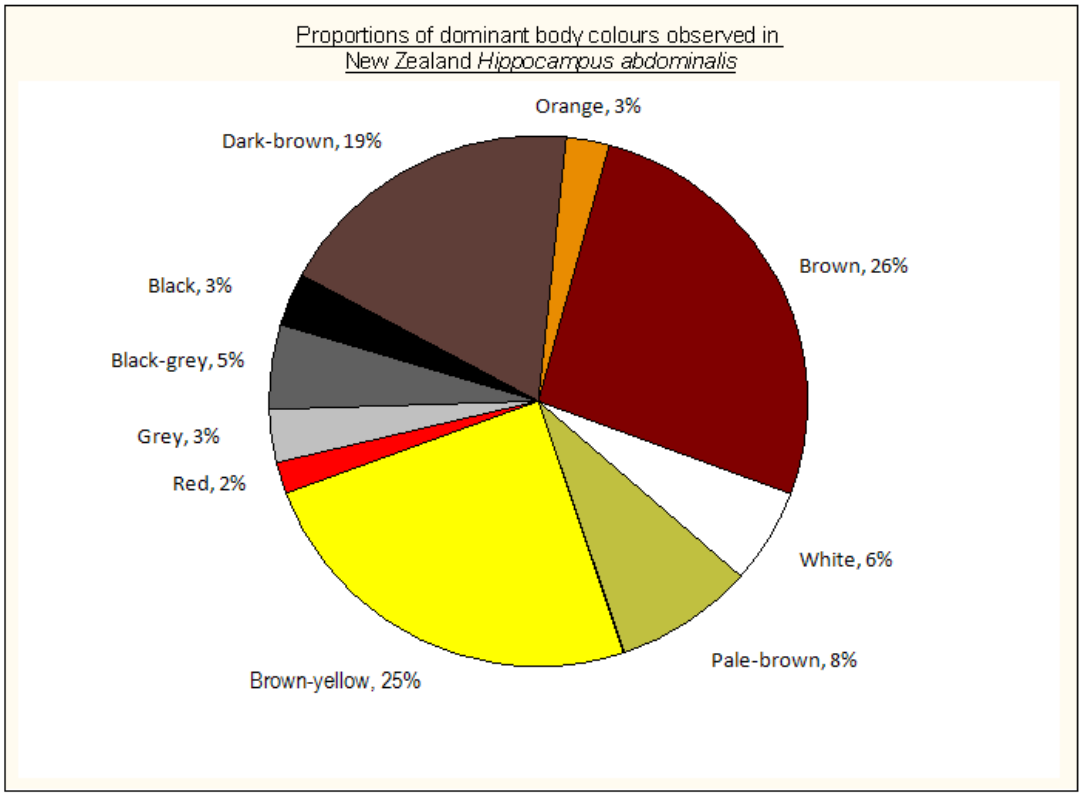


Figure 9: Pie graph showing the proportions (in percent) of the ten dominant body colours observed in New Zealand *Hippocampus abdominalis* (n=155).

Table 7: Examples of the ten dominant body colours found to be displayed in New Zealand *Hippocampus abdominalis*. Some of the individuals shown in this table also exhibit varying degrees of spotting, and the presence or absence of fronds, spines, white brood pouch and the yellow slash.

Grey: 	Orange: 	Black-grey: 
Brown: 	White: 	Brown-yellow: 
Pale-brown: 	Red: 	Dark-brown: 
Black: 		

Dominant body colouring did not vary significantly by sex (U statistic = 2858.5; p-value = 0.613657), further attested by the fact only 17% of females and 19% of males could be described as lightly coloured. Colour did slightly vary between populations though: the population in Tauranga (TAU) was observed to consist of both red and brown specimens only, and the population in Whangateau (WHA) was exclusively brown-yellow. All other populations displayed a range of three or more colours.

Other morphological aspects investigated included the presence of tail banding, the degree of spotting, and the presence of fronds and spines. Tail banding was absent in 12% of individuals, and often corresponded with solid dark colouring (i.e. a lack of contrast). The degree of spotting was evenly distributed throughout the total number of samples (i.e. roughly a third of all the samples displayed either heavy spotting, medium or light spotting). However, the degree of spotting was significantly different between males and females (U statistic = 1110.5; p-value = <0.0001). Males displayed heavy spotting on a much higher proportion than females (Figure 10) and it is uncertain why more cases were not able to be unambiguously defined (26%) than in females (5%). This aspect was not investigated between wild collected populations, yet it was noted that some aquarium specimens sometimes displayed grey spotting instead of the more common dark spotting.

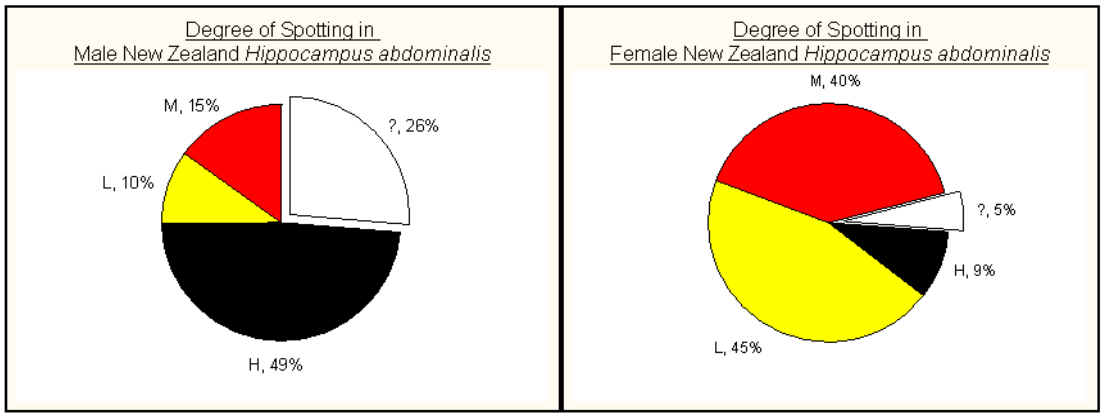


Figure 10: Pie graphs showing the varying degrees of heavy (H), medium (M) and light (L) spotting proportionally in male (left; n = 80) and female (right; n =75) New Zealand *H. abdominalis.*

Fronds were present 39% of the time in males, and 13% of the time in females (Figure 11), a significant difference (U statistic = 2237.5; p-value = 0.006365).

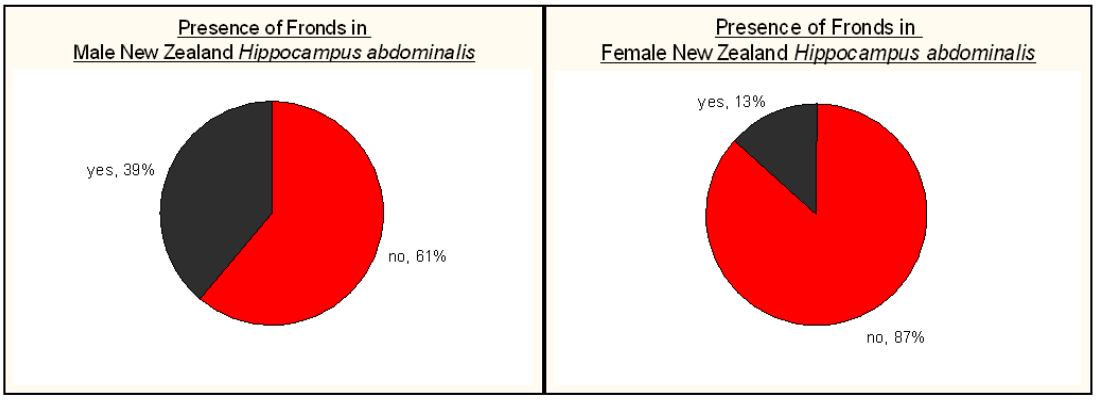


Figure 11: Pie graphs showing the proportions of presence (yes) and absence (no) of fronds in male (left; n = 80) and female (right; n = 75) New Zealand *H. abdominalis*.

Taken overall, fronds were present in 26% of the individuals sampled. It was observed that the populations in Tauranga (TAU) and Napier (NAP) did not display fronds at all, but they did display spines. If fronds were present, spines were also present 29% of the time. On the other hand, spines were present in 23% of the individuals sampled; but this did not vary significantly between males (25%) and females (21%) (U statistic = 2890; p-value = 0.695005). It was observed that the populations in Whangateau (WHA), New Plymouth (NPY) and Wellington (WEL) did not feature spines. Further information on these Mann Whitney Wilcoxon tests is available in Appendix I.

In regards to males only, 29% displayed a white brood pouch, of those 87% also showed a yellow slash above it. The yellow slash was present in 64% of males overall, and was still evident in 53% of males who did not have a white brood pouch. A white pouch could also be observed without the yellow slash but only 10% of the time.

3.2. Mitochondrial DNA sequence markers

3.2.1. Definitions of Haplotypes and their descriptive statistics

Descriptive statistics for the sequences of the three mtDNA markers are shown in Table 8. Thirty-six cytochrome b sequences, at a length of 814 base pairs, which included 20 segregating sites, yielded 16 haplotypes which differed by a maximum of 6 single nucleotide polymorphisms (SNPs) at most (i.e. 0.7% divergence). The GC content of both cytochrome b and cytochrome oxidase 1 for their most common haplotypes is 42%. Thirty-six cytochrome oxidase 1 sequences, at a length of 624 base pairs and 18 segregating sites, gave 15 haplotypes. The highest pair-wise difference between these also consisted of 6 SNPs but due to the shorter sequence length equated to 1% sequence divergence. Forty control region sequences, at a length of 404 base pairs and 19 segregating sites, revealed 14 haplotypes, with a maximum pair-wise sequence divergence of 9 SNPs, or 2.2%. The GC content of the regulatory sequence is 35%, slightly lower than that of the two aforementioned genes. All three markers had a high transition to transversions ratio, but the control region was the only marker which featured indels between haplotypes. It is very unlikely that any pseudogenes of mtDNA amplified as there were no occurrences of multiple bands, nor heterozygous sequences.

Table 8: Haplotype information for cytochrome b (CYTB) (n=36), cytochrome oxidase 1 (COX1) (n=36) and mitochondrial control region (MtCR) (n=40) of New Zealand *H. abdominalis*. Given in terms of sequence length in base pairs (bp), number of Haplotypes (H), number of segregating sites with transition to transversion ratio, number of indels, the maximum sequence divergence (msd) and the nucleotide composition (in percent) from the most common haplotype.

MtDNA Marker	Length (bp)	H	Segregating sites	Indels	msd (%)	Nucleotide composition (%) Cytosine;	Thymine;	Adenine;	Guanine
CYTB	814	16	20 (19:1)	0	0.7 %	25	32	26	17
COX1	624	15	18 (17:1)	0	1.0 %	24	33	25	18
MtCR	404	14	19 (13:4)	3	2.2 %	13	32	34	22

A common haplotype was found for each mtDNA marker, as well as many unique haplotypes at low frequencies (usually only represented by a single individual). These are defined in Table 9a-c and the full sequences are available in Appendix F. The defined haplotypes were named via the following system:

- All start with a prefix of 'JE' to identify them as originating from this study,
- then the code 'NZ' to identify their country of origin for the species,
- then the name 'Habd' to identify the species (*Hippocampus abdominalis*),
- then the identification of the marker it corresponds to (i.e. 'CB' for cytochrome b; 'CO' for cytochrome oxidase 1; or 'MC' for mitochondrial control region),
- followed by the identification number of the haplotype (1, 2, 3, etcetera),
- and finally, in brackets, the number of individuals from this study it truly represents.

To save space, the haplotypes are often referred to only by their marker and their identification number (e.g. 'CB1').

Table 9: Haplotype definition tables for a) cytochrome b (814bp), b) cytochrome oxidase 1 (624bp) and c) mitochondrial control region (404bp) of New Zealand *H. abdominalis.* Information is provided on which samples contributed each haplotype (Sample ID), their geographical origin (Origin), and the positions of DNA base substitutions. Indels are indicated by "–". Full sequences of haplotypes are available in Appendix F.

a)

Cytochrome b:			***Positions***																				
Haplotype ID	***Sample ID***	***Origin***	***48***	***93***	***129***	***153***	***159***	***222***	***288***	***327***	***350***	***351***	***364***	***441***	***447***	***525***	***543***	***616***	***647***	***651***	***742***	***750***	***792***
JENZHabdCB1(18)	AA, AJ, AL, AQ, AR, AV, AX, CN, CP, CV, DV, ZZ, FD, FF, FH, FL, FR, GA.	RAG, WHA, TAU, NPY, WEL, CHC, DUN, STW, JAB, MPQ.	A	A	C	T	A	T	A	A	C	G	T	G	T	T	C	C	T	A	C	G	G
JENZHabdCB2(1)	DB	TIB		C																			
JENZHabdCB3(1)	DA	WEL			T			C			T			A									
JENZHabdCB4(1)	CR	WEL				C	G				T							T					
JENZHabdCB5(2)	DH, DR	NAP, CHC						C			T			A									
JENZHabdCB6(1)	FO	MPQ							G														
JENZHabdCB7(1)	DJ	NAP								G													
JENZHabdCB8(1)	AF	RAG									T		C										
JENZHabdCB9(2)	AC, FX	RAG, STW									T												
JENZHabdCB10(1)	FN	MPQ	G									A											
JENZHabdCB11(1)	FS	JAB									T						T						
JENZHabdCB12(1)	AZ	TAU													C							A	
JENZHabdCB13(1)	CM	NPY														C							
JENZHabdCB14(1)	FV	JAB																	C	G			
JENZHabdCB15(2)	DD, AS	NAP, WHA																				A	
JENZHabdCB16(1)	GC	STW																					A

b)

Cytochrome oxidase 1:			***Positions***																	
Haplotype ID	***Sample ID***	***Origin***	***148***	***187***	***202***	***208***	***271***	***289***	***316***	***331***	***358***	***388***	***446***	***447***	***454***	***460***	***475***	***505***	***545***	***568***
JENZHabdCO1(22)	AO, AS, AT, AX, CE, CH, CQ, CX, DE, DG, DO, DT, FE, FM, FP, FS, FT, FU, FW, FY, GB, DB.	TAU, KTQ, NPY, WEL, NAP, CHC, DUN, PIC, JAB, STW, TIB.	A	G	C	A	T	C	T	T	T	C	A	T	A	T	A	T	C	T
JENZHabdCO2(1)	CI	NPY		A																
JENZHabdCO3(1)	CD	KTQ			T															
JENZHabdCO4(1)	BM	NAQ	G																	
JENZHabdCO5(1)	AM	RAG					C							C						
JENZHabdCO6(1)	FQ	MPQ						T												
JENZHabdCO7(1)	BA	TAU						T								C				
JENZHabdCO8(1)	CU	WEL							C	C		T	G							
JENZHabdCO9(1)	FI	DUN									C									
JENZHabdCO10(1)	AN	RAG										T								
JENZHabdCO11(1)	CY	WEL													G		G			
JENZHabdCO12(1)	ZZ	CHC																		C
JENZHabdCO13(1)	AP	WHA				C											G			
JENZHabdCO14(1)	AC	RAG																C		
JENZHabdCB15(1)	DI	NAP																	T	

c)

Mitochondrial control region:			***Positions***																		
Haplotype ID	***Sample ID***	***Origin***	***11***	***26***	***80***	***84***	***96***	***110***	***121***	***131***	***139***	***145***	***213***	***302***	***321***	***323***	***331***	***333***	***362***	***372***	***387***
JENZHabdMC1(23)	AE, AJ, AO, AP, AQ, AS, AZ, CO, CP, CW, DC, DG, DP, DS, DT, FH, FM, FQ, FT, FZ, GD, GA, DB.	RAG, WHA, TAU, NPY, WEL, TIB, NAP, CHC, MPQ, JAB, STW.	T	C	T	A	A	T	A	A	T	C	G	G	G	C	C	T	A	G	T
JENZHabdMC2(1)	AU	TAU		G			G	C				T					T				C
JENZHabdMC3(1)	FJ	DUN			C																
JENZHabdMC4(1)	DD	NAP				-													-		
JENZHabdMC5(1)	CQ	NPY				T									A						
JENZHabdMC6(1)	FF	DUN							T									F	-		
JENZHabdMC7(1)	DA	WEL								T											
JENZHabdMC8(1)	AY	TAU									C					T					
JENZHabdMC9(1)	FV	JAB									C	T									
JENZHabdMC10(1)	FN	MPQ											A								
JENZHabdMC11(1)	DM	CHC												A							
JENZHabdMC12(5)	AC, AP, FT, GD, DB	RAG, TAU, JAB, STW, TIB																	-		
JENZHabdMC13(1)	CZ	WEL																		-	
JENZHabdMC14(1)	ZZ	CHC	C																		

3.2.2. Molecular diversity and population genetics

Cytochrome b exhibited the highest haplotype diversity (*h* = 0.7524 +/- 0.0781) and mean number of pair-wise differences (*d* = 1.7159 +/- 1.0249). The highest nucleotide diversity was observed in the mitochondrial control region (π = 0.0033 +/- 0.0023) relative to the other mtDNA markers (Table 10). Selective neutrality was assessed using Tajima's D and Fu's F_S values. The Tajima's D and Fu's F_S values are all highly negative for all three mtDNA markers, indicating a very significant deviation from selective neutrality. The mismatch distribution assumes a model of sudden expansion (null hypothesis) which is modeled as a dashed line in Figure 12A-C, surrounded by 95% confidence intervals (grey dotted lines). The data from this study (i.e. the observed pair-wise differences) are represented by the solid line, showing a unimodal distribution very similar to the model, therefore signaling no rejection of the sudden population expansion model. This is further verified by the Harpending's raggedness index (HRI), with non-significant p-values.

Table 10: Molecular diversity and population genetic statistics for cytochrome b (CYTB) (n=36), cytochrome oxidase 1 (COX1) (n=36) and mitochondrial control region (MtCR) (n=40) of New Zealand *H. abdominalis* in terms of haplotype diversity (*h*), nucleotide diversity (π), and mean number of pairwise differences (*d*) and Tajima's D, Fu's F_S, and the HRI p-value (*P<0.05 and ** P<0.001).

MtDNA Marker	***h*** **(+/- SD)**	***π*** **(+/- SD)**	***d*** **(+/- SD)**	**Tajima's D**	**Fu's F_S**	**P (HRI)**
CYTB	0.7524 (0.0781)	0.0021 (0.0014)	1.7159 (1.0249)	-2.1702 *	-12.0470 **	0.9908
COX1	0.6333 (0.0954)	0.0019 (0.0014)	1.1571 (0.7650)	-2.4406 **	-14.2052 **	0.9404
MtCR	0.6628 (0.0827)	0.0033 (0.0023)	1.3397 (0.8490)	-2.4768 **	-10.2133 **	0.9192

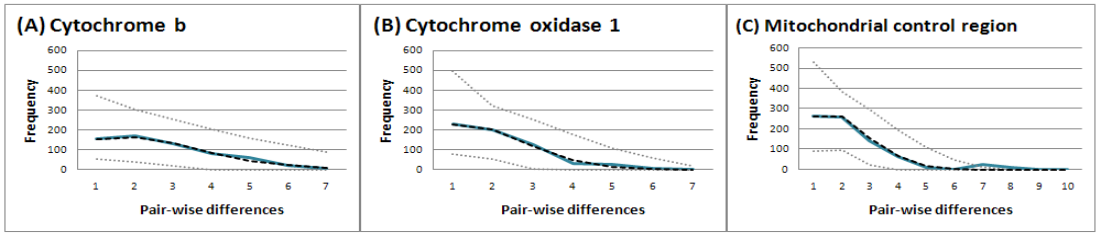


Figure 12A-C: Models of the mismatch distribution (dashed line) with 95% confidence intervals (grey dotted lines) for mtDNA data (solid blue lines) of New Zealand *H. abdominalis*: A) cytochrome b, B) cytochrome oxidase 1, C) mitochondrial control region. The number of pairwise differences are shown on the x- axis and the frequency of the mismatch is shown on the y axis.

3.2.3. Shared mitochondrial DNA haplotypes

Shared haplotypes between populations reveal evidence of migration and gene flow. The shared haplotypes for cytochrome b are displayed in Figure 12a. The most common cytochrome b haplotype (CB1) appeared in all populations except for the population in Napier (NAP). Private haplotypes were found in Raglan (CB8), Wellington (CB3, CB4), Napier (CB7), Jackson's Bay (CB11, CB14), Tauranga (CB12), New Plymouth (CB13) and Stewart Island (CB16). Three haplotypes were found to be shared between two regions (i.e. CB5 in Napier and Christchurch; CB9 in Raglan and Stewart Island; and CB15 in Napier and Whangateau). Additionally, as a few sequences were obtained from individuals in other groups also, it can be mentioned that three unique haplotypes were found: two at the Westpac Aquarium (CB6 and CB10) and in the Titahi Bay sample (CB2). However these are not shown.

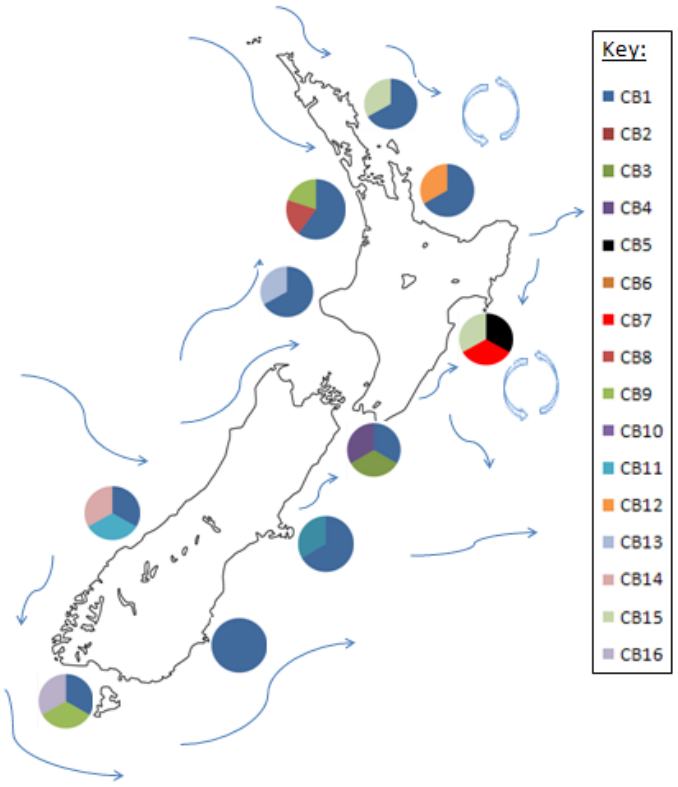


Figure 13a) Map of New Zealand with pie graphs representing the proportion of each *H. abdominalis* cytochrome b haplotype found there. The size of the pie graphs denotes how many individuals it represents. The key for the haplotypes is on the right hand side and indications of the dominant ocean currents are shown as a reminder.

