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A Molecular Study of the Mitochondrial Genome and Invasions of the Veined Rapa Whelk, Rapana venosa

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A MOLECULAR STUDY OF THE MITOCHONDRIAL GENOME AND INVASIONS OF THE VEINED RAPA WHELK, *RAPANA VENOSA*

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ABSTRACT

Rapana venosa is a predatory marine gastropod native to the coastal waters of China, Korea, and Japan. Since the 1940s, *R. venosa* has been transported around the globe and introduced populations now exist in the Black Sea, the Mediterranean Sea, the Adriatic and Aegean seas, off the coasts of France and The Netherlands, in Chesapeake Bay, Virginia, U.S.A, and in the Rio de la Plata between Uruguay and Argentina. Identifying the source population(s) of invasions has been recognized as an important step for understanding and potentially controlling the spread of invasive species. Recent studies of marine invasions have found that mitochondrial sequence data can be used to assess the genetic composition of native and introduced populations, allowing researchers to trace invasion pathways and determine source populations. However, amplifying regions of the mitochondrial genome in mollluscs is not always straightforward due to the high levels of gene rearrangement observed within the phylum. Additionally, choosing an appropriately variable mitochondrial gene region for intraspecific studies is difficult in gastropods due to limited knowledge about the variation present in mitochondrial gene regions of these animals and their lack of a non-coding, highly variable control region.

The first portion of this study entailed a sequence analysis of the whole mitochondrial genome of the marine gastropod *Rapana venosa* to determine gene order and arrangement. Each mitochondrial gene region of *R. venosa* was then compared to those of three other neogastropods to assess the extent of genetic variation present within each of the 15 mitochondrial coding regions. Mean nucleotide diversity was seen to be highest within the ND4 gene region and lowest in the COI gene region. The pattern of genetic variation between the mitochondrial gene regions was similar to that of mammals. Some gene regions, however, showed a marked difference in variability between gastropods and mammals. For example, while the ND4 gene was the most variable gene in the neogastropods, it was only the eighth most diverse gene in mammals.

The second portion of this study surveyed variation in two mitochondrial gene regions to trace the invasion pathways of *Rapana venosa*, identify likely sources for introduced populations, and evaluate current hypotheses of potential transportation vectors. Sequence data were obtained for the COI and ND2 gene regions of 178 individuals from eight native locations and 106 individuals from 12 introduced locations. Native range individuals displayed very high levels of genetic variation while all introduced populations showed a complete reduction in genetic diversity; a single haplotype was common to all introduced individuals. The genetic data supported proposed hypotheses for the origins of the introduction populations. The amount of decrease in genetic variation seen between the native range samples and the introduced samples was unexpected. Previous studies of marine invasions have documented either much smaller decreases in genetic variation or an increase in genetic variation. Although *R. venosa* populations currently appear to be thriving in their new environments, the lack of genetic variability found in this study raises questions regarding the ability of these new populations to survive in the long-term.

A MOLECULAR STUDY OF THE MITOCHONDRIAL GENOME AND INVASIONS OF THE VEINED RAPA WHELK, *RAPANA VENOSA*

INTRODUCTION

Rapana venosa Valenciennes 1846 (Gastropoda, Muricidae), the Asian veined rapa whelk, is a predatory marine gastropod native to Asian waters. Its native range extends from the Sea of Japan through the Bohai and Yellow seas and into the East China Sea (Tsi *et al.* 1983, Lai and Pan 1980, as cited in ICES 2004). However, in recent years *R. venosa* has been transported around the globe. The first known introduction occurred into the Black Sea in the 1940s (Drapkin 1963). Since then, populations of *R. venosa* have been established throughout the Aegean (Koutsoubas and Voultsiadou-Koukoura 1990, as cited in ICES 2004) and Adriatic seas (Ghistotti 1971, 1974, Mel 1976, Cucaz 1983, Rinaldi 1985, as cited in ICES 2004; Bombace *et al.* 1994), off the coasts of France (Quiberon Bay, ICES 2004) and The Netherlands (Nieweg *et al.* 2005), in the lower Chesapeake Bay, U.S.A. (Harding and Mann 1999), and in the Rio de la Plata between Uruguay and Argentina (Pastorino *et al.* 2000).