The shared haplotypes for cytochrome oxidase 1 are displayed in Figure 13b. The most common cytochrome oxidase 1 haplotype (CO1) was found in all populations except for the one in Raglan. Apart from this no haplotypes were shared between populations. Private haplotypes were found in New Plymouth (CO2), Raglan (CO5, CO10, CO14), Tauranga (CO7), Wellington (CO8, CO11), Dunedin (CO9), Christchurch (CO12), Whangateau (CO13) and Napier (CO15). Additionally, there were also three haplotypes found in individuals with uncertain origins, from the Westpac Aquarium (CO6), the National Aquarium (CO4) and Kelly Tarltons Underwater World™ (CO3) but these are not shown.

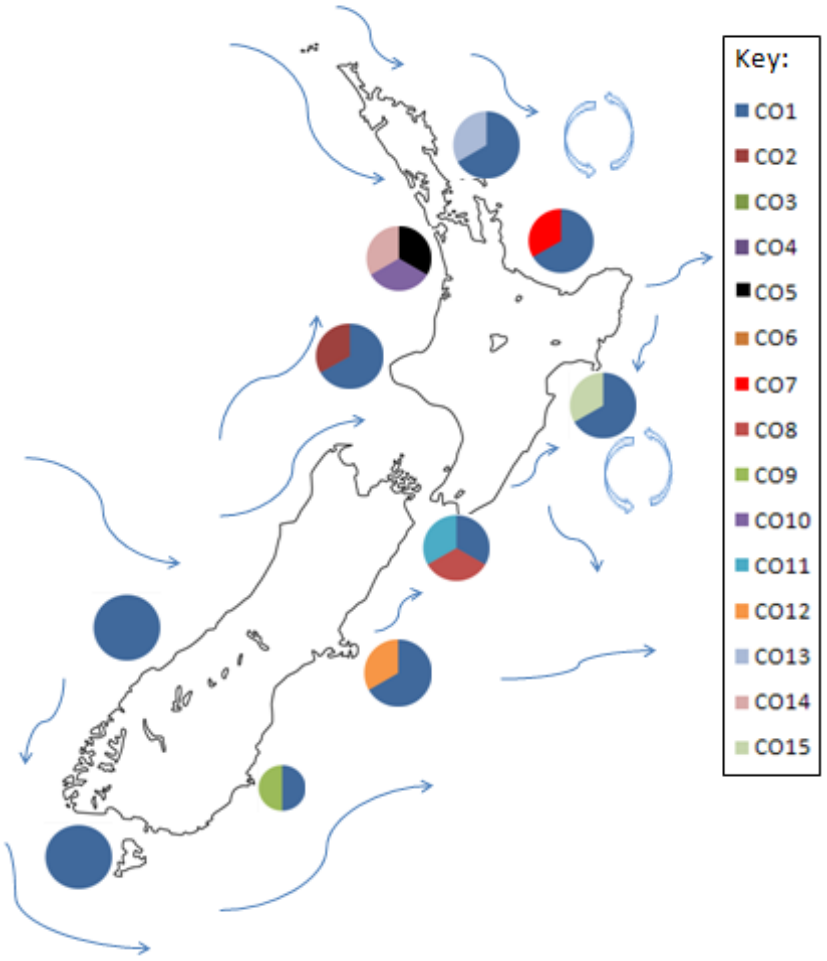


Figure 13b) Map of New Zealand with pie graphs representing the proportion of each *H. abdominalis* cytochrome oxidase 1 haplotype found there. The size of the pie graphs denotes how many individuals it represents. The key for the haplotypes is on the right hand side and indications of the dominant ocean currents are shown as a reminder.

The shared haplotypes for the mitochondrial control region are displayed in Figure 13c. The most common control region haplotype (MC1) was found in all populations except for the one in Jackson's Bay. Private haplotypes were found in Tauranga (MC2, MC8), Dunedin (MC3, MC6), Napier (MC4), New Plymouth (MC5), Wellington (MC7, MC13), Jackson's Bay (MC9) and Christchurch (MC11, MC14). The haplotype MC12 was found to be shared between Raglan, Tauranga, Jackson's Bay, Stewart Island (and Titahi Bay - not shown). One further haplotype was discovered (MC10) in an individual from the Westpac Aquarium, where the origin is uncertain (not shown).

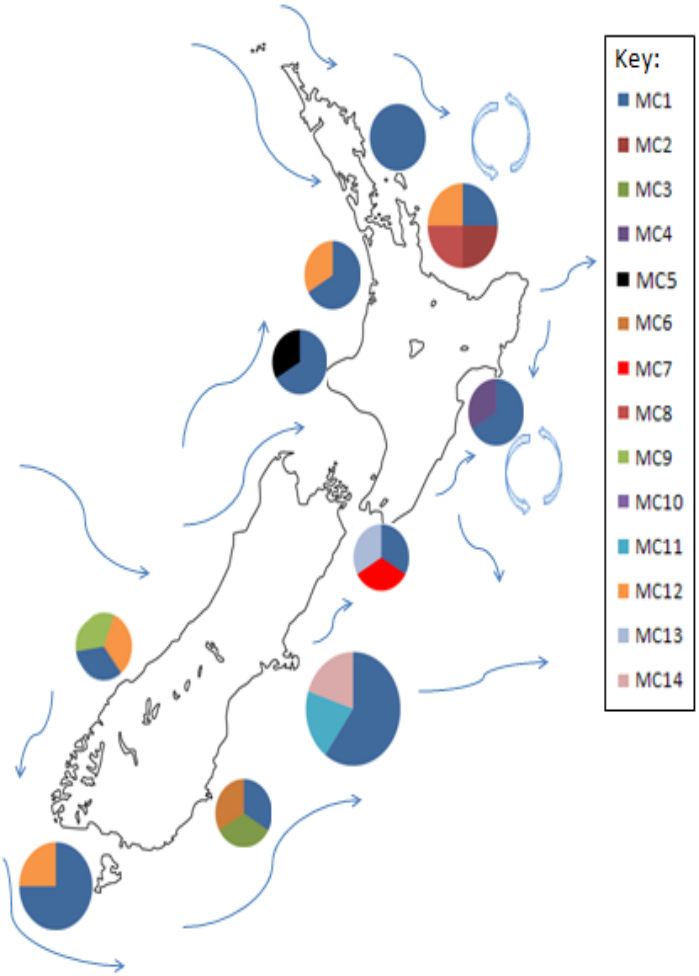


Figure 13c) Map of New Zealand with pie graphs representing the proportion of each *H. abdominalis* mitochondrial control region haplotype found there. The size of the pie graphs denotes how many individuals it represents. The key for the haplotypes is on the right hand side and indications of the dominant ocean currents are shown as a reminder.

3.2.4. Phylogenetic Relationships

The relationships between the cytochrome b haplotypes and seven Genbank derived outgroup sequences (as outlined in Section 2.2) were visualized using neighbor joining (Figure 14), maximum parsimony (Figure 15) and maximum likelihood methods (Figure 16). A pair-wise difference matrix for the cytochrome b sequence alignment is shown in table 11. Modeltest selected the Hasegawa-Kishino-Yano (HKY) model (Hasegawa et al., 1985) as the most appropriate model of evolution for cytochrome b. All estimation methods showed the three main outgroups *H. kuda*, *H. erectus and H. breviceps* (in red) basal to the *H. abdominalis* monophyletic clade, and the Australian *H. abdominalis* haplotypes (in green) sister to the New Zealand haplotypes. In all instances, the two Genbank derived New Zealand *H. abdominalis* haplotypes (in blue) are grouped with the New Zealand *H. abdominalis* haplotypes (in black) generated in this study. The neighbor joining tree was bootstrapped 1000 times, and yielded a topology identical to the maximum parsimony consensus estimation which utilized 112 informative characters to produce a tree length of 290 steps (after 1000 bootstraps) with a very high overall confidence index of 0.8586 and a retention index of 0.7735 representing a high degree of similarity and high degree of synapomorphies in the sequence. The haplotypes CB3 and CB5 (originating from Wellington, and Napier and Christchurch respectively) are consistently placed together, as well as CB12 and CB15 (originating from Tauranga, and Napier and Whangateau respectively). The resolution is lost between the remaining haplotypes in the consensus neighbor joining, maximum parsimony and maximum likelihood estimates most likely due lack of differentiation.

Table 11) Pair-wise difference matrix of cytochrome b sequences which the distances used to calculate phylogenetic relationships are based on. Interspecies differences are much larger than intraspecies differences.

	CB1	CB2	CB3	CB4	CB5	CB6	CB7	CB8	CB9	CB10	CB11	CB12	CB13	CB14	CB15	CB16	NZ40.1	NZ41.1	AUS38.1	AUS39.1	H.breviceps	H.erectus
CB1																						
CB2	1																					
CB3	4	5																				
CB4	4	5	6																			
CB5	3	4	1	5																		
CB6	1	2	5	5	4																	
CB7	1	2	5	5	4	2																
CB8	2	3	4	4	3	3	3															
CB9	1	2	3	3	2	2	2	1														
CB10	2	3	6	6	5	3	3	4	3													
CB11	2	3	4	4	3	3	3	2	1	4												
CB12	2	3	6	6	5	3	3	4	3	4	4											
CB13	1	2	5	5	4	2	2	3	2	3	3	3										
CB14	2	3	6	6	5	3	3	4	3	4	4	4	3									
CB15	1	2	5	5	4	2	2	3	2	3	3	1	2	3								
CB16	1	2	5	5	4	2	2	3	2	3	3	3	2	3	2							
NZ40.1	4	5	8	8	7	5	5	6	5	6	6	6	5	6	5	5						
NZ41.1	6	7	10	10	9	7	7	8	7	8	8	8	7	8	7	7	2					
AUS38.1	11	12	9	13	8	12	12	11	10	13	11	13	12	13	12	12	7	9				
AUS39.1	12	13	10	14	9	13	13	12	11	14	12	14	13	14	13	13	8	10	1			
H.breviceps	110	111	108	112	109	111	111	110	109	110	110	110	111	112	111	109	109	111	108	109		
H.erectus	126	126	122	128	123	127	127	126	125	126	124	124	127	127	125	125	126	128	121	122	138	
H.kuda	133	133	131	135	132	134	134	133	132	133	131	133	134	134	132	132	134	136	135	136	142	93

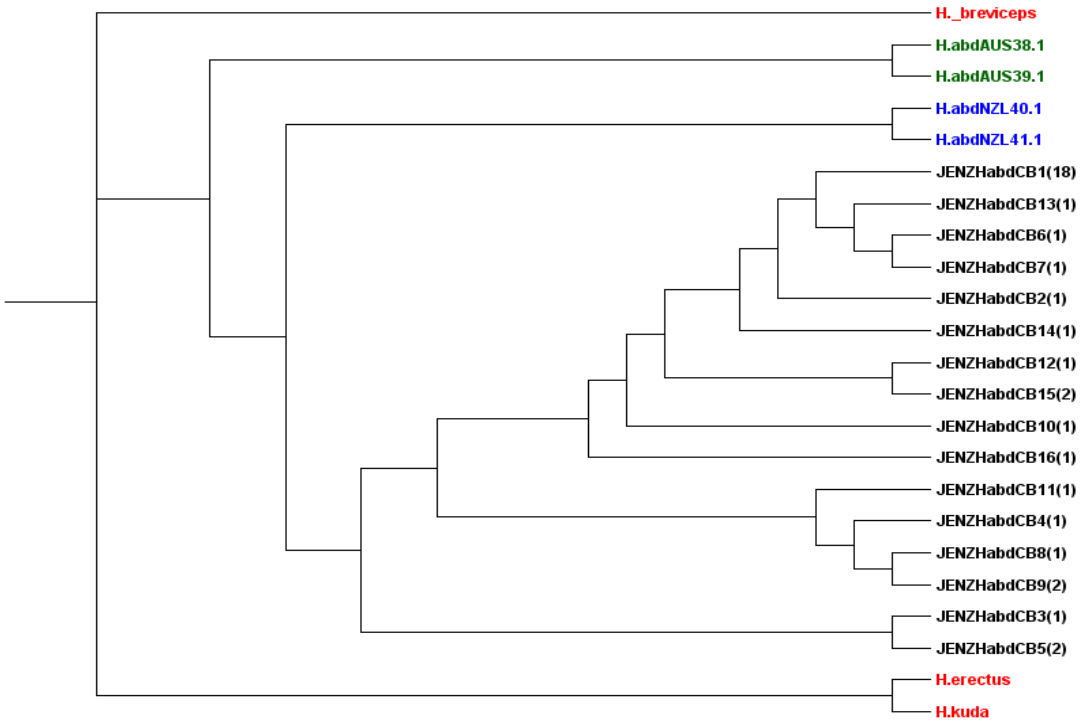


Figure 14: The initial Neighbor Joining tree produced for the cytochrome b data, showing the three other species *H. breviceps*, *H.erectus* and *H.kuda* (in red) as basal to the other haplotypes, and the Australian *H. abdominalis* sequences (green) sister to the New Zealand haplotypes.

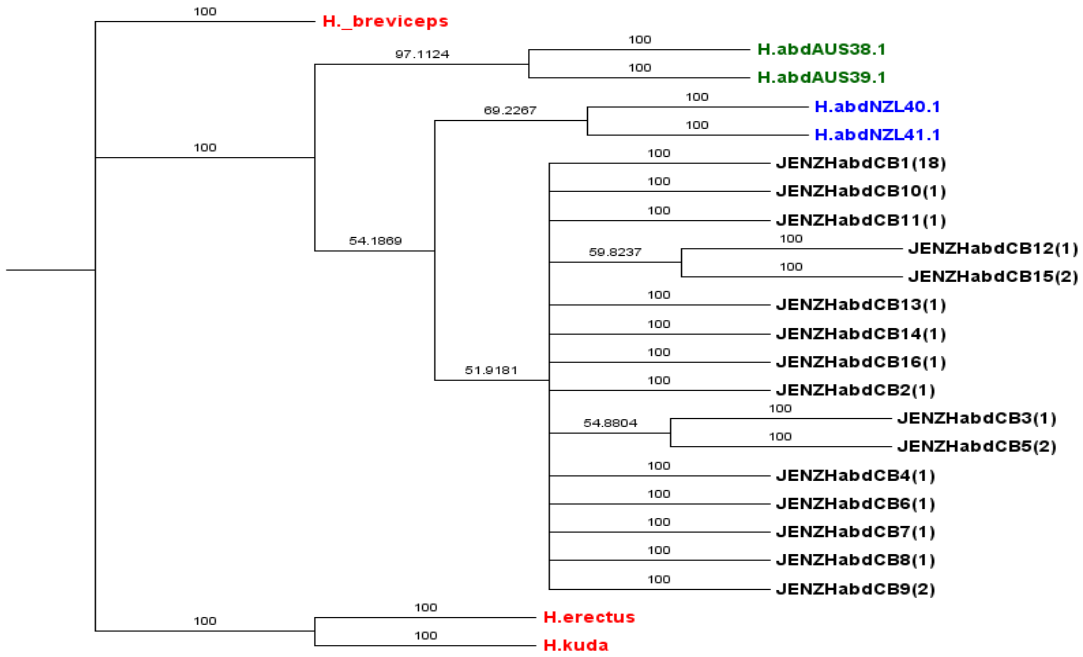


Figure 15: Consensus maximum parsimony estimation (1000 bootstraps) of 23 cytochrome b mtDNA haplotypes and outgroups, showing a monophyletic clade of New Zealand *H. abdominalis* haplotypes with a confidence index of 0.8586, retention index of 0.7735. Bootstrap support is given above branches.

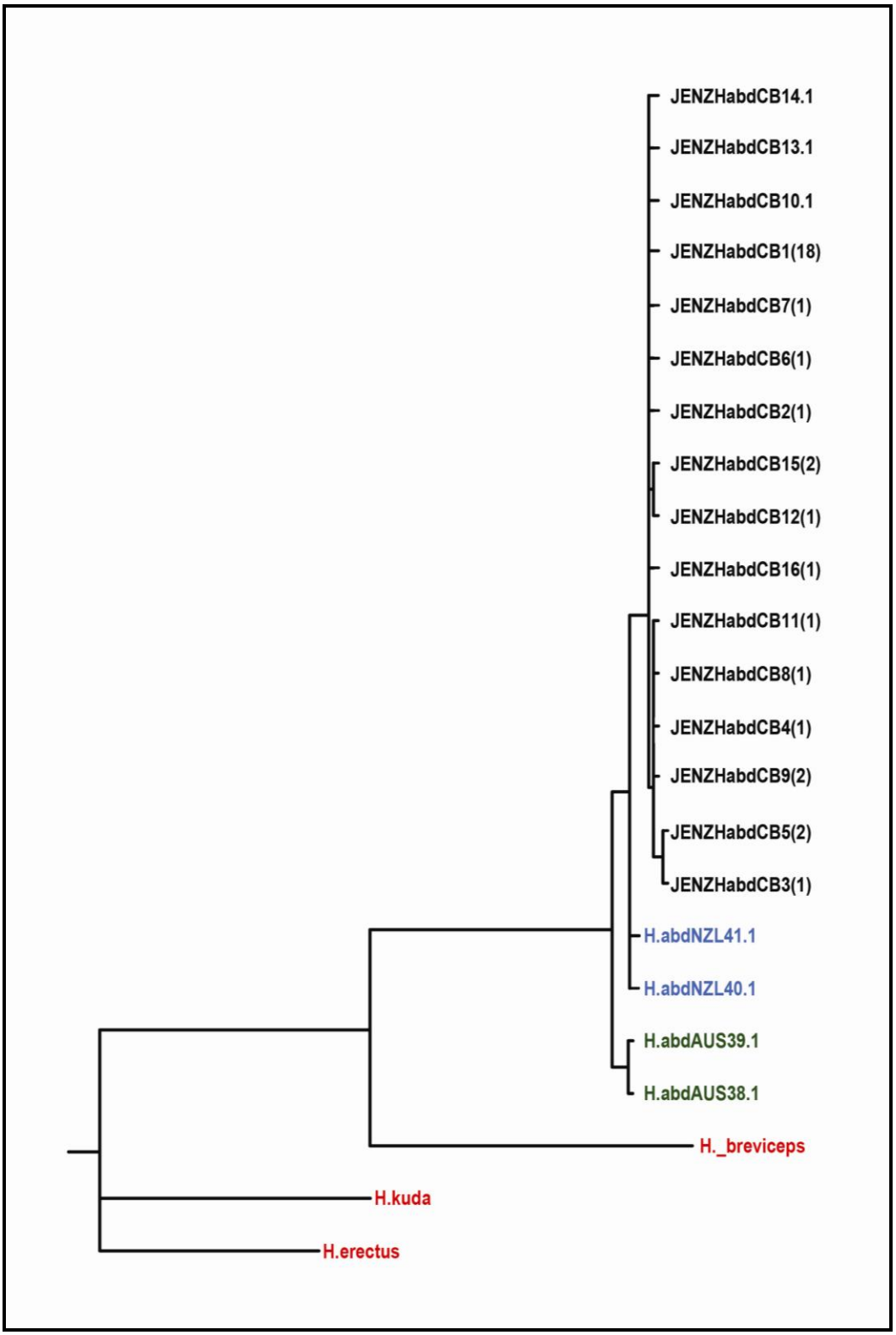


Figure 16: Maximum likelihood estimation (HKY model) of 23 cytochrome b mtDNA haplotypes and outgroups, showing New Zealand *H. abdominalis* as a monophyletic clade.

The relationships between the cytochrome oxidase 1 haplotypes and the Genbank derived *Hippocampus kuda* outgroup sequence (as outlined in Section 2.2) were visualized using neighbor joining (Figure 17), maximum parsimony (Figure 18) and the maximum likelihood method (Figure 19). A pair-wise difference matrix for the cytochrome oxidase 1 sequences is shown in Table 12. Modeltest selected the Hasegawa-Kishino-Yano (HKY) model (Hasegawa et al., 1985) as the most appropriate model of evolution for cytochrome oxidase 1. All estimation methods place *Hippocampus kuda* (in red) basal to the New Zealand *H. abdominalis* haplotypes (in black), which represent a monophyletic clade. The neighbor joining tree was bootstrapped 1000 times, and yielded a topology identical to the maximum parsimony consensus estimation which utilized 6 informative characters to produce a tree length of 99 steps (after 1000 bootstraps) with a confidence index of 0.9394 and a retention index of 0.1429. In this case there is a high degree of similarity between sequences but a low degree of synapomorphies between branches. The resolution of the consensus neighbor joining and maximum parsimony tree was very low, with only the CO11 and CO13 haplotypes (originating from Wellington and Whangateau respectively) grouping together. The initial neighbor joining tree and the maximum likelihood estimation both place the CO7 haplotype (originating from Tauranga) separate to the other haplotypes generated in this study. Following which CO6 (unknown origin) is placed separate. Furthermore, these two methods group the CO8 and CO10 haplotypes (originating from Wellington and Raglan respectively) sister to each other, as well as CO11 and CO13 as mentioned earlier.

Table 12) Pair-wise difference matrix of cytochrome oxidase 1 sequences which the distances used to calculate phylogenetic relationships are based on. Interspecies differences are much larger than intraspecies differences.

	CO1	CO2	CO3	CO4	CO5	CO6	CO7	CO8	CO9	CO10	CO11	CO12	CO13	CO14	CO15
CO1															
CO2	1														
CO3	1	2													
CO4	1	2	2												
CO5	2	3	3	3											
CO6	1	2	2	2	3										
CO7	2	3	3	3	4	1									
CO8	4	5	5	5	6	5	6								
CO9	1	2	2	2	3	2	3	5							
CO10	1	2	2	2	3	2	3	3	2						
CO11	2	3	3	3	4	3	4	6	3	3					
CO12	1	2	2	2	3	2	3	5	2	2	3				
CO13	2	3	3	3	4	3	4	6	3	3	2	3			
CO14	1	2	2	2	3	2	3	5	2	2	3	2	3		
CO15	1	2	2	2	3	2	3	5	2	2	3	2	3	2	
H. kuda	79	80	80	78	81	79	78	81	80	78	81	80	79	80	80

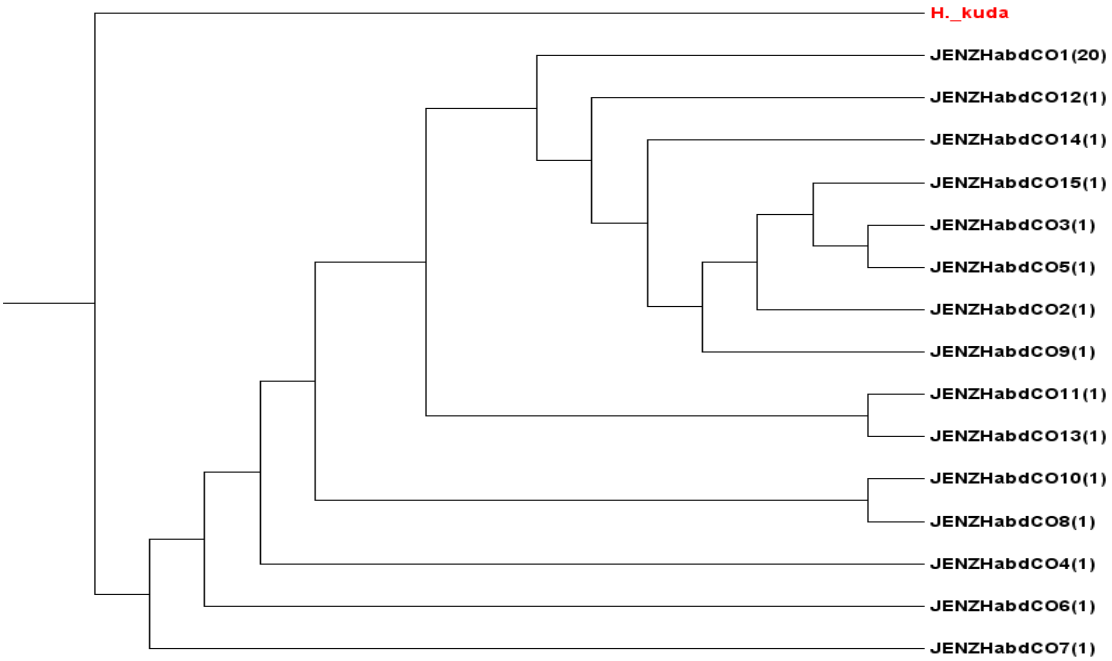


Figure 17: The initial Neighbor Joining tree produced for the cytochrome oxidase 1 data, showing the outgroup *H.kuda* (in red) as basal to the New Zealand *H. abdominalis* haplotypes.

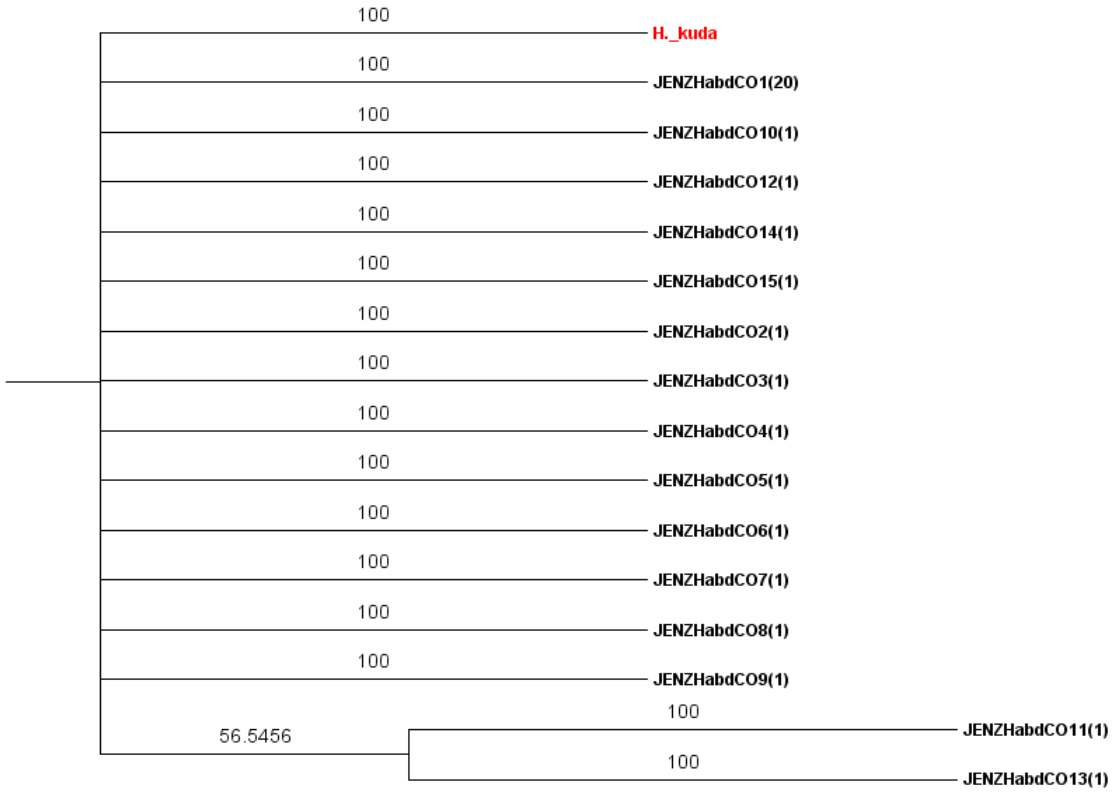


Figure 18: Consensus maximum parsimony estimation (1000 bootstraps) of cytochrome oxidase 1 mtDNA data, with the *H. kuda* outgroup (in red) basal to the New Zealand *H. abdominalis* haplotypes, with a confidence index of 0.9394, retention index of 0.1429. Bootstrap support is given above branches.

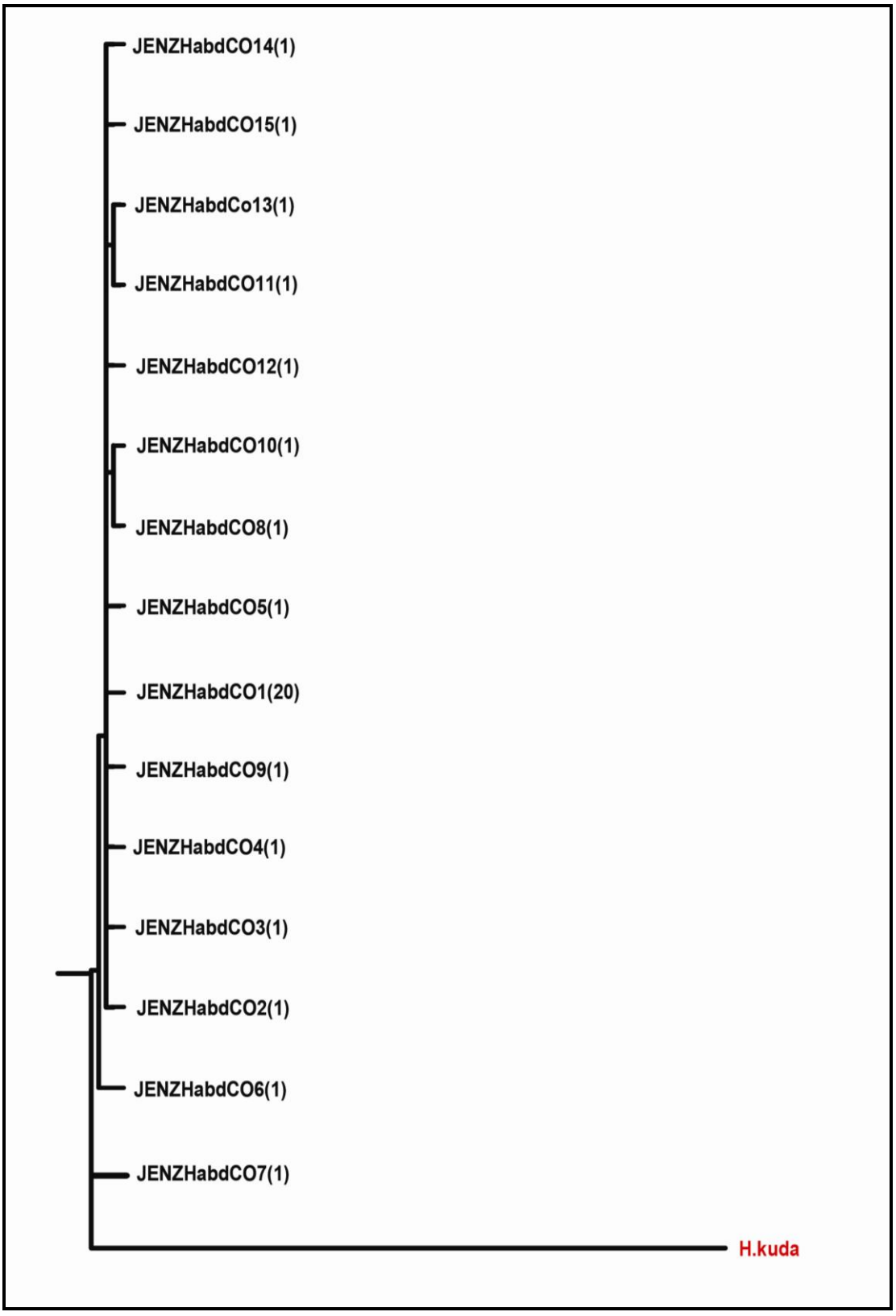


Figure 19: Maximum likelihood estimation (HKY model) of New Zealand *H. abdominalis* cytochrome oxidase 1 mtDNA haplotypes, and the *H. kuda* outgroup.

The relationships between the mitochondrial control region haplotypes and the Genbank derived *Hippocampus kuda* 'outgroup' sequence (as outlined in Section 2.2) were visualized using neighbor joining (Figure 20), maximum parsimony (Figure 21) and the maximum likelihood method (Figure 22). A pair-wise difference matrix for the control region sequences is shown in Table 13. Modeltest selected the Hasegawa-Kishino-Yano (HKY) model (Hasegawa et al., 1985) as the most appropriate model of evolution for the control region. All estimation methods place *Hippocampus kuda* (in red) basal to the New Zealand *H. abdominalis* haplotypes (in black), which represent a monophyletic clade. The neighbor joining tree was bootstrapped 1000 times, and yielded a topology identical to the maximum parsimony consensus estimation which utilized 4 informative characters to produce a tree length of 83 steps (after 1000 bootstraps) with a confidence index of 0.9398 and a retention index of 0.000, indicating a high degree of similarities between sequences and no synapomorphy between branches. There was no resolution in the consensus neighbor joining and maximum parsimony tree, but the initial neighbor joining tree and the maximum likelihood estimation both place the MC2 haplotype (originating from Tauranga) basal to the other haplotypes generated in this study. Furthermore, these two methods group the MC8 and MC9 haplotypes (originating from Tauranga and Jackson's Bay respectively) sister to each other. There is further structuring evident in the initial neighbor joining tree but this is lost in the superior maximum likelihood tree.

Table 13) Pair-wise difference matrix of mitochondrial control region sequences which the distances used to calculate phylogenetic relationships are based on. Interspecies differences are much larger than intraspecies differences.

	MC1	MC2	MC3	MC4	MC5	MC6	MC7	MC8	MC9	MC10	MC11	MC12	MC13	MC14
MC1														
MC2	6													
MC3	1	7												
MC4	2	8	3											
MC5	2	8	3	4										
MC6	3	9	4	3	5									
MC7	1	7	2	3	3	4								
MC8	2	8	3	4	4	5	3							
MC9	2	6	3	4	4	5	3	2						
MC10	1	7	2	3	3	4	2	3	3					
MC11	1	7	2	3	3	4	2	3	3	2				
MC12	1	7	2	1	3	2	2	3	3	2	2			
MC13	1	7	2	3	3	4	2	3	3	2	2	2		
MC14	1	7	2	3	3	4	2	3	3	2	2	2	2	
H. kuda	75	77	76	76	75	78	75	75	75	75	76	76	75	76

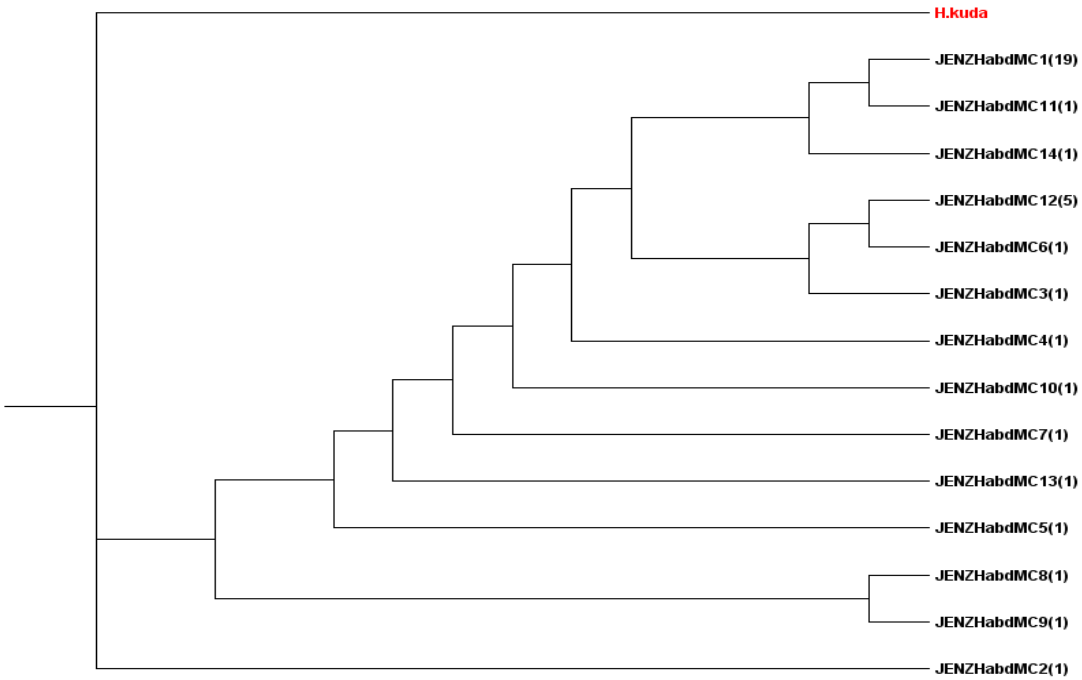


Figure 20: The initial Neighbor Joining tree produced for the mitochondrial control region data, showing the outgroup *H.kuda* (in red) basal to the New Zealand *H. abdominalis* haplotypes.

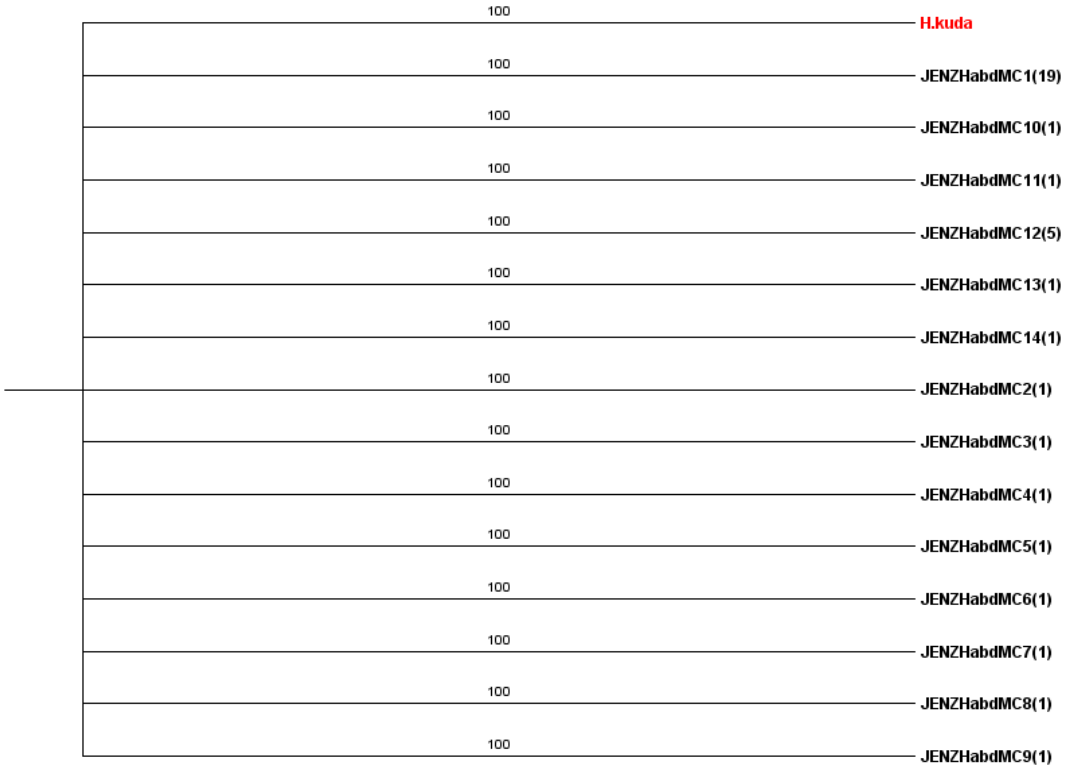


Figure 21: Consensus maximum parsimony estimation (1000 bootstraps) of New Zealand *H. abdominalis* mtDNA control region haplotypes and the H. kuda outgroup, with a confidence index of 0.9398 and a retention index of 0.0000. Bootstrap support is given above branches.

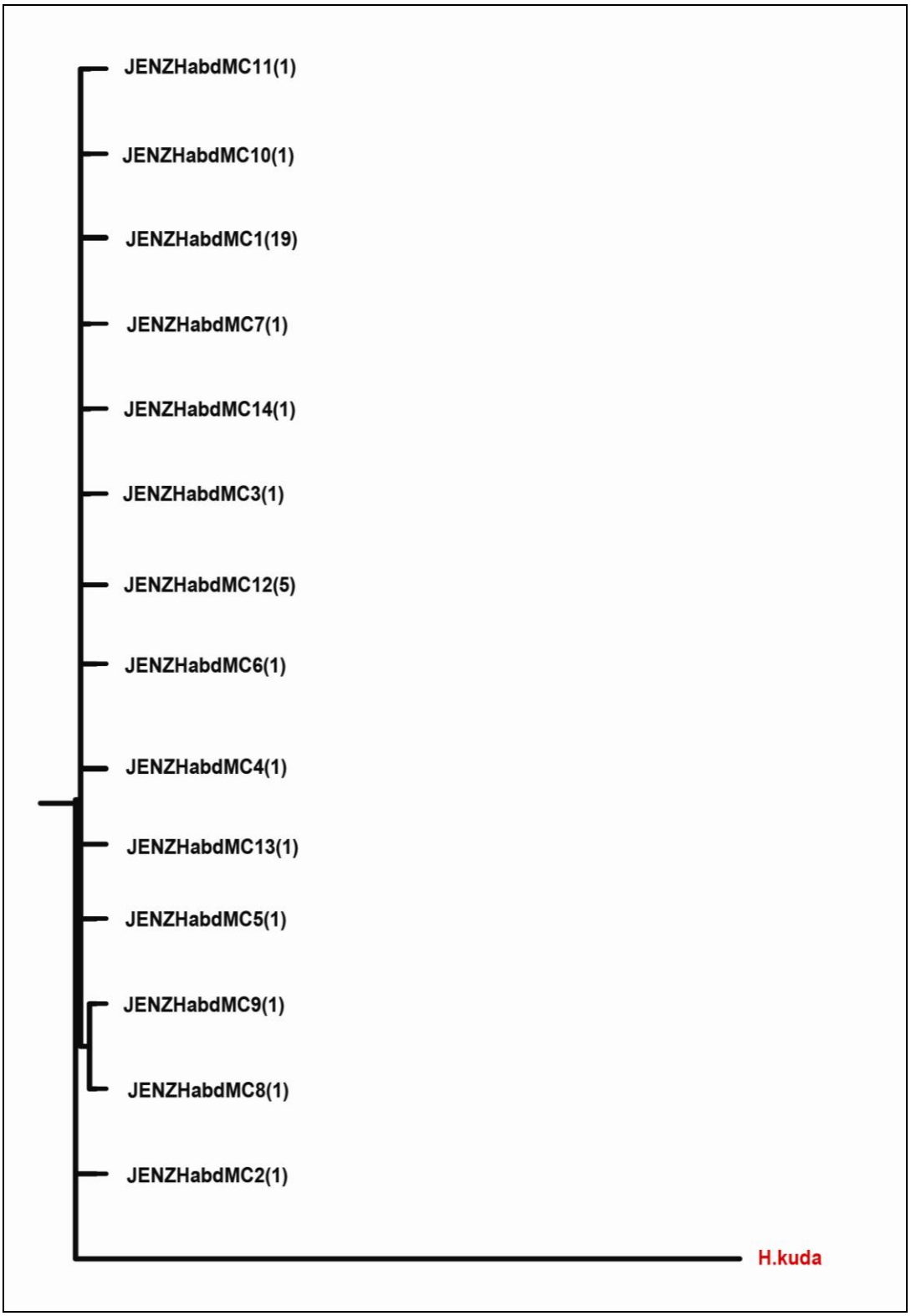


Figure 22: Maximum likelihood estimation (HKY model) of New Zealand *H. abdominalis* mitochondrial control region haplotypes, and the *H. kuda* outgroup.