Adult *Rapana venosa* can reach lengths of over 170 mm in shell length (SL) and live for approximately 15 years (J.M. Harding and R. Mann unpublished data, as cited in Mann *et al.* 2006; Harding and Mann 2003). As adults, rapa whelks live in hard sandy coastal habitats (Hwang *et al.* 1991; Chung *et al.* 1993, as cited in Harding and Mann 1999; Harding and Mann 1999) where they spend most of their lives burrowed into the substrate (Harding and Mann 1999). *R. venosa* can effectively feed, mate, and move while burrowed and laboratory observations have found rapa whelks to spend more than

95% of their time completely buried (Harding and Mann 1999). In Chesapeake Bay, whelks live in water depths of 10 to 60 meters (Harding and Mann 1999), although Wu (1988) found that *R. venosa* in Chinese waters migrate into deeper water in winter when water temperatures drop. *Rapana venosa* are capable of surviving in temperatures ranging from 4°C to 27°C (Chung *et al.* 1993, as cited in ICES 2004), and, while there are no data on the salinity tolerance of the Asian veined whelk in its native range, based on observed data from the Black Sea and laboratory data from Chesapeake Bay, salinity tolerances for adults may range from 32 ppt (Golikov 1967, as cited in Mann and Harding 2000) to salinities as low as 10 ppt (J. M. Harding unpublished data, as cited in Harding and Mann 2005, Mann and Harding 2003).

During the winter and spring months, *Rapana venosa* adults form large mating aggregations (Chung *et al.* 1993, as cited in ICES 2004). *Rapana venosa* are dioecious (ICES 2004), although imposex individuals also exist (Mann *et al.* 2006, Jestel 2003, Ware 2002, Westcott 2001). The females lay large mats of egg cases from April through June in their native range and from May through September in Chesapeake Bay (Chung *et al.* 1993, as cited in ICES 2004, Harding *et al.* 2002). The egg cases are cemented to hard substrates (Chung *et al.* 1993, as cited in ICES 2004). Each egg case contains approximately 100 to 3000 eggs (Ware 2002), and a female can lay up to 500 egg cases in each mat (Harding *et al.* 2002). Additionally, females may lay more than 10 separate egg mats per year (Ware 2002).

Depending on temperature and salinity, eggs hatch in 14 to 28 days (Harding and Mann 2003). The larvae hatch as planktonic veligers and spend three to six weeks in the water column (Harding and Mann 2003). The larvae then settle to benthic hard substrates

and metamorphose into juveniles. Juveniles remain on hard substrates until they reach a shell length of approximately 70 mm, at which point they move into deeper sand or mud bottomed habitats (Harding and Mann 2003). Individuals reach sexual maturity at about 1 year of age (Harding and Mann 2003).

Rapana venosa is one of many species that has been transported around the world by human activity. As human travel and international trade continue to increase, the rate of new invasions and the associated ecological and economic threats are also increasing (Ruiz *et al.* 1997). Currently, non-native species are considered to be the second greatest threat to native biodiversity after habitat loss (UNEP 2005) and often cause significant damage to native ecosystems. Invasive species have been deemed responsible for changes to ecological interactions, species abundance, fisheries, and for facilitating invasions of other non-native species in the same area, a process known as invasional meltdown (e.g. Carlton 1996, Vitousek *et al.* 1996, Walton *et al.* 2002, Wilcove *et al.* 1998, Simberloff and Von Holle 1999). As a result of these damages and control efforts that try to prevent damage, invasive species cost approximately $120 billion per year (Pimentel *et al.* 2005). This estimation, however, is derived mostly from costs associated with terrestrial invasive species; little is currently know about the monetary impact of invasive species on marine systems.

Rapana venosa, specifically, pose a significant ecological and economic threat to non-native regions. Within the Black Sea, rapa whelks have been cited as the major cause of the decline in abundance of several native mollusc species (Zolotarev 1996). Predation studies found *R. venosa* preferentially feed upon hard clams, *Mercenaria mercenaria*, a commercially valuable species within Chesapeake Bay, over other native

molluscs (Harding and Mann 1999). Although rapa whelks are not currently found in large abundance in areas of major hard clam beds, given their potential for range expansion, rapa whelks certainly pose a threat to the future of the hard clam populations and their lucrative fishery in Chesapeake Bay (Harding and Mann 1999).