3.3. Nuclear Microsatellite Markers

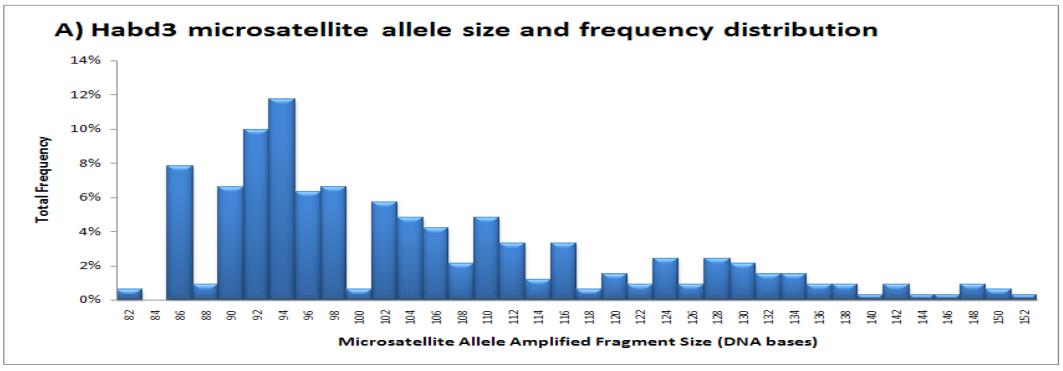
3.3.1. Descriptive statistics and allele size distributions

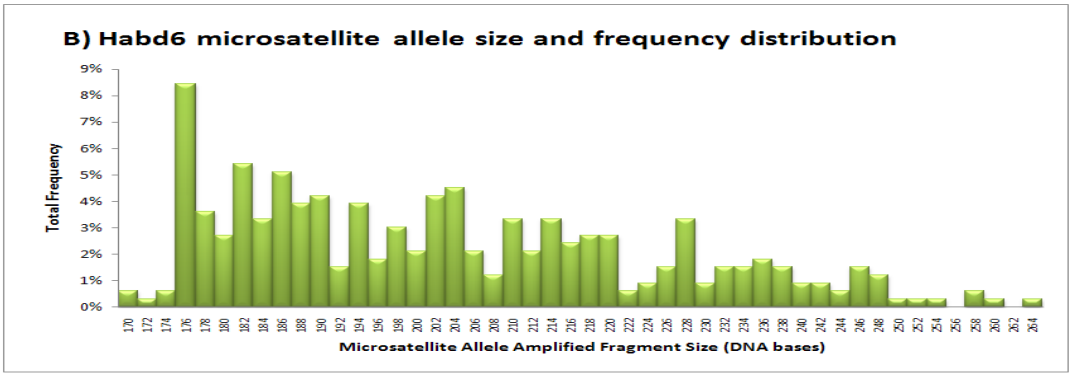
The four loci displayed an average 36.75 (+/- 6.45) alleles per locus, in various ranges of size. All allele sizes are given as PCR amplified fragment lengths (Table 14). The smallest microsatellite marker was Habd3 with a size range of 82-152 bases (mean = 104.5; s.d. = 15.71) and the largest was Habd7 with a size range of 328-404 bases (mean = 345.7; s.d. = 14.75). There are very few gaps in the allele frequency distribution (Figure 23a-d) for all loci, but Habd9 and Habd7 display some outlier allele sizes. Common allele frequencies reached over 8% of the population for Habd6 and Habd9, over 10% for Habd3 and over 15% for Habd7. The most frequent allele sizes were '94 bases', '194 bases', '244 bases' and '336 bases' for Habd3, Habd6, Habd9 and Habd7 respectively. No common multi-locus genotypes were found, strong indicator for a very high level of polymorphism.

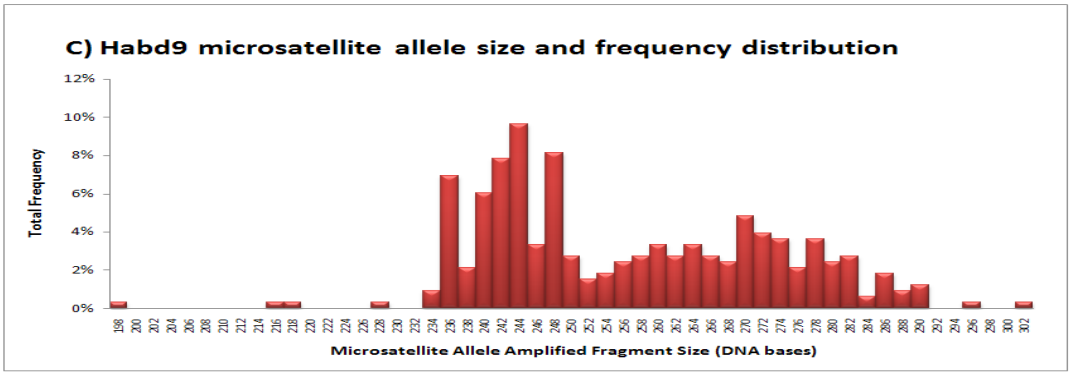
Table 14: The number of alleles for the four microsatellite markers (Habd3, Habd6, Habd9 and Habd7) and their ranges in size (all given as PCR fragment size) for New Zealand *H. abdominalis* (n=166).

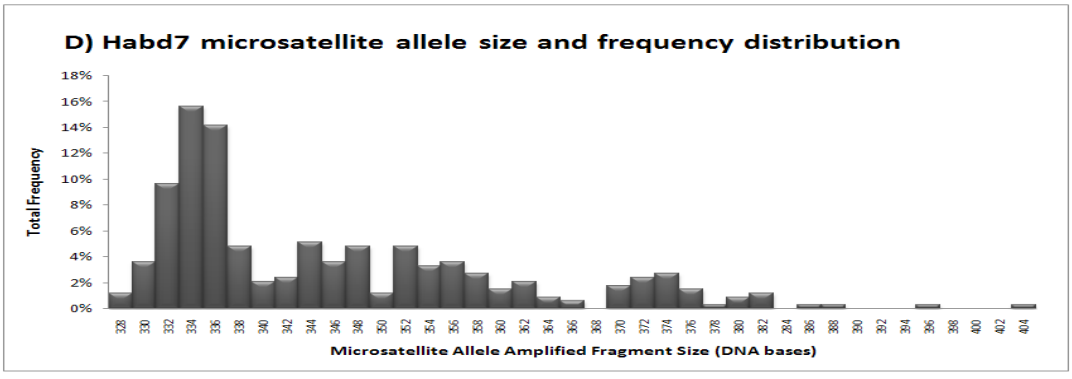
		Allele size (PCR fragment size in DNA bases)			
Marker	**Number of Alleles**	**Mean (+/- SD)**	**Minimum**	**Maximum**	**Range**
Habd3	35	104.5 (15.71)	82	152	70
Habd6	46	203.2 (21.67)	170	264	94
Habd9	35	256.4 (16.44)	198	302	104
Habd7	31	345.7 (14.75)	328	404	158
Mean (+/- SD)	36.75 (6.45)	227.5 (100.9)	194.5 (101.8)	280.5 (104.1)	106.5 (32.2)

Figure 23: Allele size and distributions of microsatellite loci of New Zealand *H. abdominalis*, shown as histograms a) Habd3, b) Habd6, c) Habd9, d) Habd7.









The allele frequencies per wild sampled population are presented on heatmap tables for Habd3 (Table15a), Habd6 (Table15b), Habd9 (Table15c) and Habd7 (Table15d). Allele size ranges did not vary extensively between populations, with the exception of the outlier allele sizes (i.e. '198 bases' and '302 bases' in Jackson's Bay, and '218 bases' in Wellington for Habd9; and '396 bases' in New Plymouth and '404 bases' in Dunedin for Habd7). All populations had a high level of polymorphism, the majority of alleles in each population are at very low frequencies. Low frequencies are shaded green and this progresses through yellow until high frequencies are found shaded in red. Frequencies that are consistently above 0.1 for a population are generally from populations with small sample sizes (n) such as Whangateau and Jackson's Bay.

Table 15a: Wild collected *H. abdominalis* populations show a wide range of allele sizes for Habd3 microsatellite marker at low frequencies (in green), more frequent alleles are shown in yellow and red. The sample size is given for each population as a reference and a total column is provided on the right hand side to show the proportion of alleles in New Zealand.

Habd3 Allele size (base pairs)	Individual Population Allele Frequencies										Total Percent (%)
	RAG	WHA	TAU	NPY	WEL	NAP	CHC	DUN	JAB	STW	New Zealand
82	0.08					0.06					1.39
84											0
86			0.06	0.05	0.15		0.04			0.06	3.54
88							0.04			0.06	0.91
90	0.04				0.15		0.07	0.06			3.22
92	0.08	0.1	0.06	0.05	0.05	0.19	0.07	0.19	0.1		8.86
94	0.19	0.2	0.06	0.2	0.05	0.13	0.14	0.13		0.11	12.09
96	0.04			0.1		0.13		0.13	0.1	0.06	5.44
98	0.04	0.1	0.06	0.2			0.11		0.1	0.11	7.19
100					0.05					0.06	1.06
102	0.08	0.2	0.13	0.05	0.1		0.07		0.2	0.06	8.79
104	0.08	0.1	0.06		0.1		0.04	0.19			5.63
106				0.05	0.1		0.04		0.1	0.11	3.97
108	0.08		0.06						0.1	0.06	2.95
110	0.04		0.06			0.06	0.04			0.17	3.66
112	0.08		0.06		0.05		0.04		0.2	0.06	4.81
114		0.1	0.06				0.04				1.98
116				0.05		0.13				0.11	2.86
118						0.06					0.63
120			0.06					0.06			1.25
122				0.05	0.05			0.06			1.63
124	0.04						0.11		0.1		2.46
126					0.05						0.5
128		0.1	0.06	0.05	0.05		0.07				3.34
130				0.05		0.13	0.04	0.06			2.73
132	0.04	0.1	0.06					0.06			2.63
134	0.04					0.06		0.06			1.63
136				0.05							0.5
138	0.04						0.04				0.74
140	0.04										0.38
142					0.05						0.5
144			0.06								0.63
146											0
148			0.06			0.06					1.25
150				0.05			0.04				0.86
											100

Table 15b: Wild collected *H. abdominalis* populations show a wide range of allele sizes for Habd6 microsatellite marker at low frequencies (in green), more frequent alleles are shown in yellow and red. The sample size is given for each population as a reference and a total column is provided on the right hand side to show the proportion of alleles in New Zealand.

Habd6 Allele size (base pairs)	Individual Population Allele Frequencies										Total Percent (%)
	RAG	WHA	TAU	NPY	WEL	NAP	CHC	DUN	JAB	STW	New Zealand
170										0.06	0.56
172	0.04										0.38
174			0.06			0.06					1.25
176	0.12		0.06			0.06	0.07	0.13		0.11	5.48
178	0.04				0.05	0.06	0.11				2.58
180	0.08	0.1					0.04	0.06	0.1	0.06	4.31
182			0.06	0.1	0.1		0.04				2.98
184			0.06	0.25		0.06					3.75
186	0.08		0.06		0.05	0.06	0.07	0.13	0.1	0.06	6.04
188			0.06			0.06	0.07				1.96
190	0.08	0.1		0.05	0.15		0.04				4.13
192				0.05	0.05	0.06					1.63
194		0.1	0.06		0.05	0.06	0.07	0.19	0.1	0.06	6.89
196	0.04			0.05	0.05	0.06			0.1		3.01
198	0.04	0.1			0.1				0.1	0.06	3.94
200	0.04	0.1	0.06	0.05			0.04				2.87
202	0.08		0.06	0.1	0.05		0.07			0.06	4.16
204		0.1		0.1		0.13	0.07		0.1		4.96
206				0.05			0.04	0.06		0.06	2.04
208					0.05		0.04	0.13			2.11
210	0.04	0.2		0.05			0.04	0.13			4.49
212	0.08				0.05	0.06		0.06			2.52
214	0.04		0.06	0.05	0.1	0.06		0.06		0.11	4.87
216								0.06		0.17	2.29
218	0.08			0.05							1.27
220			0.06				0.04		0.2		2.98
222					0.05						0.5
224										0.06	0.56
226						0.06	0.04				0.98
228											0
230			0.06		0.05	0.06					1.75
232										0.06	0.56
234		0.1									1
236					0.05	0.06	0.04				1.48
238	0.04								0.1		1.38
240	0.04			0.05			0.04				1.24
242	0.04	0.1								0.06	1.94
244											0
246	0.04								0.1		1.38
248			0.06							0.06	1.18
250											0
252			0.06								0.63
254			0.06								0.63
256											0
258			0.06				0.04				0.98
260											0
262											0
264							0.04				0.36
											100

Table 15c: Wild collected *H. abdominalis* populations show a wide range of allele sizes for Habd9 microsatellite marker at low frequencies (in green), more frequent alleles are shown in yellow and red. The sample size is given for each population as a reference and a total column is provided on the right hand side to show the proportion of alleles in New Zealand.

Habd 9 Allele size (base pairs)	Individual Population Allele Frequencies										Total Percent (%)
	RAG	**WHA**	**TAU**	**NPY**	**WEL**	**NAP**	**CHC**	**DUN**	**JAB**	**STW**	New Zealand
198									**0.1**		**1**
200											**0**
202											**0**
204											**0**
206											**0**
208											**0**
210											**0**
212											**0**
214											**0**
216											**0**
218					**0.05**						**0.5**
220											**0**
222											**0**
224											**0**
226											**0**
228											**0**
230											**0**
232											**0**
234										**0.06**	**0.56**
236	**0.12**	**0.1**		**0.1**	**0.05**	**0.06**	**0.18**		**0.1**	**0.11**	**8.18**
238					**0.05**						**0.5**
240				**0.1**	**0.15**		**0.11**			**0.06**	**4.13**
242	**0.15**	**0.1**	**0.19**	**0.05**	**0.05**	**0.06**	**0.07**	**0.13**		**0.11**	**9.11**
244	**0.12**		**0.06**	**0.1**	**0.1**	**0.19**	**0.07**	**0.13**	**0.1**	**0.22**	**10.84**
246	**0.04**	**0.2**		**0.1**				**0.06**			**4.01**
248	**0.04**			**0.05**	**0.1**	**0.06**		**0.06**	**0.1**	**0.17**	**5.8**
250	**0.04**				**0.05**			**0.06**		**0.06**	**2.07**
252	**0.04**					**0.06**		**0.06**			**1.63**
254			**0.06**			**0.13**	**0.04**				**2.23**
256	**0.04**		**0.13**		**0.05**				**0.1**	**0.06**	**3.69**
258			**0.13**	**0.05**			**0.04**		**0.2**		**4.11**
260			**0.06**	**0.05**	**0.05**	**0.06**	**0.04**			**0.06**	**3.16**
262	**0.04**			**0.1**		**0.06**	**0.07**				**2.72**
264	**0.04**	**0.2**					**0.07**			**0.11**	**4.21**
266			**0.06**	**0.05**		**0.13**	**0.04**				**2.73**
268		**0.1**	**0.06**				**0.04**				**1.98**
270				**0.05**			**0.07**	**0.13**			**2.46**
272	**0.08**				**0.05**	**0.06**		**0.06**			**2.52**
274	**0.12**	**0.2**			**0.05**	**0.06**					**4.28**
276	**0.04**			**0.05**				**0.13**	**0.1**		**3.13**
278	**0.12**			**0.05**	**0.1**		**0.04**	**0.06**	**0.1**		**4.64**
280			**0.13**	**0.05**	**0.05**		**0.04**				**2.61**
282			**0.06**	**0.05**	**0.05**		**0.04**	**0.06**			**2.61**
284		**0.1**									**1**
286						**0.06**					**0.63**
288								**0.06**			**0.63**
290			**0.06**				**0.07**				**1.34**
292											**0**
294											**0**
296											**0**
298											**0**
300											**0**
302									**0.1**		**1**
											100

Table 15d: Wild collected *H. abdominalis* populations show a wide range of allele sizes for Habd7 microsatellite marker at low frequencies (in green), more frequent alleles are shown in yellow and red. The sample size is given for each population as a reference and a total column is provided on the right hand side to show the proportion of alleles in New Zealand.

Habd7 Allele size (base pairs)	Individual Population Allele Frequencies										Total Percent (%)
	RAG	WHA	TAU	NPY	WEL	NAP	CHC	DUN	JAB	STW	New Zealand
330	0.08					0.06		0.06	0.1	0.06	3.57
332	0.15		0.13	0.05		0.13			0.4		8.54
334	0.19	0.1	0.06	0.15	0.15	0.13	0.14	0.25	0.1	0.17	14.39
336	0.19	0.1	0.31	0.1	0.15	0.06	0.29	0.19	0.1	0.06	15.46
338		0.2	0.06	0.05	0.05		0.18	0.06			6.04
340										0.11	1.11
342	0.08	0.1	0.06	0.05		0.06		0.06			4.14
344	0.04	0.1	0.06	0.05	0.1	0.13	0.11		0.1	0.11	7.94
346	0.04			0.05	0.1	0.13		0.13			4.38
348					0.2	0.13	0.04	0.06	0.1	0.11	6.34
350					0.05		0.04			0.06	1.41
352	0.04	0.1	0.13	0.1	0.05					0.06	4.69
354	0.08	0.1		0.1			0.04				3.13
356	0.04	0.1		0.05		0.06	0.04				2.87
358	0.04			0.05	0.05			0.06	0.1	0.06	3.57
360						0.06		0.06			1.25
362			0.06		0.05		0.04			0.11	2.59
364			0.06	0.05							1.13
366				0.05	0.05						1
368											0
370	0.04									0.06	0.94
372										0.06	0.56
374						0.06	0.07				1.34
376		0.1		0.05			0.04				1.86
378											0
380			0.06								0.63
382											0
384											0
386											0
388											0
390											0
392											0
394											0
396				0.05							0.5
398											0
400											0
402											0
404								0.06			0.63
											100

3.3.2. Molecular diversity, population genetics and linkage

The expected number of alleles under the infinite allele model (A_{IAM}) varied from 44-75, slightly larger than the observed number of alleles (31-46). The expected number of alleles under the stepwise mutation model are not reported as they produced underestimations (i.e. expected < observed). The observed heterozygosity ranged from 0.87 to 0.98, with the expected heterozygosity estimated to be slightly higher for all loci except for Habd6. Habd6 was the only marker found to display a negative value for the fixation index (F_{IS}) and all other values were close to zero giving an average of 0.02 (+/- 0.03). None of the loci deviated from Hardy-Weinberg equilibrium, although Habd6 came close with a low p-value of 0.08174. The Garza-Williamson (G-W) statistic, can range between zero (if the population has experienced a recent genetic bottleneck) and one (if the population is stationary). In regards to these loci it ranged in the middle with an average value of 0.38 (+/- 0.13) across all loci.

Table 16: Molecular diversity indices were calculated for the four microsatellite Loci for New Zealand *Hippocampus abdominalis* (n=166).This included the number of observed alleles (A), number of expected alleles under the infinite allele model (A_{IAM}), observed and expected heterozygosity, the p-value testing deviation from Hardy-Weinberg equilibrium (HWE) (*P<0.002), the fixation index F_{IS} and the G-W statistic.

Marker	A	A_{IAM}	Ho	He	HWE p-value	F_{IS}	G-W statistic
Habd3	35	52.04	0.921687	0.944728	0.52704*	0.02295	0.49296
Habd6	46	75.54	0.981928	0.967987	0.08174*	-0.01596	0.48421
Habd9	35	60.21	0.939759	0.955065	0.35192*	0.01456	0.33333
Habd7	31	43.46	0.873494	0.929349	0.13386*	0.05877	0.21384
Mean (+/- SD)	36.75 (6.45)	57.81 (13.65)	0.929217 (0.05)	0.949282 (0.02)	0.27364 (0.21)	0.02008 (0.03)	0.381085 (0.13)

Weak but significant linkage was observed between Habd6 and Habd9 (p=0.04268) under a significance level of 0.05. The fact Habd6 comes close to deviating from Hardy-Weinberg equilibrium will have influenced the detection of linkage.

The mean molecular diversity (i.e. an average across all loci) is reported for each wild collected population (Table 17). A table featuring the breakdown for the separate loci can be viewed in Appendix K. The populations displayed very similar levels of polymorphism. The number of alleles from an average of 8.25-15 and the expected number of alleles under the infinite allele model range from 8.25-16, suggesting that almost all of the alleles truly present in each population were observed. The mean observed heterozygosity reaches the maximum value of 1 in Tauranga and Jackson's Bay, closely followed by Whangateau (0.950) and Stewart Island (0.945). The lowest mean observed heterozygosity was in Christchurch (0.857). The highest overall expected heterozygosity was in the Napier population (0.963) and the lowest again was the population in Christchurch (0.939). None of the populations came close to deviating from Hardy-Weinberg equilibrium and the inbreeding coefficient F_{IS} was never far from zero but Christchurch had the highest (i.e. most inbred) value of 0.074, whilst Jackson's Bay had the lowest value at -0.128.

Table 17: Averaged molecular diversity indices (includes all four microsatellite loci) for wild collected populations of New Zealand *Hippocampus abdominalis*. This included the sample size (n) number of observed alleles (A), number of expected alleles under the infinite allele model (A_{IAM}), observed and expected heterozygosity, the p-value testing deviation from Hardy-Weinberg equilibrium (*P<0.002), the fixation index F_{IS} and the G-W statistic.

Population	N	A	A_{IAM}	H_O	H_E	HWE p-value	F_{IS}
RAG	13	15	16.38	0.865	0.944	0.311	0.067
WHA	5	8.25	8.58	0.950	0.961	0.776	-0.048
TAU	8	13	13.11	1.000	0.960	0.941	-0.081
NPY	10	14	14.25	0.900	0.951	0.511	0.030
WEL	10	13.5	14.40	0.875	0.954	0.631	0.063
NAP	8	12	12.79	0.906	0.963	0.539	0.028
CHC	14	16	17.28	0.857	0.939	0.299	0.074
DUN	8	10.5	11.35	0.875	0.940	0.572	0.040
JAB	5	8.25	8.24	1.000	0.945	1.000	-0.128
STW	9	12	12.98	0.945	0.949	0.662	-0.025

3.3.3. Phylogenetic Relationships

Nei's standard genetic distance (Nei, 1978) was used to compute phylogenetic relationships between populations (Figure 24) and individuals (Figure 25) using neighbor joining. No outgroups were used in the construction of these trees. The populations in Napier and Dunedin show some affinity with each other but are segregated away from the other populations which form a monophyletic clade. Within this, the populations in Jackson's Bay and Stewart Island appear to be sister to each other, and the same can be said for those in Raglan and Christchurch.

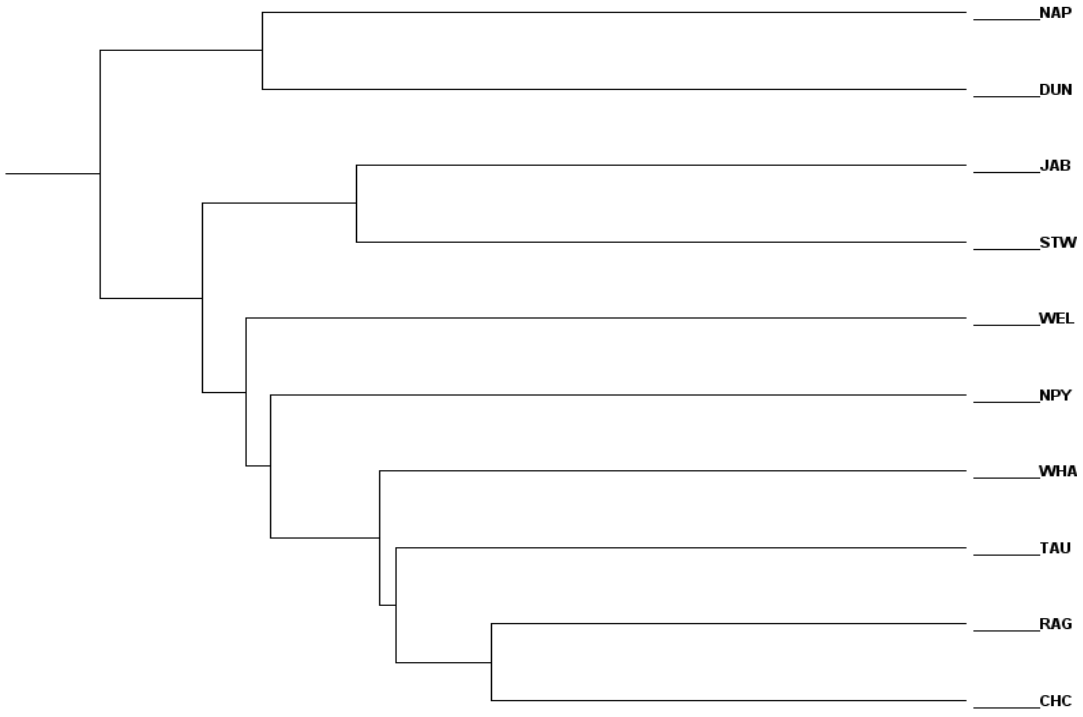


Figure 24: Neighbor joining tree showing the phylogenetic relationships for wild populations of New Zealand *H. abdominalis* (n=90), computed using data from four microsatellite loci (Habd3, Habd6, Habd9, and Habd7).