Rapa whelks also pose a threat to the ecological balance of Chesapeake Bay and other ecosystems into which they have been introduced. Although young rapa (below 40 mm in shell length) are consumed by native blue crabs, *Calinectes sapidus* (Harding 2003), and perhaps other native crustaceans, adult whelks (over 100 mm in shell length) in Chesapeake Bay apparently have no native predators (Harding and Mann 1999). Sea turtles are able to prey on the native whelks, the knobbed whelk, *Busycon carica*, and channeled whelk, *Busycon canaliculatum*, in the Bay (Harding and Mann 1999), and perhaps on young *Rapana venosa* (40 to 100 mm SL). However, the shell of the adult rapa whelk is three to six times thicker than that of the native whelks (Harding *et al.* 2002) and the rapa whelk is more box-shaped, decreasing the number of vulnerable spots on the shell that sea turtles could crush (Harding and Mann 1999). This lack of predation together with the huge reproductive output of these animals may allow rapa whelks to proliferate in Chesapeake Bay and other introduced areas, and to out compete a variety of native organisms.

Rapana venosa also threaten areas adjacent to their immediate areas of introduction. Mann and Harding (2003) suggested that rapa whelks, due to their temperature and salinity tolerances, may be capable of extending their range from Chesapeake Bay north to Cape Cod and south to Charleston, SC, thus, threatening the majority of the U.S. eastern coastline.

Rapana venosa within Chesapeake Bay are numerous and are actively reproducing (Mann and Harding 2000). Additionally, the range of *R. venosa* within the Bay is increasing (Harding and Mann 2005). Based on the criteria set forth by Kolar and Lodge (2001), rapa whelks in Chesapeake Bay can be characterized as established and, due to their ongoing spread throughout the lower Bay, invasive.

Identifying potential source population(s) of introduced species is an important step in understanding and controlling the spread of invasive species. First, existing knowledge of an organism's biology and ecology in source locations can provide clues as to how to better control the damage and predict the spread of an organism in its new location (Bond *et al.* 2002). Second, finding the source populations can lead us to a deeper understanding of the characteristics needed for and mechanisms of a successful invasion (Vermeij 1996). Finally, within marine environments, non-native species can be transported to new areas in a variety of ways. Organisms can be carried in ballast water and sea chests or as fouling organisms attached to hulls, drilling platforms, dry docks, buoys, marina floats, seaplanes, recreational equipment, or floating marine debris. They can be accidentally or intentionally released from public or private aquaria, research or educational institutions, aquaculture or fishery operations, or restoration endeavors (Carlton 2001). With so many possible vectors existing for marine transport, identifying the source of an invasive population may help us determine which of these vectors are most important in transporting organisms and, therefore, need more regulation.

When a species introduction occurs, typically a small subset of individuals from the native population arrives in the receptor location. These individuals likely do not represent the full range of genetic diversity maintained within the source population, an

event referred to as the founder effect (Conner and Hartl 2004). If a new subset of these individuals is transported to yet another receptor location, there should be a secondary reduction of genetic diversity in the newest population. The reduction in genetic diversity, as well as the genetic relatedness of native and introduced populations, can be assessed using molecular genetic techniques to determine the most probable path of introductions.

Sequence analysis of mitochondrial DNA gene regions has proven particularly useful for studies investigating potential introduction pathways and source populations of marine introduced species. These studies have surveyed a variety of mitochondrial regions including: cytochrome c oxidase I (Kelley *et al.* 2006, May *et al.* 2006, Simon-Bouhet *et al.* 2006, Bachelet *et al.* 2004, Martel *et al.* 2004, Hänfling *et al.* 2002, Cristescu *et al.* 2001), srRNA (12S) (Martel *et al.* 2004), lrRNA (16S) (Martel *et al.* 2004), NADH dehydrogenase subunit 5 (Cristescu *et al.* 2001), and intergenic non-coding mitochondrial regions (Voisen *et al.* 2005, Azzurro *et al.* 2006). Sequence data from the mitochondrial genome is useful for intraspecific populations studies because mitochondrial genes, on average, evolve faster than those of the nuclear genome (Brown 1979). This faster rate of evolutions is due to two factors. First, mitochondrial genes have a higher mutation rate due to the high levels of free radicals and the low fidelity of DNA polymerases and DNA editing systems in the mitohcondria (Brown 1979, Avise 2004). Second, the mitochondrial genome exhibits a smaller effective population size than the nuclear genome, causing the mitochondrial genome to be more effected by genetic drift (Birky *et al.* 1983). The result of these processes is increased variability in the mitochondrial genome that allows for the detection of subtle differences in genetic

structure between populations, and in the case of studies of invasive species, between native and introduced populations.