The phylogenetic tree based on multilocus genotypes of wild collected individuals has been colour coded to show which populations each belongs to, with a trend of colder colours for southernmost populations, and warmer colours for those originating from the northern parts of New Zealand. Whilst some clustering is evident, there are no distinct patterns in a phylogeographical context.

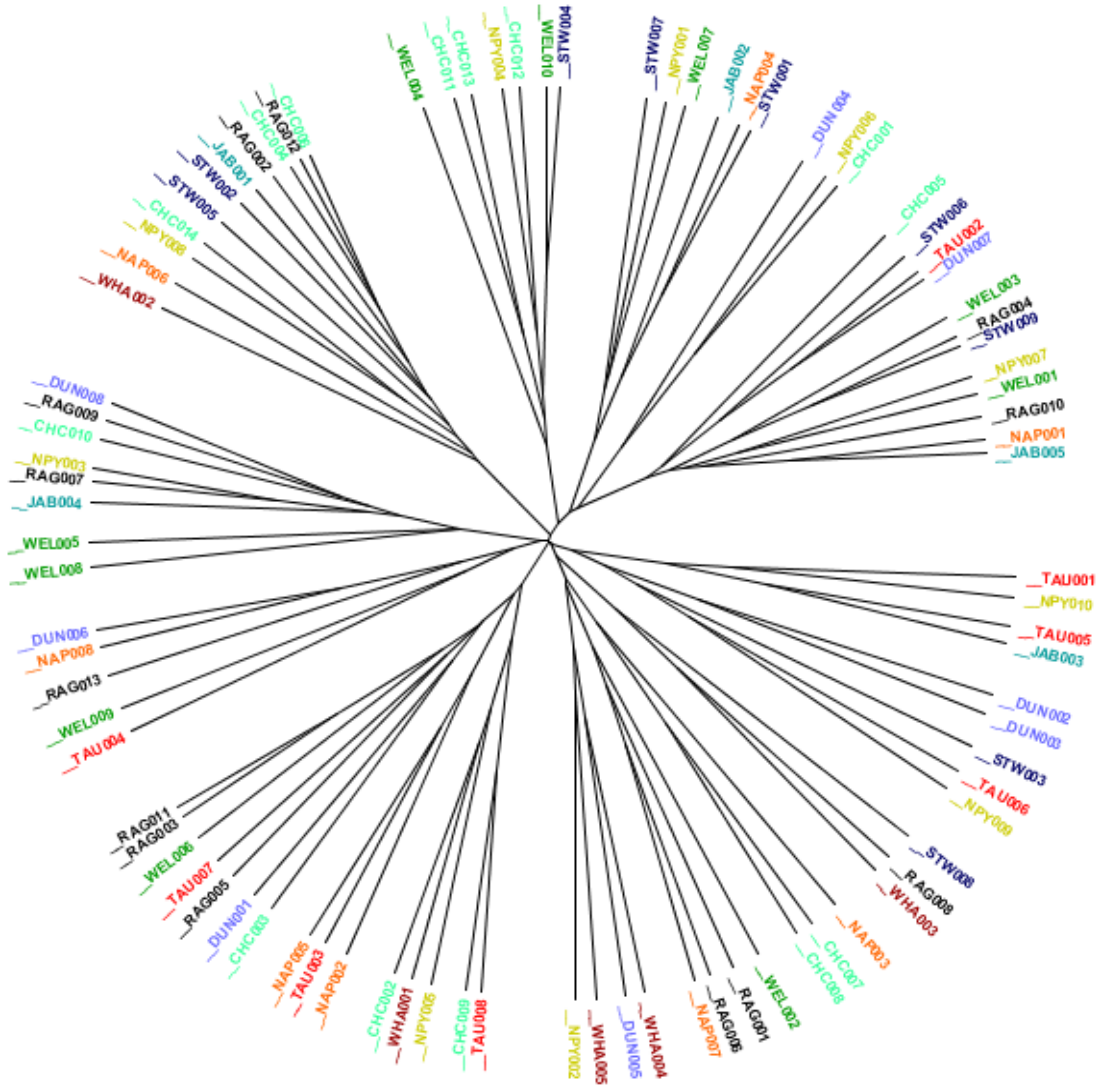


Figure 25: Star-shaped Neighbor Joining tree to show the phylogenetic relationships between 90 wild collected New Zealand *H. abdominalis* individuals using their multilocus microsatellite genotypes. All belong to one of ten wild collected populations: Raglan (RAG; black), Whangateau (WHA; maroon), Tauranga (TAU; red), Napier (NAP; orange), New Plymouth (NPY; yellow), Wellington (WEL; green), Christchurch (CHC; turquoise), Dunedin (DUN; light blue), Jackson's Bay (JAB; medium blue) or Stewart Island (STW; dark blue).

3.3.4. Population pair-wise F_{ST}

A matrix of global population pair-wise F_{ST} values shows that there is very low differentiation between populations. Positive values are emphasized in bold. This is very likely due to the overriding power of high polymorphism within those populations. The significance p-values are reasonably high across all populations. This data suggests that the South Island populations display more genetic structuring than the North Island populations. The highest values are found between the population at Jackson's Bay and Whangateau (F_{ST} = 0.016; p-value = 0.1163) and Dunedin (F_{ST} = 0.015; p-value = 0.1908). Locus specific matrices are available in Appendix L.

Table 18: Wild collected population pair-wise differentiation F_{ST} (below diagonal) and p-values (above diagonal) for New Zealand *H. abdominalis*, combined for all four microsatellite loci. Positive values are emphasized in bold. P-values were calculated through 1023 permutations.

	RAG	WHA	TAU	NPY	WEL	NAP	CHC	DUN	JAB	STW
RAG		0.9447	0.8725	0.6238	0.6823	0.8691	0.7241	0.9386	0.7832	0.5715
WHA	0.000		0.6822	0.8870	0.4848	0.4455	0.9091	0.6471	0.1163	0.2184
TAU	0.000	0.000		0.5960	0.5894	0.4880	0.8883	0.3533	0.6014	0.2640
NPY	0.000	0.000	0.000		0.5251	0.8313	0.6765	0.3170	0.2292	0.2384
WEL	0.000	0.000	0.000	0.000		0.6639	0.6926	0.6647	0.3696	0.7579
NAP	0.000	0.000	0.000	0.000	0.000		0.4154	0.8422	0.5605	0.8925
CHC	0.000	0.000	0.000	0.000	0.000	**0.001**		0.4327	0.1958	0.2181
DUN	0.000	0.000	**0.002**	**0.004**	0.000	0.000	**0.001**		0.1908	0.4033
JAB	0.000	**0.016**	0.000	0.010	**0.004**	0.000	**0.011**	**0.015**		0.3638
STW	0.000	**0.009**	**0.006**	**0.006**	0.000	0.000	**0.007**	**0.001**	**0.005**	

4. Discussion

4.1. Sampling and abundance

Hippocampus abdominalis are found along the coasts of the North and South Island of New Zealand, mainly in low density populations which are sparse in distribution and that sightings outside these populations are rare (personal observation). Van Dijken (2001) only observed seahorses at 6 out of 49 sites (North Island) during the Summer of 1999/2000, causing him to conclude that either seahorses require highly fastidious habitats, or that they are rare in abundance, or that they simply hadn't been found. (Van Dijken, 2001) was not able to find a relationship between habitat characteristics (such as depth, temperature, visibility, percentage of silt, holdfasts, substratum and exposure) and abundance, but one characteristic observed in this study was that seahorses were only ever seen in locations that harboured high bioproductivity. (Van Dijken, 2001) came close to this notion by recording the number of fish species present during surveys but this does not include seahorse prey or the type of biodiversity which attracts seahorse prey, therefore it is recommended that this characteristic is included in future abundance studies.

The population found in Raglan was the only one, out of the nine wild sample sites examined, where *H. abdominalis* were observed in high density. This location was sampled twice, once in February and again in November of 2008, and on each visit the SCUBA divers estimated there to be between 30-50 individuals present. In the February expedition, there was only enough equipment to sample 13 individuals, whereas in November there was no limitation. Genotyping of these samples made it apparent that no specimen was sampled twice, suggesting that either the previously sampled individuals had migrated, or died (although this is very unlikely to have occurred due to our disturbance as these specimens were handled in exactly the same manner as all the others during the study, over a third of which were held in

Aquariums, and no mortalities were reported), or it may have been simple coincidence. There may be other high density populations elsewhere in New Zealand, but these must be very inaccessible or their whereabouts not widely disclosed.

4.2. Morphology

155/169 photographs were of sufficient quality so that the samples they depicted could be evaluated for morphological length measurements and other features sometimes displayed by this species. It is acknowledged that one of the three cameras used did not have a high enough megapixel capability to consistently produce the detail required for photographic analysis and that future studies need to make this a chief priority, especially if more detailed morphological features are investigated. A multiple number of photos were taken for each sample, this turned out to be highly useful and is also recommended for future studies if this method was employed. This study assumes that taking three independent measurements per photo would have created a final score of the same quality as taking the measurement from a live and struggling fish, as the quality of the measurement via either method depends heavily on the Researcher's aptitude. In either case, the quality of the measurement depends on the researchers' aptitude. Considering the advances in digital photography, morphological feature analysis from photographs is an underutilized method for scientific studies, as this provides the opportunity to reanalyze a sample and keep a record noninvasively (i.e. without the deposit of voucher specimens).

The "Guide to the Identification of Seahorses" by (Lourie et al., 2004), is the most recent and accepted version of seahorse taxonomy. It claims *H. abdominalis* display a head length to snout length ratio between 2 and 5.1 (with the most common value being 2.6). Whilst it is not disputed that across the entire geographic range and age groups the head length to snout length ratio can be of this size, this study only found a range of 1.9-3.9 (with the median equaling 2.6 and the average being a value of 2.7). It is still not known what factors determine snout length in seahorses. Unfortunately, Armstrong (2001) calculated a snout length to head length ratio percentage, and therefore the data from his study was not comparable. Francis (1988) postulated that New Zealand *Hippocampus abdominalis* are larger than those

found in southeast Australia. This study has now provided a representative version of descriptive statistics, at least for New Zealand, so this theory can now be tested. It is worthy to note that the smallest seahorse sampled was a male of 192 mm, this is still far above the 8-9cm point at which this species reaches sexual maturity which means all samples in this study qualify as adults. Armstrong (2001) measured the 'height' of 32 *H. abdominalis* individuals across the entire geographical range and observed a range of 55.5-226.7 mm, although the small sample size is unlikely to be a good representation for the entire species. Wilson and Martin-Smith (2007) sampled three *H. abdominalis* populations in southeast Australia (in the Derwent Estuary, Northwestern Bay, and the Huon River with sample sizes of 20, 27 and 65 respectively) and reported the 'height' of each sample in millimeters. As 'height' is a different measure (tip of the coronet to tip of the tail) to 'standard length' (the sum of head length, trunk length and tail length) the data is not comparable at this stage, but it is unlikely that there is any significant size difference between the two countries. The other morphological variables assessed do not have any comparable counterparts as this was the first time some of these traits (e.g. presence of fronds, colour) have been quantified and some others are often assessed differently (e.g. 'number of tail rings' rather than 'tail length in millimeters'). Lourie (Lourie et al., 2004) measured many other morphological traits (e.g. number of trunk rings, number of dorsal fin rays, etcetera) for the purpose of identification, this study had a different focus and therefore such specialized measurements were sacrificed in place of other indicators of diversity such as proportions of frond, spine, and colour display. However, this provides a good baseline data set for any future studies that choose to record the variables in this manner.

The sexual dimorphism of *H. abdominalis* size has been assessed previously though, finding that the males display shorter snouts and longer tails than females(Foster, Vincent, 2004). This study measured 80 males and 75 females of New Zealand *H. abdominalis* and discovered with the use of the Mann Whitney Wilcoxon test that the snout length, head length to snout length ratio, and the trunk length differed significantly ($P<0.05$). Thus the claim that the snout length is significantly shorter in

males is reinforced. The fact that the tail length was not found to be significantly different between sexes was surprising as it is often claimed to be longer in males to support the brood pouch. Instead the trunk length was named to be significantly shorter in males. When the summary statistics were consulted it is obvious that the tail length is longer in males, but this difference is confounded or weakened (and reinforced in the case of trunk length) due to the amount of large females that were found (i.e. average standard length in males was 192.6 mm compared to 195.6 mm in females). Although standard length was not found to be a significant deviator between males and females, indicating that there must be no strong advantage for this to vary between sexes. In all cases, there was a large amount of overlap between the ranges meaning none of the significances found are sexually distinguishing features.

The dependence of all continuous variables on standard length has limited their applicability for population comparisons within this study, as standard length had some mutually exclusive ranges between populations and this would have created a strong bias. The trait that would be least influenced by this is the head length to snout length ratio, but as this was found to be significantly different between males and females it could not be used to represent populations. The Stewart Island Aquarium had held their seahorses for many years and so these had been fattened excessively in captivity, but the same sizes are obviously achievable in the wild as seen in the population sampled from the Napier mussel farm. It is not clear why the population in Tauranga consisted only of small individuals, more studies will need to be carried out on their population ecology and site fidelity.

H. abdominalis was found to exhibit a range of colours which could be classified into ten categories and described proportionately. These categories were sufficient to provide baseline data for the species, and allows for the declaration that generally 1 in 5 *H. abdominalis* specimens are lightly coloured. Colour is an ambiguous variable to measure as it varies so widely in Teleost fish, which is why there was no effort made to delineate this further with the use of colour cards. Brash *et al.* (2008) puts this variation down to the fact Teleost fish have multiple copies of the pigmentation

genes found in tetrapods, which they claim is most likely due to the fish-specific whole genome duplication event that occurred around 320-350 million years ago (Braasch et al., 2008). Due to the high variety in colouring, it is not surprising that a few colours conceal other traits this species is known for such as tail banding. 12% of all individuals did not display obvious tail banding, and this was usually when the body colouring was dark or the degree of spotting very low (reducing contrast also). This reinforces Lourie *et al.*'s (2004) observation that the tail is 'often' striped, but not always. Across all samples, the degree of spotting was very evenly distributed between the categories of 'heavy', 'medium' and 'low'. 37% of these samples originated from recent Aquarium conditions, which needs to be noted as the artificial lighting may produce unusual spotting colours (Mass, 2007). Fronds and spines were each observed in a quarter of specimens sampled, so it could be said that these features are relatively uncommon. It is still not known what causes the incidence of either of these traits. Brood pouches occur in males only, and these were observed to be lightly coloured in nearly a third of all males, more often than not a yellow slash was present also. The incidence of a yellow slash was much higher than the incidence of a white brood pouch but neither occurred consistently. There is no known correlation for these traits either and as no similar quantitative assessments have been published for a *Hippocampus* species this data could not be readily compared with other studies. Lourie *et al.* (2004) stated that another sexually dimorphic trait is the heightened degree of spotting on the upper body of male *H. abdominalis*. This claim can be supported by the data generated in this study. Another trait that varied was the presence of fronds, with males displaying these more often (39%) than females (13%) overall. The presence of spines and the proportions of each body colour displayed did not vary by sex. Colour, spine and frond presence did vary between populations however, and it is very likely that it is mainly influenced by environmental factors (see (Mass, 2007)) but a more detailed ecological study with larger sample sizes would be required to find trends in regards to these traits. On the whole, whilst there is very high morphological diversity, none of these characters are distinguishing sexual dimorphic characters or verified population identifiers due to this.

4.3. Mitochondrial DNA markers

This study provided new DNA sequences for three mtDNA markers - cytochrome b (814 base pairs), cytochrome oxidase 1 (624 base pairs) and the control region(404 base pairs) - which revealed a maximum sequence divergence of 0.7%, 1% and 2.2% between haplotypes respectively.

The cytochrome b sequences generated in this study had 20 informative sites over 814 base pairs, with nucleotide frequencies very similar to those found for the majority of the genus by Teske *et al.* (2004) and a nucleotide diversity ($\pi = 0.0021$) similar to what was found for *H. trimaculatus* ($\pi = 0.0017$) by Lourie and Vincent (2004). However the haplotype diversity was considerably lower for New Zealand *H. abdominalis* ($h = 0.75$) than for *H. trimaculatus* ($h = 0.94$). In regards to the sequence divergence, the study by Armstrong is most appropriate for comparison. In Armstrong's (2001) study, based on 357 base pairs of the cytochrome b gene, a maximum sequence divergence of 0.28-2.24% was found within the *H. abdominalis* species, and 8.68-22.13% between *Hippocampus* species. This molecular data was required after Kuiter's (2000) morphologically based conclusions questioned the number of species in this area. The genetic data of this study, with the aid of the morphological data, reconfirms the pronouncement by Armstrong (2001) that New Zealand *H. abdominalis* is one species. Nevertheless, the relationship of *H. abdominalis* across the Tasman Sea still requires further study. As the current study investigated a larger portion of the cytochrome b gene, and found much lower sequence divergence, it is very likely that the majority of genetic diversity uncovered by Armstrong (2001) came from the samples that originated from Australia. Even though more data is required, it can be speculated that the New Zealand *H. abdominalis* population may have spawned from the Australian population via a founder effect of a few individuals crossing the Tasman Sea at some point in history, most likely during a period of low sea level. The direction of the current flow in the

Tasman Sea and the fact that Australia is central to seahorse biodiversity underpin this theory.

This is the first study to examine cytochrome oxidase 1 sequences in *Hippocampus*; therefore the revealed characteristics can not be compared to any other species in the genus. However, Ward *et al*. (2005) examined cytochrome oxidase 1 sequences of 207 species of fish using the same primers as utilized in this study, and concluded that Teleost cytochrome oxidase 1 sequences generally have a GC content of 47.1 % (+/- 0.2%) and it can be noted that the most common haplotype in Cytochrome oxidase 1 of New Zealand *H. abdominalis* is slightly lower at 42%. It is expected that more cytochrome oxidase 1 sequences will be generated in the future, mainly for the universal 'barcode of life' initiative (Ratnasingham, Hebert, 2007). Thus, it is possible that these sequences have a high potential for future use.

Seahorse population structure has most often been investigated with the use of mitochondrial control region sequences. The nucleotide diversity (π) of the New Zealand *H. abdominalis* mitochondrial control region is 0.0033 and the haplotype diversity (h) is 0.66. Both values fall within the ranges described by Teske *et al*. (2005) from when they investigated the global *H. kuda* complex (π = 0.0017-0.0076; h = 0.48-0.084) and the same can be said for *H. capensis* (π = 0.0030-0.0046; and h = 0.48-0.84 also). The study on *H. capensis* Teske *et al.* (Teske et al., 2003) also commented on the transition transversion ratio and the mean number of pair-wise differences (d), said to be 8:1 and 1.2-1.86 respectively. The transition transversion ratio for the control region in New Zealand *H. abdominalis* is slightly lower at 13:4 but the mean number of pair-wise differences (d = 1.34) fell within their range.

The 122 new mtDNA sequences uncovered by this study are an immense contribution to the current understanding of *H. abdominalis* genetics overall. Unfortunately, as there were three markers and ten wild collected sample populations to be represented, only 2-5 sequences were generated to represent each marker for each population, which does not constitute as high representation. Furthermore, these

were sequenced from samples randomly, and therefore only eight individuals had two out of three markers sequenced, and only two individuals had all three markers sequenced. Initially, the intention of this was to spread the participation of individuals but it has resulted in the fact that sequences could not be concatenated and therefore the statistical analyses regarding the diversity of wild collected populations could not be computed with high statistical power, and no breakdown was given of molecular diversities at the wild collected population level. This could have been avoided had the suggestions of Ryman *et al.* (2006) been followed which state that statistical power and alpha should be assessed before launching a study, either through simulation or comparisons with estimates from similar investigations. In hindsight, more robust analyses could have been achieved with less markers and higher sample sizes per population for the mitochondrial DNA investigation. However, due to resource constraints the number of individuals to be sequenced was always limited, as was the number of fieldtrips to collect wild individuals.

Unsurprisingly, the mtDNA of *H. abdominalis* appears to be similar in nature to that of other species in the genus but with a relatively low level of diversity relative to what has been observed in terms of sequence divergence for *H. abdominalis* overall (Armstrong, 2001) and well below levels that are thought to constitute species delineation. Cytochrome b had the highest number of haplotypes and mean number of pair-wise differences between them but this marker about a quarter longer than cytochrome oxidase 1 and over twice the length in sequence to the control region marker which gave the highest nucleotide diversity. Overall all three markers can be considered to be very similar in diversity even though cytochrome b and cytochrome oxidase 1 sequences are under slightly different selective pressures than the regulatory control region (a trend flaunted by the relative nucleotide compositions) but by and large they are linked in the evolution of the mtDNA. Measures such as Tajima's D, Fu's F_S concurred across all three markers. All values were negative due to an excess of low frequency polymorphisms, which may be due to a recent population expansion, genetic drift and/or positive selection (Tajima, 1989). Fu (1997) states that Fu's F_S and Tajima's D are very powerful for testing population

growth and genetic hitchhiking, and that Fu and Li's D (Fu, Li, 1993) values are better for testing background selection. In future, it is recommended that all tests be employed to discriminate between the possibilies. Supplementary to these results, signatures of population expansion were also suggested by the strictly unimodal nature of the observed data fitting to the mismatch distribution model and the corresponding Harpending's raggedness index which was not significant, indicating that the sudden expansion model was not rejected.