Sequencing mitochondrial genes in molluscs is not always a straightforward process. Previously, gene order within the mitochondrial genome was thought to be highly conserved within most phyla (Boore and Brown 1994). Gene rearrangements, however, have been observed in arthropods, annelids, nematodes, and molluscs and a consistent mitochondrial gene order has only been observed in vertebrates (Saccone *et al.* 1999). Based on this conserved gene order within the Phylum Chordata, "universal" primers have been developed that amplify a variety of mitochondrial gene regions in almost all vertebrates (i.e. Martin *et al.* 1992). These primers are designed to target genes flanking either side of the gene of interest. Yet, in organisms with gene rearrangements such as gastropods, many of the previously developed "universal" primers do not successfully amplify the mitochondrial gene regions for which they were designed (E. Chandler, unpublished data). In these species, knowledge of the gene order is imperative to designing primers that target the correct genes, those that are adjacent to the region of interest. While very little information currently exists about the *Rapana venosa* mitochondrial genome (Yoon *et al.* 1996), preliminary sequencing efforts have found that *R. venosa* also displays a mitochondrial gene order distinct from that of vertebrates (E. Chandler, unpublished data).

In addition to the problems often encountered in sequencing mitochondrial genes in gastropods, choosing a gene region that is appropriately variable for intraspecific population studies is also problematic. When conducting intraspecific population studies, it is often useful to survey the most variable gene region possible. The various

mitochondrial gene regions exhibit different evolutionary rates (Aquadro and Greenberg 1983, Cann *et al.* 1984), and in vertebrates, the most variable portion of the mitochondrial gene region is usually the non-coding control region, an area of the genome that controls replication and transcription (Avise 2004). Due to the fact that this region is non-coding, it is under limited selective pressure, resulting in high genetic variability. The mitochondrial genomes of most gastropods, however, do not contain sizable non-coding regions. The largest non-coding region of most gastropods range from 42 basepairs (bp) as seen in the pulmonate snail *Albinaria coerulea* (Hatzoglou *et al.* 1995) to 165 bp as seen in the marine snail *Conus textile* (Bandyopadhyay *et al.* 2007). Thus, there has been no region of the mitochondrial genome that has been recognized in gastropods to be a standard for use in intraspecific studies and the variability of the other mitochondrial gene regions within gastropods has not been assessed.

The goal of this project was, first, to amplify and map the mitochondrial genome of *Rapana venosa* and to compare the mitochondrial sequence data of *R. venosa* with that of other neogastropods to assess the genetic variability in each of the mitochondrial gene regions of these animals; these results are presented in Chapter 1. The second aim of the project was to use mitochondrial sequence data from the cytochrome c oxidase I (COI) and NADH dehydrogenase subunit 2 (ND2) gene regions to assess the population structure of *R. venosa* in its native range and in several introduced populations. The genetic relationships among populations were used to identify the most probable pathway of the invasions and likely source populations of each invasion, and to evaluate whether the genetic data are consistent with the current hypotheses regarding the likely vectors for each introduction. The results of this population study are presented in Chapter 2.

CHAPTER 1:
THE MITOCHONDRIAL GENOME OF THE RAPA WHELK (*RAPANA VENOSA*) AND THE GENETIC VARIABILITY OF THE MITOCHONDRIAL CODING REGIONS IN THE NEOGASTROPODS

INTRODUCTION

Metazoan mitochondrial genomes code for thirteen protein subunits (referred to here as protein coding regions), two ribosomal RNAs (rRNAs), and 22 transfer RNAs (tRNAs) (Chomyn *et al.* 1986, Chomyn *et al.* 1985, Anderson *et al.* 1981). With few exceptions, this content is conserved over a wide range of taxa (Mortiz *et al.* 1987). The order in which these genes occur in the circular mitochondrial genome was thought to be conserved at high taxonomic levels (Boore and Brown 1994); however, this has only been observed within the chordates (Saccone *et al.* 1999). Within the arthropods, annelids, nematodes, and molluscs, the mitochondrial gene order is much more variable (reviewed in Saccone *et al.* 1999).

Currently there are 33 sequenced molluscan mitochondrial genomes, and it is apparent that gene rearrangements have occurred many times within this phylum. For example, Wilding *et al.* (1999) compared the genomes of the chiton *Katharina tunicata*, the marine snail *Littorina saxatilis*, and the land snail *Capaea nemoralis*. They found that one protein coding gene rearrangement and three tRNA rearrangments separated the gene orders of *K. tunicate* and *L. saxatilis* and at least seven protein coding gene rearrangements separated *L. saxatilis* from *C. nemoralis*. Within the class gastropoda, the mitochondrial gene order has been determined for 12 species; within these 12 species, six mitochondrial genomes display protein coding gene rearrangement and nine genomes

show transfer RNA rearrangement (Genbank, National Center for Biotechnology Information, Bethesda, MD).

In addition to the large number of gene rearrangements seen in gastropods, the mitochondrial genomes of these animals often differ from those of vertebrates in their lack of a non-coding control region. In vertebrates, this non-coding control region is approximately 1000 basepairs (bp) in length and includes sites that control replication and transcription of the mitochondrial genome (Avise 2004). Non-coding regions of sizes comparable to that of vertebrates have been found in only two gastropods: a 935 bp putative control region in the abalone *Haliotis rubra* (Maynard *et al.* 2005) and a 1500 bp repeat region in the limpet *Lottia digitalis* (Simison *et al.* 2006). Within the majority of gastropod mitochondrial genomes, the longest length of non-coding nucleotides ranges from 42 bp in the pulmonate snail *Albinaria coerulea* (Hatzoglou *et al.* 1995) to 165 bp in the marine snail *Conus textile* (Bandyopadhyay *et al.* 2007). In vertebrates, the control region does not contain any coding sequence and thus, is under limited selective pressure, resulting in higher variability as compared to other regions of the mitochondrial genome (Aquadro and Greenberg 1983, Cann *et al.* 1984). The increased variability within the control region facilitates intraspecific genetic analysis and has been used extensively in intraspecfic population studies (Hillis *et al.* 1996).

Without a sizeable control region, researchers investigating population structure in gastropods do not have a standard highly variable mitochondrial gene region to survey, and there is currently no consensus on which mitochondrial gene region should be used in gastropod population studies. Previous studies investigating intraspecific genetic diversity within gastropods have often used nucleotide sequence data from the COI gene

region (e.g. Simon-Bouhet *et al.* 2006, Bachelet *et al.* 2004, Kyle and Boulding 2000) despite the fact that this gene is one of the most conserved mitochondrial gene regions in mammals (Saccone *et al.* 1999). The utility of COI data in these intraspecific studies has often been limited due to low levels of variation. For example, Bachelet *et al.* (2004) examined populations of *Cyclope neritea* and found limited genetic diversity within the COI gene; the populations displayed only 19 polymorphic sites in a 553 bp region and only five distinct haplotypes in a total of 93 individuals. With no knowledge of the amount of genetic variation within each mitochondrial gene region of gastropods, researchers have no basis for choosing appropriate gene regions for studies. A survey of the variation of the mitochondrial genes would provide information regarding which gene regions are most variable and thus, should be used to illustrate intraspecific variation in the absence of a control region. Additionally, identification of conserved genes of the mitochondrial genome would help researchers chose appropriate regions for use in interspecific phylogeny studies.

In the last year, the complete mitochondrial genomes of three neogastropod molluscan species, *Lophiotoma cerithiformis* (Bandyopadhyay *et al.* 2006), *Ilyanassa obsoleta* (Simison *et al.* 2006), and *Conus textile* (Bandyopadhyay *et al.* 2007), have been sequenced. This study presents a fourth, the mitochondrial genome of the Asian veined rapa whelk *Rapana venosa*. In addition, the 13 protein coding gene regions and the two ribosomal coding regions of the four neogastropods are compared in order to assess the genetic variability of each of these mitochondrial regions and identify variable regions that may be useful in future intraspecific population and interspecific phylogeny studies of neogastropods.