The most common haplotype for each marker was found in almost all wild collected populations around the coast, the exception for cytochrome b was the Napier population, for cytochrome oxidase 1 the Raglan population and for the control region the Jackson's Bay population. As these populations are located in the northwest, northeast and southwest (i.e. all over) New Zealand, there is no consistent or obvious pattern of barriers to gene flow. The high number of private alleles observed in populations could indicate that migration between populations is gradual or stepwise, but as some other haplotypes are observed in Raglan and Stewart Island (i.e. CB9), or between Raglan, Tauranga, Jackson's Bay, Stewart Island and Titahi Bay (i.e. MC12), this could just be due to a lack of coverage and in fact the genetic diversity overwhelms the sampling strategy. The relationship between haplotypes was investigated via neighbor joining, maximum parsimony and maximum likelihood analyses. As the single nucleotide polymorphisms were distributed evenly throughout the markers, no gamma correction was applied. Since the bootstrapped neighbor joining trees were identical in topology (and very similar in values) to the maximum parsimony trees, only the latter was provided.

Maximum likelihood is a parametric method considered to be superior to the other two methods as it fits the data to the most appropriate model of sequence evolution for analysis. The Hasegawa-Kishino-Yano (HKY) model (Hasegawa et al., 1985) assumes that all bases have different equilibrium base frequencies and that transitions evolve at different rates to transversions. Jones *et al.* (2003) also found the HKY model to be the most appropriate (in their case whilst working on cytochrome b).

Most of the phylogenetic trees were presented as cladograms as to show their detail, but the maximum likelihood trees were displayed as phylograms to show relative branch lengths. For cytochrome b, seven outgroup sequences were available from Genbank, and these rooted each tree independent of the analysis method used. Very little genetic structuring was observed after bootstrapping and with the maximum likelihood method, the haplotypes that were sister to each other originated from locations that shared major current systems (i.e. CB3 and CB5 share the south-eastern currents and CB12 and CB15 share the north-eastern currents). Cytochrome oxidase 1 and control region haplotypes showed some structuring also with the phylogenetic analyses but without an obvious current connection, which could indicate that the cytochrome b haplotype current connection is simply a coincidence. There was only one outgroup available for cytochrome oxidase 1, from the *H. kuda* whole mtDNA genome sequence (Kawahara et al., 2008), and whilst there were other inter-species control region sequences available there was no need for more than one.

4.4. Nuclear DNA microsatellite markers

Even though the microsatellite loci primers were designed based on Australian *H. abdominalis* specimens (Wilson, Martin-Smith, 2007), all amplified successfully for all 169 samples, implying a low degree of divergence for the flanking regions of each locus between populations across the Tasman Sea. Difficulties in sequencing the fragments means the exact number of repeats are not certain, thus this was not reported (chromatograms are available in Appendix E). Two programs were used to aid in the identification of genotyping errors such as null alleles, large allele dropout, and typographic errors; but none were found. Whilst Microsatellite Analyzer version 4.05 (Dieringer, Schlötterer, 2003) calculated the number of expected alleles under both the stepwise mutation model (SMM) and the infinite allele model (IAM), only the latter was reported as the SSM model gave underestimations below the true values observed. Thus it appears that the true mutational mechanism of these loci is likely to have more affinity to the IAM than the popular SMM. All four microsatellite loci displayed distributions which were skewed to right (i.e. shorter alleles occur more frequently) and Habd7 had the largest range in allele size with a range extending 158 bases (or 79 repeats). This is another verification of the concept that long alleles have higher degrees of variation. Long microsatellite alleles often have higher error rates (Hoffman, Amos, 2005), therefore extra scrutiny was directed towards the Habd7 marker. This included occasional re-amplification and verification though independent genotype runs when results were ambiguous but even despite this the locus has a tendency to exhibit an excess of homozygotes (H_O = 0.87) when compared with its expected heterozygosity (H_E = 0.93). A reassuring factor was that Wilson and Martin-Smith (2007) found similar observed heterozygosity values for this locus (H_O = 0.867; H_E = 0.92) in the Australian *H. abdominalis* populations. The most polymorphic locus was Habd6 (46 alleles) with a higher observed (H_O = 0.98) than expected (H_E = 0.97) heterozygosity. Overall, very high levels of polymorphism were observed, to the extent of 31-46 alleles per locus (i.e. Habd3 = 35; Habd6 = 46; Habd9 = 35; Habd7 = 31), with no identical multi-

locus haplotypes observed over 166 individuals. Wilson and Martin-Smith (2007) generated genotyping data for 112 Australian *H. abdominalis* specimens and observed 33 alleles for Habd3, 36 for Habd6, 26 for Habd9 and 27 for Habd7. The reason that Wilson and Martin-Smith (2007) detected a lower number of alleles is most likely due to the fact that their sample size is a whole third smaller than of this study. It should be noted that the PCR amplified fragment for the Habd7 allele was much larger in this study (range = 328-404 bases) in comparison to the Australian *H. abdominalis* (the cloned product was reported to be 250 bases in length). A broader across the entire geographic range would be required to determine whether there is any merit to this observation. Jones *et al.* (2003) worked with a similar sample size to Wilson and Martin-Smith (2007) using three microsatellite loci in *H. subelongatus* and uncovered 24-42 alleles per locus. Also, Galbusera *et al.* (2007) used 15 loci whilst working with *H. capensis* from both wild and captive populations and found a range of 3-18 and 2-8 alleles per locus respectively. However, these populations can be expected to have lower diversity as *H. capensis* as the entire study was to verify a recent genetic bottleneck. This study did not compare wild collected populations to Aquarium sourced populations as most of the stock in Aquariums was relatively recently acquired from the wild and as actual information on origins was minimal. Whilst Galbusera et al. (2007) proved that their 15 loci cross amplified in *H. abdominalis,* these were not used due to resource constraints and as they were only verified in one sample.

High polymorphism is a common feature of microsatellite loci, DeWoody and Avise (2000) genotyped 524 microsatellite loci across 78 species of fish (12 of which were marine), and revealed that marine fish have a much higher allelic diversity (20.6 +/- 11.8) and mean heterozygosity (0.79 +/- 0.26) than non-marine fish. In comparison to these figures, *H. abdominalis* had excessively high allelic diversity (36.75 +/- 6.45) and mean heterozygosity (0.94 +/- 0.02) across all four loci. Whilst diversity is high, differentiation between populations is very low; the most common allele frequency reached over 15% of all samples and only a few private alleles were

observed. There was a fairly even distribution of alleles across the wild sampled populations, with no obvious trends, an indication that gene flow is extensive.

Wilson and Martin-Smith (2007) observed significant linkage (LRT; $p<0.05$) at Habd6/7 within the Sydney population, Habd 6/9 in the Huon River population, and Habd 3/6 in the Northwestern Bay population. A weak but significant linkage was observed for New Zealand *H. abdominalis* between Habd6 and Habd9 ($p=0.04268$), this may have just managed to slip under the significance threshold as the likelihood ratio test assumes all loci are in Hardy-Weinberg equilibrium and Habd6 gets very close to deviating from this with a p-value of 0.082. It is unlikely that a recent genetic bottleneck has occurred in the New Zealand population as the distribution of alleles has very few gaps at intermediate lengths (bottleneck events cause for a faster reduction in the number of alleles than the allele size range), and this is supported by the G-W statistic also.

The neighbor joining tree for the microsatellite populations shows no patterns in genetic structure and even the individual tree has no clustering by geographical locations. The intra-population diversity is so high that it is overpowering the degree of population differentiation. Due to this, no isolation by distance effect was investigated. Marine species often display low F_{ST} values due to the high level of gene flow that is facilitated by the open nature of the ocean (Waples, 1998). Ward *et al.* (1994) reviewed fish studies of population genetics and found the mean F_{ST} for marine species to be 0.062, with over 60% having values lower than 0.03. Each comparison also had very high p-values, this is most likely due to the fact that even with the microsatellite data the sample sizes were not large enough for precise estimation of genetic distances. Generally sample sizes of over 50 individuals are best, but this varies with the number of alleles and their range (Ruzzante, 1998).

4.5. Threats and conservation

It is hoped that this data will contribute to lifting *H. abdominalis* from the data deficient listing. This study and the study by Van Dijken (2001) are the only investigations into *H. abdominalis* abundance in New Zealand, and both have concluded that this species can be found consistently in a few locations but are very rare to be seen in between. When Van Dijken (2001) was monitoring populations, one site had a very significant decline in population numbers, it was hypothesized that this may have occurred to collection (either by Aquariums or hobbyists) or an environmental perturbation (which was not noticeable) or migration. During the sampling process of this study it became evident that hobbyist seahorse collectors are plentiful in New Zealand, especially in Northland and Raglan. Although in Raglan the collections are often for traditional medicine instead of 'backyard aquariums' (personal observation). Such threats facing *H. abdominalis* are unfortunately not yet recognized and are still understudied in New Zealand. The effect of bycatch is unknown as the reporting is still inadequate. Recently, *H. abdominalis* was rejected from entry into the quota management system and also removed from Section 4C of the Fisheries Act of 1996 (Ministry of Fisheries, 2008), meaning in the future, even less attention will be paid to this species. So unlike Australia which is increasingly protecting Syngnathids, New Zealand is taking steps backwards in their monitoring and protection of the species, mainly under the claim of insufficient data.

Due to the desirable characteristics that *H. abdominalis* possesses, the inadequate protection afforded to them under New Zealand law and the declining nature of seahorse species worldwide, it is very likely that this species will increase in abundance on the traditional medicine market. In case further forensic work such as that by Sanders *et al*. (2008) is carried out, 112 new sequences are available for comparison purposes.

There were many reports of seahorses taken and dried as curios by fishermen (N. Ryan, personal communication; S. Friar, personal communication; R. Adams, personal communication). The only continual and registered occurrence of this is the supply for a business named 40 Degrees South Fishing Company Ltd. (a licensed fish receiver with the Ministry of Fisheries) which collects seahorses caught as bycatch from licensed commercial fishermen and dries them for local sale and export for between $510-800 per kilogram (J. Hall, personal communication).

Fortunately *H. abdominalis* is a more resilient species than many others, with large brood sizes (from mature males), the capability of travelling a few hundred meters a day and now this can also be affirmed by the claim that this species exhibits a high genetic diversity. Furthermore, with no or little population structuring, dispersal can be referred to as unlimited, and this would many any future conservation initiative easier (Neigel, Avise, 1993).

4.6. Recommended future research

Future avenues of research include more intensive surveying of *H. abdominalis* abundance in quantitative measures the Ministry of Fisheries can make use of. Both this study and the study by Van Dijken (2001) demonstrate that *H. abdominalis* are not very commonly found, suggesting that these organisms may go under the radar if data is not generated. Whilst this study was able to infer that gene flow between all populations is high, the process of how this occurs (i.e. the means of dispersal, and whether it is active or passive, at the juvenile and the adult stage) is still not certain. Microsatellites are very fast evolving DNA markers, but did not provide enough detail in this regard, so it is recommended that *in situ* population ecology studies are employed. The use of SCUBA diving clubs in such research is recommended as these groups often involve proactive environmentally friendly individuals who know local marine habitats well and this would curb sampling costs. A similar claim was made by (Goffredo et al., 2004). This study found that one of the characters desired in the traditional medicine trade (i.e. light colouring) and some of the characters desired in the aquarium trade (i.e. fronds, spines) occur relatively rarely and an investigation into the causes of these desirable traits would be interesting not only to these trades but for evolutionary biology in general.

4.7. Conclusions

This study utilized mitochondrial DNA and microsatellite DNA markers, as well as some morphological data, to re-confirm that there is only one species of *H. abdominalis* in New Zealand and to quantify how diverse this species is in regards to these factors. As *H. abdominalis* is likely to be facing a heightened degree of exploitation in the future, with little protection within New Zealand as of date, it is hoped that the results from this study will make the implementation of a conservation management plan easier to implement in the future.

New Zealand *H. abdominalis* are very morphologically diverse, displaying large ranges in all the variables measured, and it was possible to quantify these as representative for the New Zealand population as a whole. Ten dominant body colours were identified, and features such as fronds and spines were found to be relatively uncommon overall. Sexually dimorphic characteristics identified include a shorter snout length and trunk length in males, which also display a higher occurance of fronds, higher degrees of spotting and of course the presence of a brood pouch (which displayed a yellow slash most of the time but was only lightly coloured in about a third of the samples). Whilst some population differentiation was observed most likely due to environmental influences, these features were not distinguishing.

The mitochondrial DNA markers - cytochrome b (814 bp), cytochrome oxidase 1 (624 bp) and the mitochondrial control region (404 bp) – revealed sequence divergences of 0.7%, 1% and 2.2% respectively from sample sizes of 36-40. 112 new sequences were generated in total for the species yielding 14-16 haplotypes per marker, and it is hoped that these will be referred to in future studies. The sequences representing *H. abdominalis* exhibit nucleotide and haplotype diversities that fall within previously established ranges of Hippocampus diversity and estimates of selective neutrality and population expansion state that the New Zealand population has very likely recently undergone population expansion, perhaps due to a

founder effect linked to Australia. 14-16 haplotypes were found per marker, one being very common and there being many other less frequent, and these had very close phylogenetic relationships even thought they originated from varying origins around the country, suggesting that gene flow is very high, and this was reinforced by very low F_{ST} values.

The microsatellite markers exhibited very high levels of polymorphism with an average of 36.75 +/- 6.45 alleles per locus, with relatively large and even distributions in size for New Zealand as a whole and across the ten wild collected populations. As the stepwise mutation model underestimated the expected number of alleles, it is proposed that the evolution of these loci has more affinity with the infinite allele model. None of the loci deviated from Hardy-Weinberg equilibrium and only slight linkage disequilibrium was detected between Habd6 and Habd9. The high diversity of these markers in combination with the G-W statistic which found no strong signatures of recent population bottlenecks, suggests that the possible founder event proposed by the relative low diversity of the mtDNA data (when compared to Armstrong's (2001) data for the entire geographic range) occurred a very long time ago. All of the populations showed similar values of diversity, and private alleles were rare. The high degree of intra-population variation seems to have overwhelmed any inter-population differentiation that was investigated, also corroborated by very low F_{ST} values and the lack of population structuring displayed in the phylogenetic trees.

In conclusion, New Zealand *H. abdominalis* show a very high diversity in morphology and in regards to microsatellite and mitochondrial DNA markers, but with very low differentiation between populations, suggesting that the individuals from all coasts are in the population genetic extreme of high connectivity among populations. The data generated from this study should be considered as an indicative baseline representing the New Zealand population of *Hippocampus abdominalis.*

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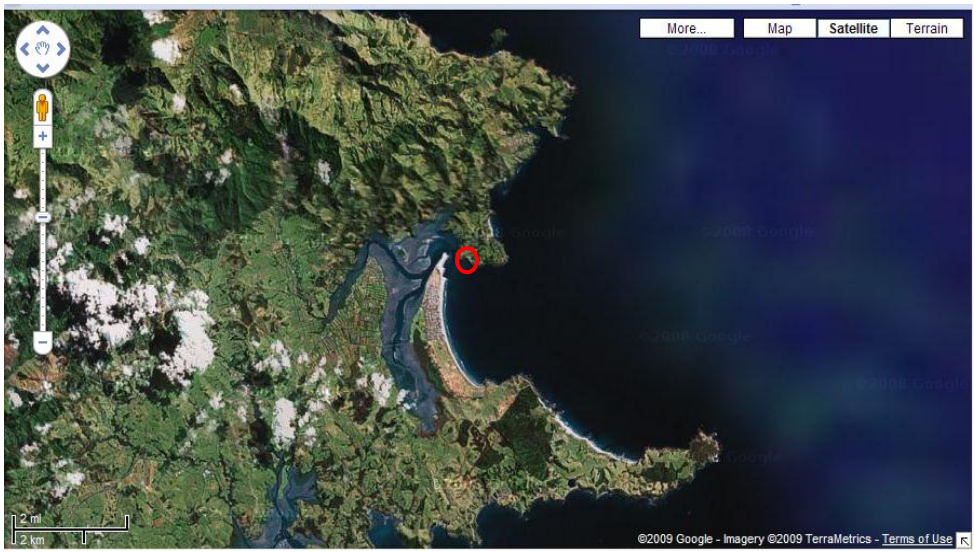
Appendix A – *In situ* Sample sites

RAGLAN (RAG and RAG2):

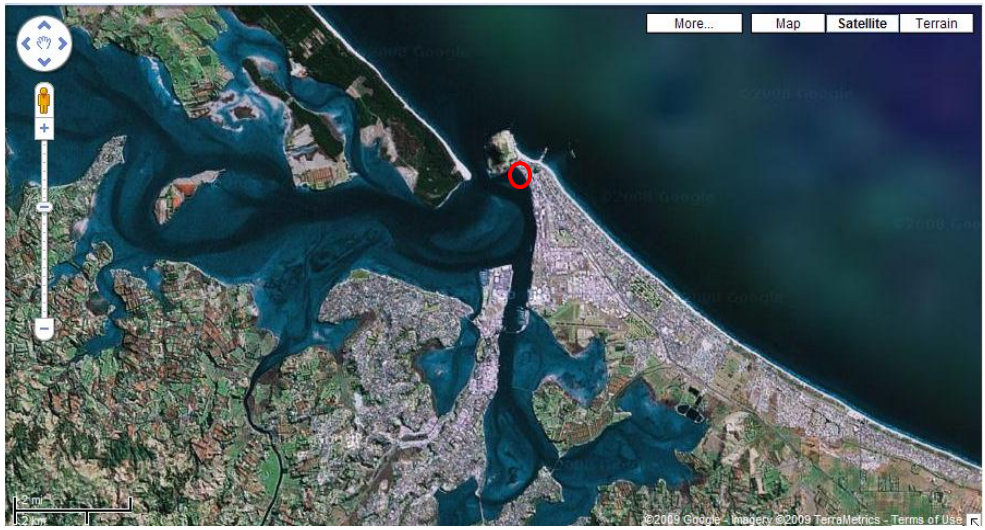
Raglan harbour is a small township located on the west coast of the upper North Island. Thirteen seahorses (although more were present) were sampled (ID: AA, AC-AN) on the 4th of February 2008, and thirty-two were sampled (ID: DX-FC) on the 23rd of October 2008 from under the main fishing wharf and the immediate surrounding area (red circle).

WHANGATEAU (WHA):



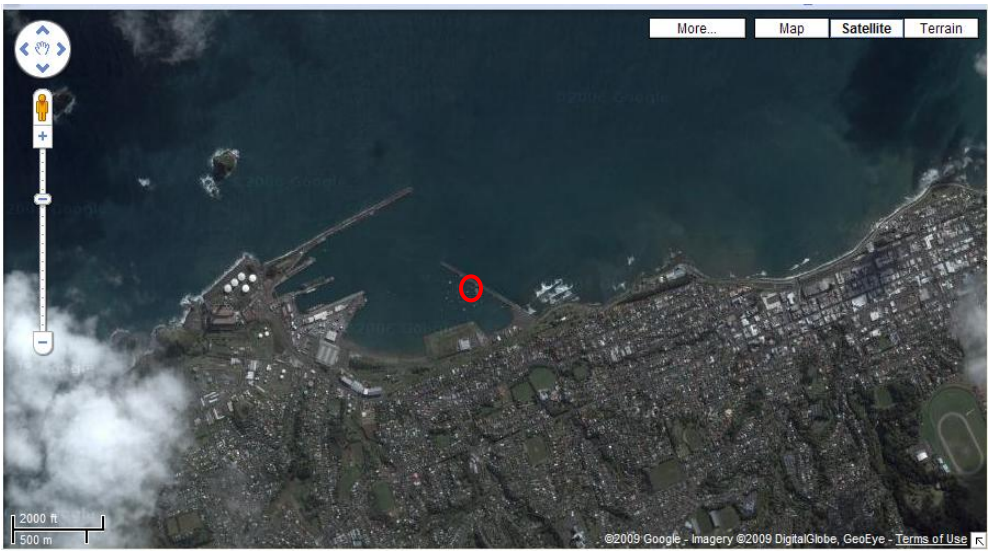
Whangateau harbour is located on the northeastern coast of the North Island and has a high biodiversity of fish species due to nearby marine reserves. Five seahorses (ID: AO-AS) were found on the 24th of March 2008 to the left and right hand side of Ti point jetty (red circle) swimming in plumes of macroalgae.

TAURANGA (TAU):



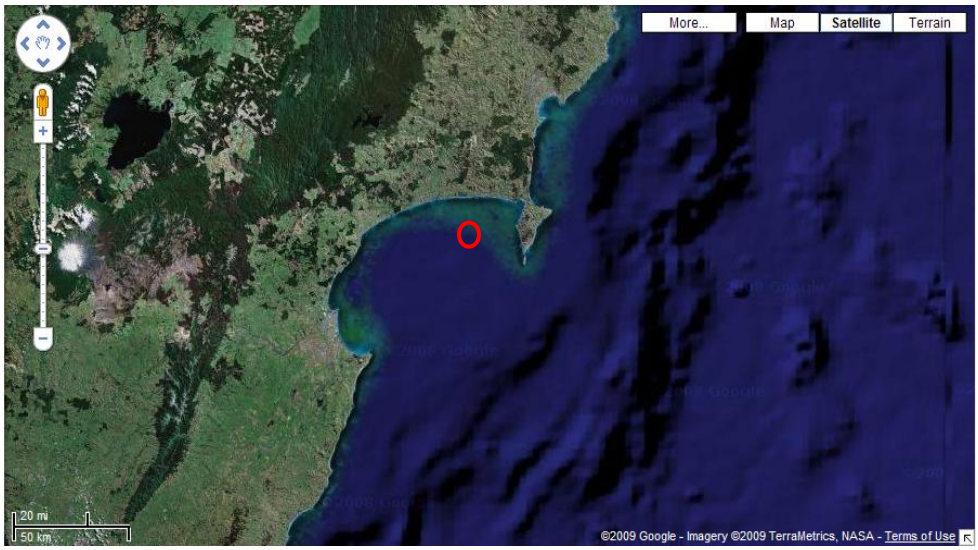
Tauranga is located on the northeastern coast of the North Island of New Zealand. Eight seahorses (ID: AT-BA) were collected on 28th of March 2008 from under Salisbury wharf outside Mount Maunganui (red cicle). These were found attached to ropes, wires and the pilons under the wharf.