MATERIALS AND METHODS

Total genomic DNA was extracted from preserved foot tissue using a Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. A variety of primers - universal primers, primers developed for closely related molluscan species, and primers specific to *Rapana venosa* gene sequences - were used in various combinations in the polymerase chain reaction (PCR) to amplify the entire mitochondrial genome (Table 1). All primers were provided by Invitrogen Corporation (Carlsbad, CA). Each primer pair was run in a 10 µl PCR reaction consisting of 1 µl 10X PCR buffer plus 15 mM $MgCl_2$, 0.2 µl 10 mM dNTP, 0.1 µl 100 pmol/µl forward primer, 0.1 µl 100 pmol/µl reverse primer, 0.2 µl 10 mg/ml bovine serum albumin (BSA), 0.05 µl 5 units/µl *Taq* polymerase, 8.25 µl sterile water, and 0.1 µl *R. venosa* genomic DNA. PCR reactions were optimized on a temperature gradient using a BioRad iCycler thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA) under the following conditions: 94°C for 5 minutes; 35 cycles of 94°C for 1 minute, 45.6°C to 65°C for 1 minute, and 72°C for 2 minutes; 72°C for 7 minutes and a 4°C hold. Annealing temperatures within the temperature gradient were set at 45.6°C, 48.2°C, 52.0°C, 57.3°C, 61.0°C, 63.5°C, and 65.0°C. Five µl of each reaction were electrophoresed on a 1% agarose gel. The gel was soaked in an ethidium bromide bath and visualized on a UV transilluminator to verify the success of the amplification.

Primer pairs that yielded products were re-amplified at the optimal annealing temperature (Table 2) in a 25 µl PCR reaction consisting of 2.5 µl 10X PCR buffer plus

15 mM $MgCl_2$, 0.5 µl 10 mM dNTP, 0.25 µl 100 pmol/µl forward primer, 0.25 µl 100 pmol/µl reverse primer, 0.5 µl 10 mg/ml BSA, 0.125 µl 5 units/µl *Taq* polymerase, 20.625 µl sterile water, and 0.25 µl template *R. venosa* total DNA. Five µl of this re-amplified reaction were visualized as above.

The remaining 20 µl of PCR product was cleaned either with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA), according to the manufacturer's protocol, or with ExoSAP reagents (USB Corporation, Cleveland, OH) by adding 0.32 µl 10 units/µl exonuclease 1, 0.8 µl 1 unit/µl shrimp alkaline phosphatase (SAP), and 1.6 µl 10x SAP buffer to 20 µl PCR product and incubating at 37°C for 45 minutes, followed by 15 minutes at 80°C. The concentration of each PCR product cleaned with the QIAquick PCR Purification Kit was quantified on a BioMate™ 3 Series Spectrophotometer (ThermoSpectronic, Rochester, NY). The concentration of each PCR product cleaned using ExoSAP reagents was estimated by visualization on a 1% agarose gel.

Cleaned PCR products were prepared for sequencing using the ABI Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) at a 1/8 dilution and sequenced on an ABI 31300x*l* genetic analyzer (Applied Biosystems, Foster City, CA). Standard chromatographic format (SCF) curves of each forward and reverse sequence were created using Sequencing Analysis v. 5.1.1 software (Applied Biosystems, Warrington, UK). The SCF curves for each sample were aligned and edited using Sequencher 4.2.2 (Gene Codes Corp., Ann Arbor, MI). Sequencher 4.2.2 was also used to align overlapping amplified sequence fragments from the different primer-pair combinations. MacVector 7.2 (Oxford Molecular Ltd, Madison, WI) was used to

determine open reading frames and translate coding portions of the alignment. The alignment was then compared in pieces to genetic sequences registered in the NCBI gene database using the NCBI ntBLAST and BLASTx programs (National Center for Biotechnology Information, Bethesda, MD) to determine the identity of each fragment. Coding regions of each gene were delineated by comparing the translated sequence to the results of the BLASTx searches set for the invertebrate mitochondrial genetic code. tRNAs were identified using tRNAscan-SE (Lowe and Eddy 1997) with the Source set to Mito/Chloroplast and the Genetic Code set to Invertebrate Mito. ntBLAST and multiple sequences alignments with other neogastropod sequences were used to find tRNA sequences that tRNA-scanSE failed to detect, and to identify the small rRNA and large rRNA subunits. The complete *Rapana venosa* mitochondrial genome was submitted to GenBank. The circular genome was drawn using Redasoft Visual Cloning software v 3.2 (Redasoft Corporation, Whitehead Institute for Biomedical Research, Cambridge, MA).

Nucleotide sequences for the 13 protein coding genes and the two ribosomal rRNAs of *Lophiotoma cerithiformis* (NC_008098; Bandyopadhyay *et al.* 2006), *Ilyanassa obsoleta* (NC_007781; Simison *et al.* 2006), and *Conus textile* (NC_008797; Bandyopadhyay *et al.* 2007) were obtained from Genbank (National Center for Biotechnology Information, Bethesda, MD). A multiple alignment of nucleotide sequences for each gene region was created from these organisms and *Rapana venosa* in MUSCLE (Edger 2004). Diversity statistics for each gene region, including the number of polymorphic sites (S), number of transitions (ti), number of transversions (tv), mean number of pairwise differences (k), and mean nucleotide sequence diversity (π) (Nei 1987), were calculated in Arlequin 3.01 (Excoffier *et al.* 2005).

RESULTS AND DISCUSSION

Rapana venosa Mitochondrial Amplification and Gene Order Determination

The mitochondrial genome of *Rapana venosa* is 15,272 bp in length and contains the 13 protein coding genes, 2 ribosomal RNA (rRNA) coding regions, and 22 transfer RNAs (tRNA) usually present within metazoan mitochondrial genomes (Figure 1, Table 3, Appendix 1). The length of the genome is remarkably similar to the lengths of three other published gastropod mitochondrial genomes: 15,264 bp of *Ilyanassa obsoleta* (Simison *et al.* 2006), 15,380 bp of *Lophiotoma cerithiformis* (Bandyopadhyay *et al.* 2006), and 15,562 bp of *Conus textile* (Bandyopadhyay *et al.* 2007). These three marine gastropods occur in the same suborder, Neogastropoda, as *R. venosa.*

The 13 protein coding genes, two rRNAs, and 14 of the 22 tRNAs are transcribed from the same strand. The remaining eight tRNAs are coded on the reverse strand. Based on protein similarity to other gastropod sequences, the start and stop nucleotide positions were determined for all protein coding genes, except ND4. All protein coding regions, except ND4, begin with the start codon ATG and all, including ND4, end with either a TAG or TAA stop codon. These start and stop codons were also seen in the mitochondrial genome of *Lophiotoma cerithiformis* (Bandyopadhyay *et al.* 2006) and the marine snail *Littorina saxatilis* (Wilding *et al.* 1999).

Based on sequence alignment with the other neogastropods, the starting location of the ND4 gene could not be fully determined. BLASTx results showed the protein alignment beginning at base pair 9325. This starting location gives a start codon of CTG, which has not been identified in other gastropods. This start position would result in a

seven base pair overlap with the ND4L gene, an overlap that also occurs in *Conus textile* (Bandyopadhyay *et al.* 2007) and in vertebrates (Wolstenholme 1992). However, the ND4 gene may begin six nucleotides prior to this location, at position 9319, where there is an ATA codon. This codon is known to act as a start codon for the ND4 gene in *Lophiotoma cerithiformis* (Bandyopadhyay *et al.* 2006). This starting location at ATA is the location noted in Table 3.

The exact beginning and end positions of the small (s) rRNA and large (l) rRNA coding regions were estimated based on nucleotide sequence similarity with the other neogastropods. The srRNA is estimated to begin adjacent to the 3' end of the $tRNA^{Glu}$ and end adjacent to the 5' end of the $tRNA^{Val}$. With these boundaries, the srRNA is 972 bp in length, the same length as has been estimated for the srRNA in *Lophiotoma cerithiformis* (Bandyopadhyay *et al.* 2006) and similar to those found in *Ilyanassa obsoleta* (886 bp, Simison *et al.* 2006) and *Conus textile* (958 bp, Bandyopadhyay *et al.* 2007). The lrRNA coding sequence is also estimated to be defined by its adjacent tRNA sequences; the 5' end begins adjacent to $tRNA^{Val}$ and the 3' end stops adjacent to $tRNA^{Leu\text{-}CUN}$. The predicted lrRNA is 1358 bp in length and is comparable in length to the lrRNA coding region reported for *C. textile*, *L. cerithiformis*, and *I. obsolete*: 1378 bp, 1366 bp, and 1304 bp, respectively.