NEW PLYMOUTH (NPY):



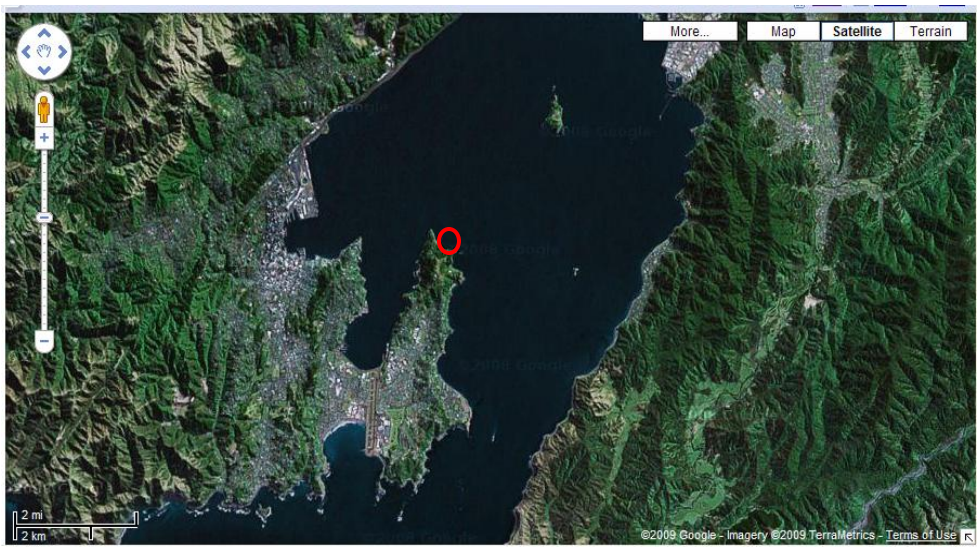
New Plymouth is located approximately 15 km north of mount Taranaki on the central west coast of the North Island. A breakwater wall built for the marina has become a popular place to spot seahorses. Ten seahorses (ID: CH-CQ) were collected here (red circle) on the 25th of May 2008 only a few meters into the water, attached on various manmade objects and macroalgae.

NAPIER (NAP):



Napier is located on the central east coast of the North Island of New Zealand. Eight seahorses (ID: DC-DJ) were found on the 1st of August 2008 at the base of a mussel farm (red circle) at a depth of 34 meters approximately 45 km from Napier city, near Mahia peninsular. This location has often been used to stock the recently closed Seahorse Farm (A. Harry, personal communication).

WELLINGTON (WEL):

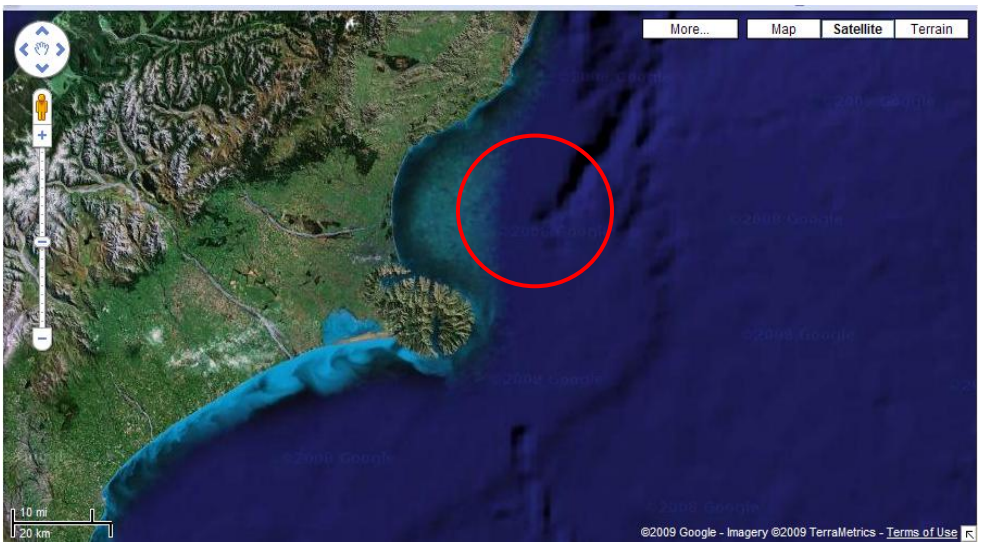


Wellington is the capital city of New Zealand and features a large harbour where seahorses are commonly found sheltering from the rough Cook Strait conditions. Ten seahorses (ID: CR-DA) were collected from within Kau Bay (red circle) on the 23rd of May 2008, only a few meters offshore, within macroalgae.

TITAHI BAY (TIB):

Titahi Bay is located on the western coast of the lower North Island approximately 25 km north of Wellington. The area searched (red circle) is very rocky with a large amount of macroalgae. SCUBA divers searched for one hour in choppy conditions, 23rd May 2008. Only one seahorse (ID: DB) was found, only a few meters offshore, attached to be base of a plume of macroalgae.

CHRISTCHURCH (CHC):



Christchurch is nestled in the northern inner side of Banks peninsular on the eastern side of the South Island. Fourteen seahorses (ID: DK-DW) were collected from Pegasus Bay (red circle) floating seaweed between 10-80 km offshore on the 28th of February 2008. These were held at the Southern Encounter Aquarium and sampled for this on the 1st of September 2008. One was provided as frozen specimen due to an unexpected death in early 2008.

JACKSON BAY (JAB):

Jackson Bay is located 30 minutes drive below the township of Haast, on the southwest coast of the South Island. It is one of the only semi-sheltered bays along this coast. Five seahorses (ID: FR-FV) were found under the main wharf (red circle) on the 30th of October 2008.

DUNEDIN (DUN):

Dunedin harbour is a large low exposure inlet on the southeast coast of the South Island. Four seahorses (ID: FD-FG) were found in the seaweed beds on the inner side of Aramoana mole (red circle) and sampled on the 29th of October 2008. Another four seahorses (ID: FH-FK) were sampled at the Portobello Marine Science Aquarium and are said to have originated from various locations within the harbour.

STEWART ISLAND (STW):

Stewart Island is located immediately below the South Island of New Zealand. In 2001, Ron Dennis collected seahorses from a variety of sites within and around Halfmoon Bay (red circle) and had put them on display in the Stewart Island Aquarium. All were fed daily with fresh mysid shrimps which accounts for their incredibly large size. Throughout the years, all juveniles produced were released back into the Bay, as were the fully grown individuals when the Aquarium closed at the end of 2008. The specimens (ID: FW-GE) were sampled on the 2nd of November 2008.

Appendix B – Genetic marker guide and microsatellite scores

Blackout boxes (left) represent DNA sequences acquired and values (right) represent allele scores.

		Mitochondrial Markers			Microsatellite Markers							
Sample ID	**Origin ID**	**CYTB**	**COX1**	**MtCR**	**Habd3**		**Habd6**		**Habd9**		**Habd7**	
AA	RAG	■	■		94	94	190	190	246	272	330	332
AC	RAG	■	■	■	102	124	176	218	236	278	336	370
AD	RAG				104	108	212	214	242	274	334	336
AE	RAG			■	86	104	178	202	244	244	334	334
AF	RAG	■			86	112	186	202	242	256	354	354
AG	RAG		■		90	112	200	238	272	274	332	334
AH	RAG				102	138	180	210	262	278	332	336
AI	RAG				94	140	242	240	248	264	342	342
AJ	RAG	■		■	94	134	172	180	242	278	356	358
AK	RAG	■			92	98	198	218	244	276	344	352
AL	RAG	■			108	132	176	246	242	274	336	346
AM	RAG	■	■		92	110	176	212	236	236	330	336
AN	RAG		■		94	96	186	196	250	252	332	334
AO	WHA		■	■	94	114	180	210	242	268	338	338
AP	WHA		■	■	94	102	210	234	236	284	352	356
AQ	WHA	■			92	132	190	198	264	274	336	342
AR	WHA	■			98	128	200	204	246	264	334	354
AS	WHA	■	■	■	102	104	194	242	246	274	344	376
AT	TAU		■		102	120	182	258	256	258	334	352
AU	TAU			■	98	104	194	248	242	244	336	344
AV	TAU	■			112	128	174	200	242	254	362	364
AW	TAU				86	110	252	254	282	290	332	336
AX	TAU	■	■		144	148	184	220	258	280	332	336
AY	TAU			■	102	114	202	214	260	280	336	352
AZ	TAU	■		■	108	132	186	230	242	266	342	380
BA	TAU		■		92	94	176	188	256	268	336	338
BB	NAQ				86	120	218	246	240	242	334	340
BC	NAQ				?	?	?	?	?	?	?	?
BD	NAQ				116	136	176	214	274	286	332	346
BE	NAQ				110	116	170	176	238	266	372	374
BF	NAQ				110	146	182	204	238	268	386	388
BG	NAQ				94	96	182	190	242	270	336	382
BH	NAQ				86	96	186	190	242	270	336	356
BI	NAQ				90	138	204	236	244	256	338	352
BJ	NAQ				94	110	186	190	248	270	336	382
BK	NAQ				90	106	196	226	236	282	334	360
BL	NAQ				86	92	180	182	240	260	336	334
BM	NAQ		■		120	152	188	250	282	248	340	372
BN	NAQ				92	96	176	184	250	270	330	338
BO	NAQ				88	94	204	234	244	244	348	360
BP	NAQ				86	90	188	194	248	268	356	374
BQ	NAQ				90	112	188	194	242	260	332	334
BR	NAQ				108	136	180	210	236	252	336	344
BS	NAQ				110	116	176	202	250	248	372	334
BT	KTQ				92	94	176	228	244	248	380	382
BU	KTQ				86	98	176	228	244	248	356	374
BV	KTQ				90	130	182	218	238	240	356	354
BW	KTQ				90	96	182	188	238	240	332	356
BX	KTQ				?	?	?	?	?	?	?	?
BY	KTQ				94	110	176	188	248	270	334	382
BZ	KTQ				86	86	176	228	236	244	356	354

CA	KTQ				86	90	190	202	278	280	330	336
CB	KTQ				90	130	188	194	248	270	334	370
CC	KTQ				?	?	?	?	?	?	?	?
CD	KTQ				90	116	204	228	248	252	332	336
CE	KTQ				90	134	192	218	246	266	336	360
CF	KTQ				94	94	186	222	244	286	328	336
CG	KTQ				98	106	186	192	242	270	372	372
CH	NPY				96	150	202	206	246	248	334	396
CI	NPY				94	98	196	200	246	262	336	376
CJ	NPY				94	130	210	218	262	278	334	354
CK	NPY				92	128	184	202	240	266	334	358
CL	NPY				102	136	192	204	242	282	338	346
CM	NPY				98	106	184	204	244	270	336	344
CN	NPY				86	94	184	190	240	244	332	342
CO	NPY				94	116	182	240	236	236	354	366
CP	NPY				96	122	182	214	276	280	356	364
CQ	NPY				98	98	184	184	260	258	352	352
CR	WEL				86	106	182	236	240	244	344	362
CS	WEL				90	92	194	230	256	272	336	344
CT	WEL				86	90	190	208	244	260	334	366
CU	WEL				90	126	212	222	240	250	338	350
CV	WEL				94	106	190	192	238	278	348	348
CW	WEL				104	104	186	214	242	280	346	358
CX	WEL				86	142	190	198	236	248	334	334
CY	WEL				112	128	178	182	218	278	346	352
CZ	WEL				102	122	196	198	274	282	348	348
DA	WEL				100	102	202	214	240	248	336	336
DB	TIB				86	118	178	224	246	268	370	374
DC	NAP				92	130	176	174	244	244	332	344
DD	NAP				82	94	200	204	254	286	332	374
DE	NAP				96	96	188	198	266	266	334	344
DF	NAP				92	116	186	236	244	248	348	360
DG	NAP				134	148	204	226	242	254	342	346
DH	NAP				94	118	178	196	236	260	334	346
DI	NAP				110	116	184	230	272	274	330	356
DJ	NAP				92	130	214	212	262	252	336	348
DK	CHC				86	90	204	208	262	270	336	336
DL	CHC				88	112	178	236	242	264	338	338
DM	CHC				90	130	176	190	242	262	362	376
DN	CHC				110	138	200	206	236	236	336	344
DO	CHC				92	92	176	202	244	254	336	374
DP	CHC				98	102	194	220	236	236	336	336
DQ	CHC				124	124	240	258	264	290	334	344
DR	CHC				94	128	210	264	272	290	334	344
DS	CHC				94	106	188	194	260	268	338	338
DT	CHC				94	98	180	186	278	282	354	374
DU	CHC				128	150	178	204	240	280	336	338
DV	CHC				102	124	188	202	240	272	334	356
DW	CHC				104	114	178	226	240	258	334	336
ZZ	CHC				94	98	182	186	236	244	348	350
DX	RAG2				96	102	178	226	248	266	348	354
DY	RAG2				86	86	178	218	238	256	332	338
DZ	RAG2				92	106	228	232	240	258	332	338
EA	RAG2				94	98	216	244	242	272	332	336
EB	RAG2				92	110	190	232	244	248	332	336
EC	RAG2				98	112	176	246	266	276	334	334
ED	RAG2				98	142	204	228	246	274	332	334
EE	RAG2				104	112	202	226	248	258	334	348
EF	RAG2				92	142	182	214	234	266	330	354

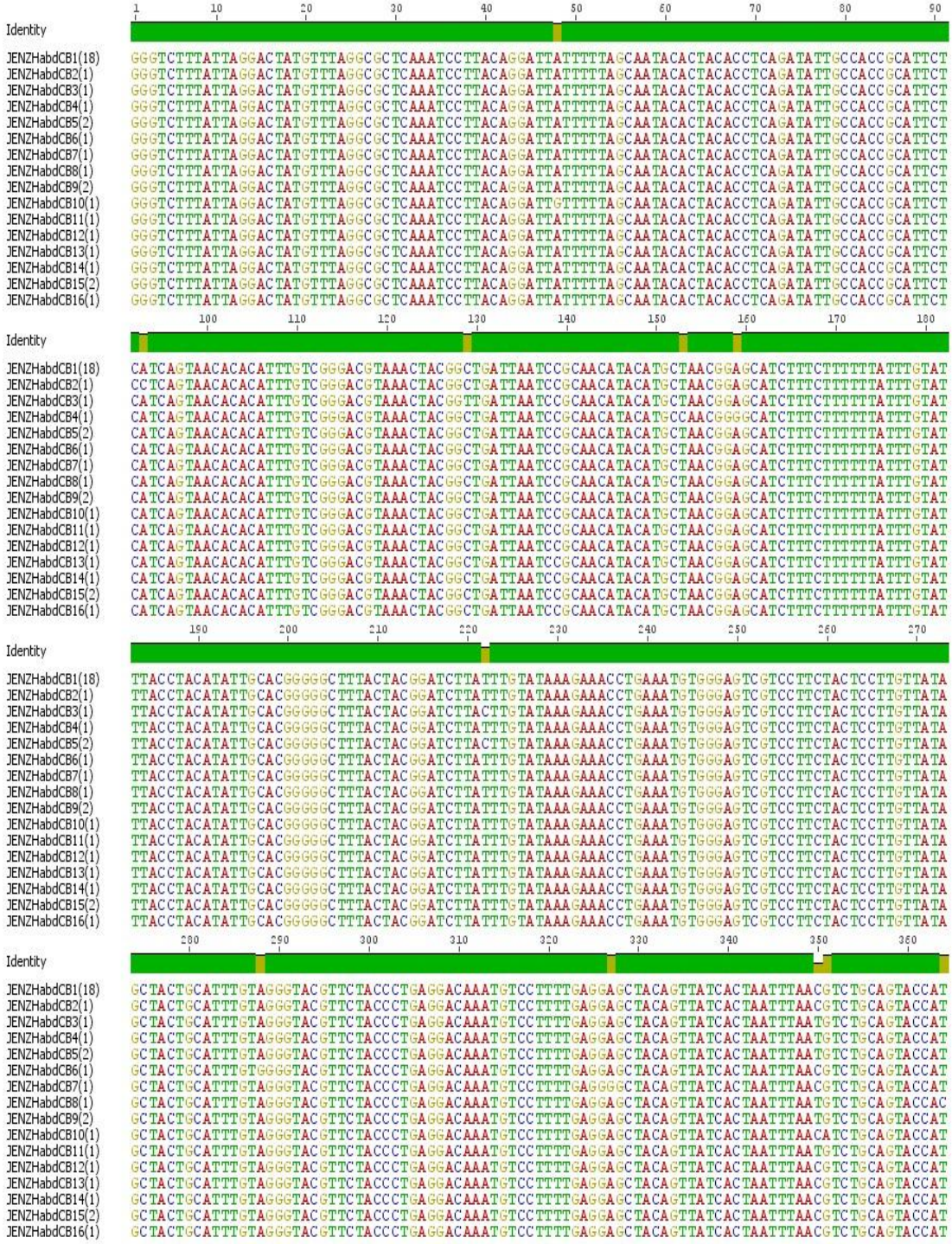
EG	RAG2				86	126	176	198	234	262	338	340
EH	RAG2				104	106	176	220	240	280	334	344
EI	RAG2				86	104	220	234	244	284	334	348
EJ	RAG2				86	96	182	210	260	270	334	352
EK	RAG2				92	134	182	198	270	272	334	358
EL	RAG2				92	126	204	220	262	272	346	370
EM	RAG2				90	94	176	228	242	262	332	334
EN	RAG2				94	104	178	206	246	250	330	376
EO	RAG2				92	98	182	184	274	288	334	350
EP	RAG2				106	124	178	228	236	240	330	334
EQ	RAG2				120	132	216	232	260	278	334	380
ER	RAG2				86	94	178	216	264	268	336	354
ES	RAG2				96	128	216	220	268	282	376	378
ET	RAG2				96	98	176	192	240	254	334	334
EU	RAG2				110	124	210	220	272	286	246	374
EV	RAG2				92	108	176	206	248	274	334	334
EW	RAG2				94	102	188	260	260	272	332	336
EX	RAG2				82	116	176	238	242	270	352	372
EY	RAG2				92	100	182	238	236	288	352	362
EZ	RAG2				98	102	204	210	228	250	340	358
FA	RAG2				94	98	232	248	264	272	336	352
FB	RAG2				116	148	234	248	240	286	332	348
FC	RAG2				92	92	212	216	236	242	370	372
FD	DUN				92	132	176	194	242	250	330	404
FE	DUN				92	96	186	214	272	276	346	360
FF	DUN				122	134	194	194	270	276	334	346
FG	DUN				94	130	176	208	248	270	338	342
FH	DUN				90	104	208	210	246	282	334	334
FI	DUN				92	120	206	218	252	288	336	348
FJ	DUN				104	104	210	212	242	244	336	336
FK	DUN				94	96	180	186	244	278	334	358
FL	MPQ				90	110	184	224	248	290	332	352
FM	MPQ				86	92	186	244	244	244	328	336
FN	MPQ				94	94	190	206	216	296	328	332
FO	MPQ				104	106	184	212	236	276	332	332
FP	MPQ				90	90	186	246	264	280	330	356
FQ	MPQ				86	102	228	238	258	278	328	364
FR	JAB				98	106	196	204	236	256	336	358
FS	JAB				92	96	186	238	248	276	332	348
FT	JAB				108	112	194	220	258	302	330	332
FU	JAB				102	112	180	198	198	278	332	334
FV	JAB				102	124	220	246	244	258	332	344
FW	STW				96	110	214	216	244	248	334	348
FX	STW				88	94	180	216	236	242	336	340
FY	STW				96	110	194	214	250	260	344	372
FZ	STW				92	108	176	202	240	248	352	370
GA	STW				112	116	170	176	236	264	344	358
GB	STW				92	106	206	216	242	244	330	348
GC	STW				86	102	242	248	248	256	334	340
GD	STW				106	110	186	232	234	264	362	362
GE	STW				98	116	198	224	244	244	334	350
GF	MSQ				96	106	182	218	240	270	332	352
GG	MSQ				96	114	182	236	240	274	334	352
GH	MSQ				102	104	188	198	244	270	358	362
GI	MSQ				92	124	202	228	248	250	332	336
GJ	MSQ				90	98	176	188	238	264	336	374
GK	MSQ				94	128	176	228	282	286	340	344
GL	MSQ				96	110	200	236	248	246	334	346
GM	MSQ				92	94	202	234	248	254	332	342

Appendix C – Laboratory Solutions

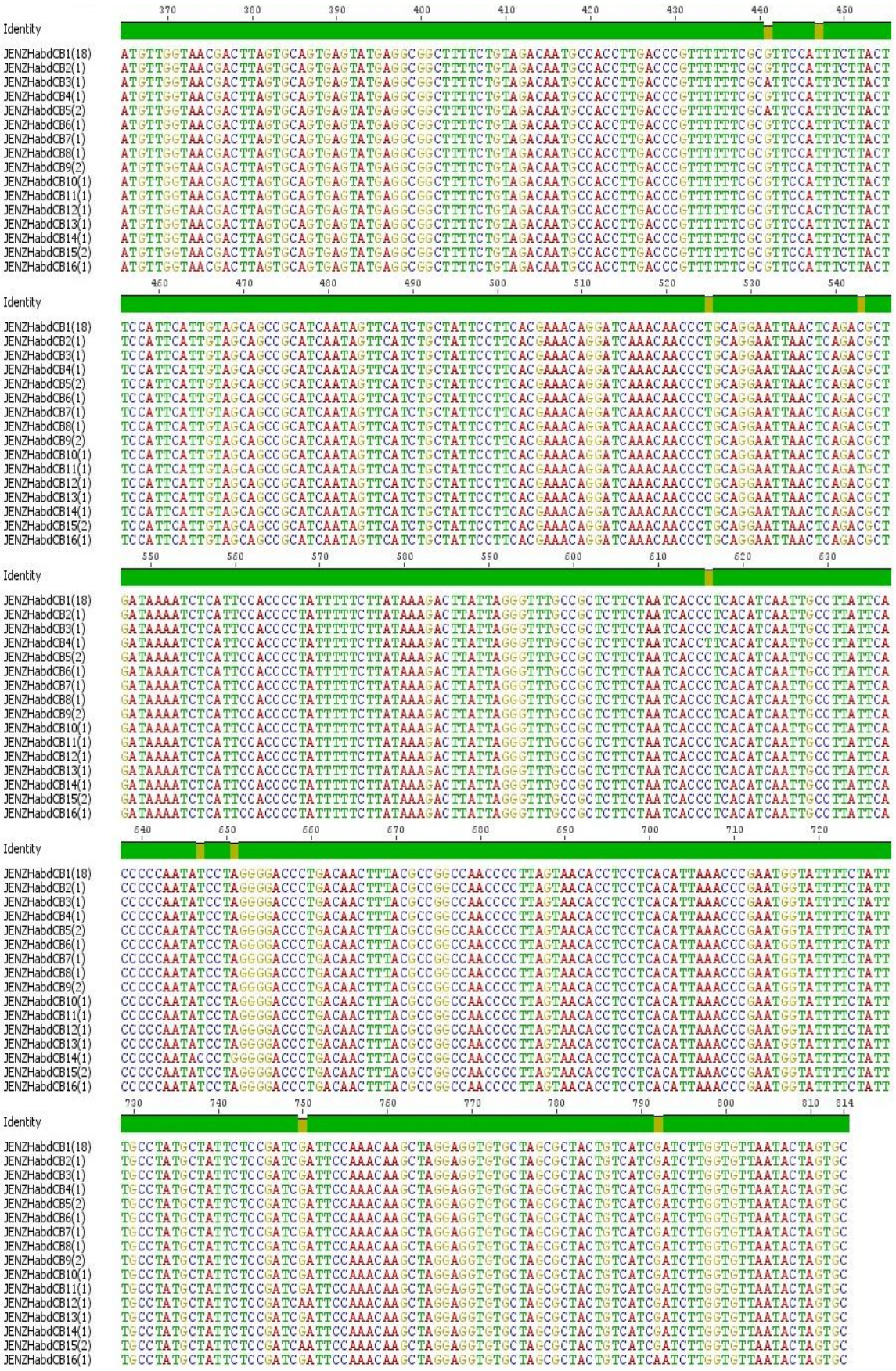
SDS-EDTA-Tris lysis solution:	1M Tris pH 8 10% SDS (sodium dodecyl sulfate) 0.5M EDTA (Ethylene-diamine-tetra-acetic acid)
Tris-EDTA storage solution:	1 mM Tris pH8 10 mM EDTA (Ethylene-diamine-tetra-acetic acid)
Polyethylene glycol-sodium: chloride	20% PEG (Polyethylene Glycol) 8000 2.5 M $NaCl_2$ (Sodium Chloride)
Gel loading dye:	15% Ficoll (Type 400) 0.25% Bromophenol Blue 0.25% xylene cyanol
Saline Borate buffer:	0.45 M Borate (Boric acid) 0.125 M $NaCl_2$ (Sodium Chloride)

Appendix F – MtDNA haplotype alignments

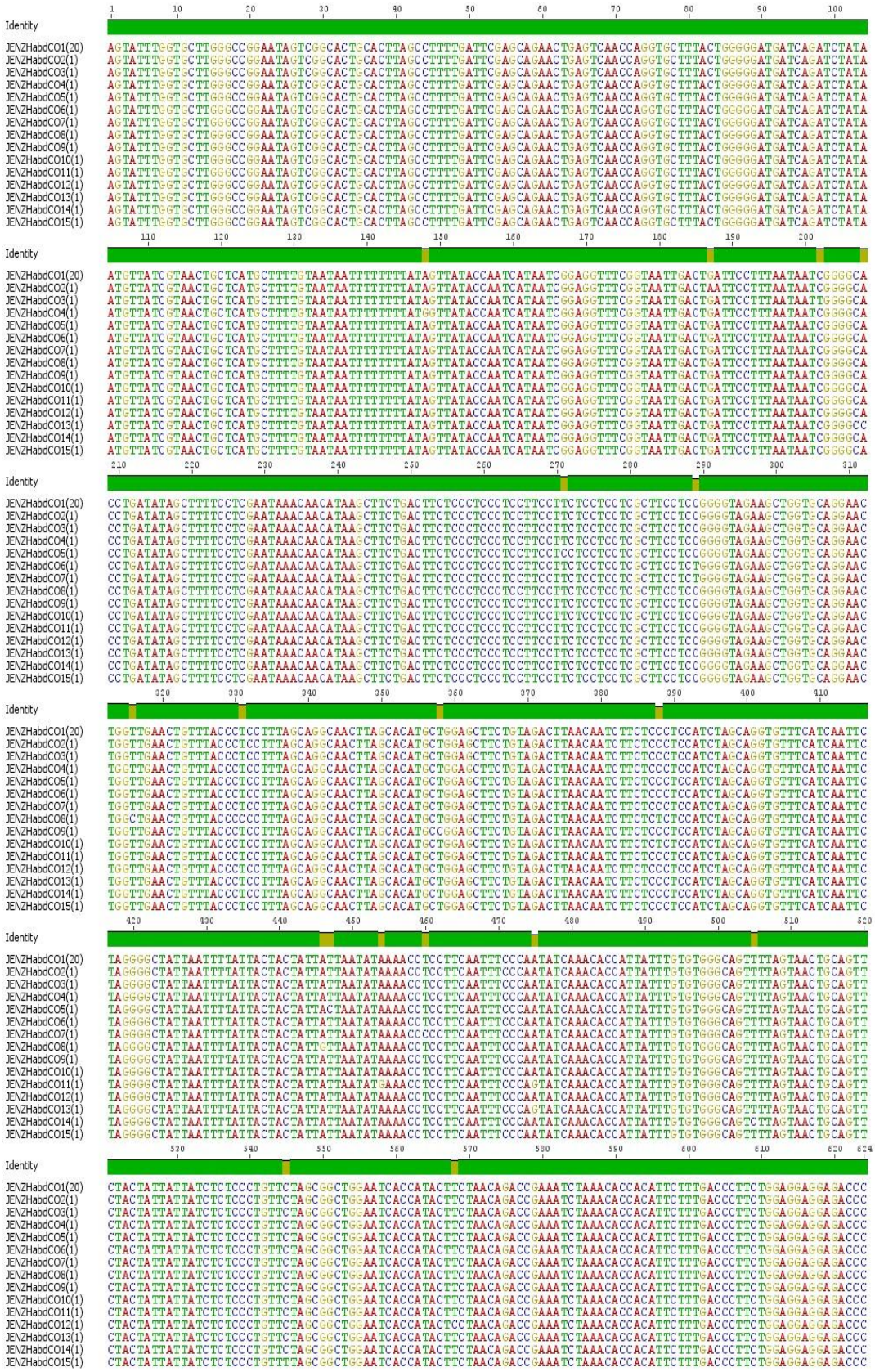
Cytochrome B:



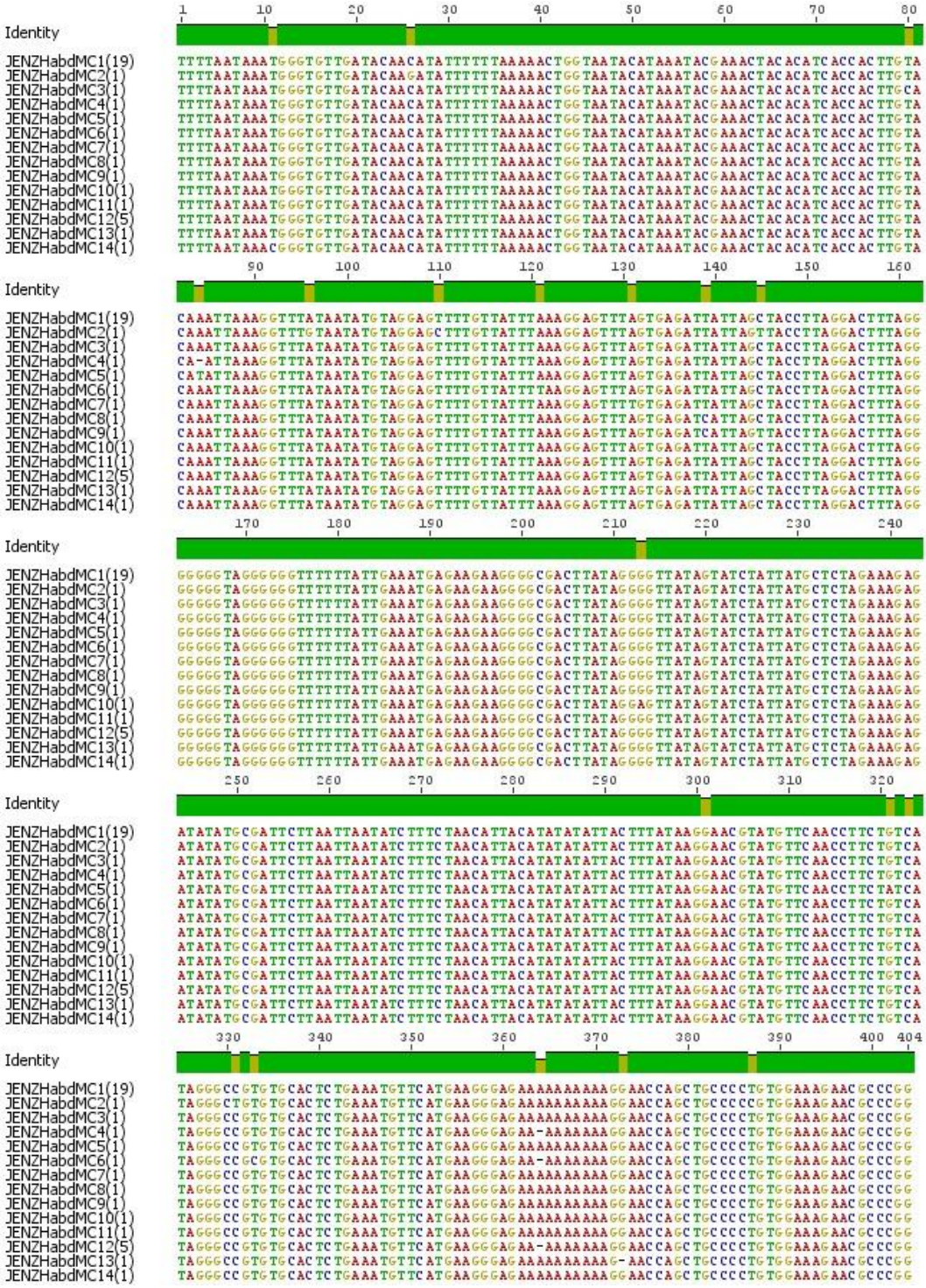
Cytochrome b continued…



Cytochrome oxidase 1:



Mitochondrial control region:



Appendix H – Continuous variables summary

Full summary statistics (all in millimeters) of the continuous morphological variables snout depth (SnD), snout length (SnL), head length (HL), head depth (HD), trunk length (TrL), tail length (TaL), standard length (StdL) and the head length to snout length ratio (HL:SnL) for all 155 assessed samples of New Zealand *Hippocampus abdominalis*.

Continuous variable	**Mean**	**Median**	**Minimum**	**Maximum**	**Lower quartile**	**Upper quartile**	**Standard deviation**
SnD	4.05	3.99	2.47	6.39	3.41	4.62	0.82
SnL	11.32	10.65	4.67	22.95	8.25	13.97	3.82
HL	29.65	29.31	14.06	48.29	25.48	34.11	6.87
HD	14.09	14.26	6.35	20.42	11.80	16.14	2.90
TrL	46.57	46.08	24.31	84.57	38.79	53.75	11.23
TaL	117.81	114.96	60.44	191.11	97.27	136.17	28.64
StdL	194.03	192.60	105.08	313.22	161.75	221.11	44.46
HL:SnL	2.73	2.64	1.92	3.94	2.36	3.11	0.46

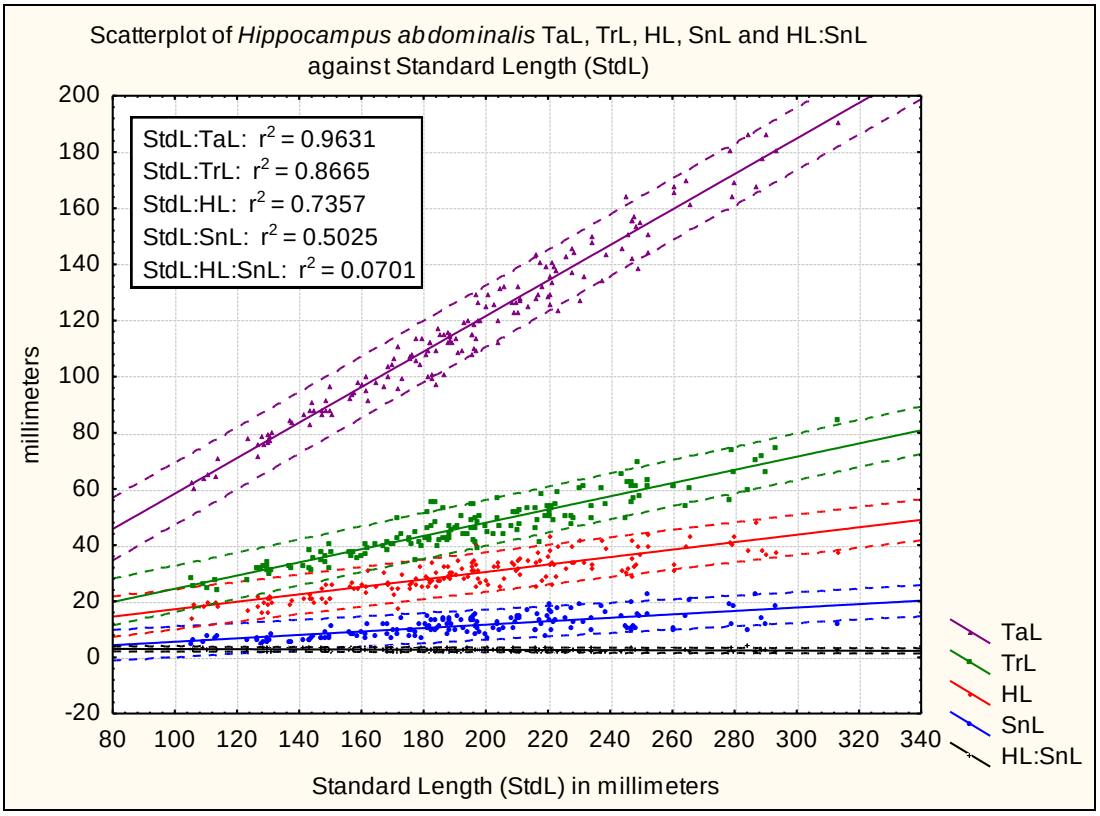
Appendix I – MWW test results

Full results of the Mann Whitney Wilcoxon test for (continuous and discrete) variables that could possibly be significantly different between males (n=80) and females (n=75). The significant values are indicated in red ($P<0.05$).

variable	Rank Sum M	Rank Sum F	U	Z	p-level	Z adjusted	p-level
SnD	5920.000	6170.000	2680.000	-1.14399	0.252627	-1.14399	0.252627
SnL	5453.000	6637.000	2213.000	-2.81612	0.004861	-2.81612	0.004861
HL	5707.000	6383.000	2467.000	-1.90666	0.056566	-1.90666	0.056566
HD	6181.000	5909.000	2941.000	-0.20946	0.834086	-0.20946	0.834086
TrL	5511.000	6579.000	2271.000	-2.60845	0.009096	-2.60845	0.009096
TaL	6474.000	5616.000	2766.000	0.83606	0.403119	0.83606	0.403119
StdL	6108.000	5982.000	2868.000	-0.47085	0.637751	-0.47085	0.637751
HL:SnL	7105.000	4985.000	2135.000	3.09541	0.001966	3.09541	0.001966
Colour	6381.500	5708.500	2858.500	0.50486	0.613657	0.51548	0.606220
Spotting	8129.500	3960.500	1110.500	6.76370	0.000000	7.01396	0.000000
Fronds	7002.500	5087.500	2237.500	2.72840	0.006365	3.57129	0.000355
Spines	6350.000	5740.000	2890.000	0.39207	0.695005	0.53605	0.591925

Appendix J – Dependent variable regression

A regression of tail length (TaL), trunk length (TrL), head length (HL), snout length (SnL), and the head length to snout length ratio (HL:SnL) against standard length (StdL)shows a positive correlation between all associations. 95% prediction lines are included to attempt to encapsulate the variation of the dependent variables.



Appendix K – Locus specific diversity indices

Table of wild collected populations molecular diversity indices and population genetic statistics broken down per (microsatellite) locus. This includes the number of samples (n), the number of observed alleles (A), number of expected alleles under the infinite allele model (A_{IAM}), observed heterozygosity (H_O), expected heterozygosity (H_E), the deviation from Hardy-Weinberg equilibrium p-value, and the fixation index (F_{IS}).

Population	Marker	A	A_{IAM}	H_O	H_E	HWE p-value	F_{IS}
RAG (n=13)	Habd3	16	16.7	0.923	0.951	0.64846	0.010
	Habd6	18	19.7	0.923	0.972	0.38481	0.032
	Habd9	14	15.8	0.846	0.942	0.10413	0.085
	Habd7	12	13.3	0.769	0.911	0.10750	0.141
WHA (n=5)	Habd3	8	8.4	1.000	0.956	1.00000	-0.111
	Habd6	9	9.1	1.000	0.978	1.00000	-0.084
	Habd9	7	7.8	1.000	0.933	1.00000	-0.139
	Habd7	9	9.1	0.800	0.978	0.10526	0.143
TAU (n=8)	Habd3	15	15.1	1.000	0.992	1.00000	-0.043
	Habd6	16	16.0	1.000	1.000	1.00000	-0.034
	Habd9	11	11.8	1.000	0.950	1.00000	-0.091
	Habd7	10	9.5	1.000	0.900	0.76427	-0.154
NPY (n=10)	Habd3	13	12.6	0.900	0.932	0.71927	0.009
	Habd6	13	12.6	0.900	0.932	0.70725	0.009
	Habd9	15	16.2	0.900	0.974	0.26956	0.053
	Habd7	15	15.6	0.900	0.968	0.34654	0.047
WEL (n=10)	Habd3	13	14.1	0.900	0.953	0.52021	0.031
	Habd6	15	15.6	1.000	0.968	1.00000	-0.061
	Habd9	15	15.6	1.000	0.968	1.00000	-0.061
	Habd7	11	12.3	0.600	0.926	0.00250	0.341
NAP (n=8)	Habd3	10	11.4	0.875	0.942	0.11679	0.041
	Habd6	15	15.1	1.000	0.992	1.00000	-0.043
	Habd9	12	12.4	0.750	0.958	0.03922	0.196
	Habd7	11	12.4	1.000	0.958	1.00000	-0.081
CHC (n=14)	Habd3	17	18.5	0.857	0.958	0.05388	0.090
	Habd6	20	21.5	1.000	0.976	1.00000	-0.044
	Habd9	16	17.5	0.857	0.950	0.10279	0.082
	Habd7	11	11.6	0.714	0.873	0.03945	0.169
DUN (n=8)	Habd3	10	10.9	0.875	0.933	0.59051	0.032
	Habd6	10	11.4	0.875	0.942	0.49322	0.041
	Habd9	12	12.9	1.000	0.967	1.00000	-0.071
	Habd7	10	10.2	0.750	0.917	0.20254	0.159
JAB (n=5)	Habd3	8	8.4	1.000	0.956	1.00000	-0.111
	Habd6	9	9.1	1.000	0.978	1.00000	-0.084
	Habd9	9	9.1	1.000	0.978	1.00000	-0.084
	Habd7	7	6.4	1.000	0.867	1.00000	-0.233
STW (n=9)	Habd3	12	13.2	1.000	0.954	0.45748	-0.081
	Habd6	14	14.3	1.000	0.967	1.00000	-0.066
	Habd9	10	11.2	0.889	0.922	0.73292	0.007
	Habd7	12	13.2	0.889	0.954	0.45686	0.042

Appendix L – Locus specific pair-wise F_{ST}

Spreadsheet of locus specific F_{ST} values between wild collected populations of New Zealand H. abdominalis. None of the p-values were significant. Positive values are emphasized in bold..

Populations		HA3	HA6	HA9	HA7
RAG	**WHA**	0.000	0.000	0.000	0.000
RAG	**TAU**	0.000	0.000	**0.012**	0.000
RAG	**NPY**	0.000	0.000	0.000	0.000
RAG	**WEL**	0.000	0.000	0.000	**0.002**
RAG	**NAP**	**0.003**	0.000	0.000	0.000
RAG	**CHC**	0.000	0.000	0.000	**0.013**
RAG	**DUN**	0.000	0.000	0.000	0.000
RAG	**JAB**	0.000	0.000	0.000	0.000
RAG	**STW**	0.000	0.000	0.000	**0.005**
WHA	**TAU**	0.000	0.000	**0.039**	0.000
WHA	**NPY**	0.000	**0.016**	**0.008**	0.000
WHA	**WEL**	0.000	0.000	**0.032**	0.000
WHA	**NAP**	**0.006**	0.000	**0.019**	0.000
WHA	**CHC**	0.000	0.000	**0.012**	0.000
WHA	**DUN**	0.000	0.000	**0.029**	0.000
WHA	**JAB**	0.000	0.000	**0.040**	**0.046**
WHA	**STW**	**0.011**	**0.008**	**0.030**	0.000
TAU	**NPY**	0.000	0.000	0.000	0.000
TAU	**WEL**	0.000	0.000	**0.009**	**0.003**
TAU	**NAP**	**0.004**	0.000	0.000	**0.022**
TAU	**CHC**	0.000	0.000	**0.008**	0.000
TAU	**DUN**	0.000	0.000	**0.010**	**0.008**
TAU	**JAB**	0.000	0.000	0.000	**0.036**
TAU	**STW**	0.000	0.000	**0.021**	**0.026**
NPY	**WEL**	**0.021**	**0.018**	0.000	0.000
NPY	**NAP**	**0.001**	0.000	0.000	0.000
NPY	**CHC**	0.000	**0.019**	0.000	**0.003**
NPY	**DUN**	**0.012**	**0.046**	0.000	0.000
NPY	**JAB**	**0.007**	**0.031**	0.000	**0.026**
NPY	**STW**	**0.002**	**0.037**	0.000	0.000
WEL	**NAP**	**0.034**	0.000	0.000	0.000
WEL	**CHC**	0.000	0.000	0.000	0.000
WEL	**DUN**	**0.007**	**0.013**	0.000	0.000
WEL	**JAB**	0.000	**0.005**	0.000	**0.023**
WEL	**STW**	**0.010**	**0.009**	0.000	0.000
NAP	**CHC**	**0.008**	0.000	0.000	**0.022**
NAP	**DUN**	0.000	**0.008**	0.000	0.000
NAP	**JAB**	**0.019**	0.000	0.000	0.000
NAP	**STW**	0.000	**0.001**	0.000	0.000
CHC	**DUN**	**0.005**	0.000	**0.009**	0.000
CHC	**JAB**	0.000	0.000	0.000	**0.071**
CHC	**STW**	0.000	**0.006**	**0.001**	**0.021**
DUN	**JAB**	**0.024**	0.000	0.000	**0.042**
DUN	**STW**	**0.015**	0.000	0.000	0.000
JAB	**STW**	0.000	**0.008**	0.000	**0.034**