The distance between coding features in the *Rapana venosa* mitochondrial genome ranges from -13 bp to 55 bp. There are five incidents of gene overlap: three bp between $tRNA^{Trp}$ and $tRNA^{Gln}$, one bp between $tRNA^{Gly}$ and $tRNA^{Glu}$, -13 bp between ND4L and ND4, one bp between ND5 and $tRNA^{Phe}$, and one bp between COIII and $tRNA^{Lys}$. Evidence of gene overlap has been observed in *Littorina saxatilis* (Wilding *et*

al. 1999) and the other neogastropods *Ilyanassa obsoleta* (Simison *et al.* 2006), *Lophiotoma cerithiformis* (Bandyopadhyay *et al.* 2006), and *Conus textile* (Bandyopadhyay *et al.* 2007). All of these organisms show the $tRNA^{Trp}$ / $tRNA^{Gln}$ overlap seen in *R. venosa*, and *L. saxatilis* also shares the $tRNA^{Gly}$ / $tRNA^{Glu}$ overlap. The overlaps observed at the end of the ND5 and COIII genes in *R. venosa* occur only if these genes end in a complete stop codon (TAG in both cases). It has been suggested, however, that an overlap in a protein coding gene may not be a true overlap; instead, these genes may end with an incomplete stop codon of TA, as seen here, or just T. Post-transcriptional poly-adenylation adds the remaining adenines to the end of the sequence to form a complete TAA stop codon (Ojala *et al.* 1981). Evidence of these incomplete stop codons has been found in the opisthobranch gastropods *Pupa strigosa*, *Roboastra europeae*, *Albinaria coerulea*, and *Biomphalaria glabrata* (Kurabayashi and Ueshima 2000, Grande *et al.* 2002, Hatzoglou *et al.* 1995, DeJong *et al.* 2004).

Sixteen of the 22 tRNAs were identified by tRNAscan-SE (Lowe and Eddy 1997). The other six tRNAs were identified by multiple sequence alignments with *Ilyanassa obsoleta, Lophiotoma cerithiformis,* and *Conus textile.* Anticodons found for *Rapana venosa* tRNAs are the same as those found in *L. cerithiformis* and other gastropods (e.g. Bandyopadhyay *et al.* 2006, Maynard *et al.* 2005, Kurabayashi and Ueshima 2000, Hatzoglou *et al.* 1995).

The *Rapana venosa* mitochondrial genome exhibits no obvious control region. Within the genome there are 19 regions of non-coding basepairs, ranging in length from one bp to 55 bp, for a total of 217 non-coding basepairs. The total amount of non-coding nucleotides found in *R. venosa* is similar to that in *Lophiotoma cerithiformis*

(Bandyopadhyay *et al.* 2006), which showed a total of 320 non-assigned nucleotides. *Lophiotoma cerithiformis*, however, displays a larger stretch of non-coding nucleotides, 139 bp, between the $tRNA^{Phe}$ and COIII genes. The largest stretch of non-coding nucleotides found in *R. venosa,* while much shorter at 55 bp, is located at the same position as the long sequence found in *L. cerithiformis.* Interestingly, 11 of the 12 gastropods for which the mitochondrial genomes have been sequenced also display a non-coding region adjacent to the COIII gene (*C. textile*, *I. obsolete, L. cerithiformis, Roboastra europaea*, Grande *et al.* 2002; *Cepaea nemoralis*, Terrett *et al.* 1996; *Albinaria coerulea*, Hatzoglou *et al.* 1995; *Euhadra herklotsi*, Yamazaki *et al.* 1997; *Biomphalaria glabrata*, DeJong *et al.* 2004; *Pupa strigosa,* Kurabayashi and Ueshima 2000; *Aplysia californica*, Knudsen *et al.* 2006; *Haliotis rubra*, Maynard *et al.* 2005). The lengths of these non-coding regions range from 42 to 158 bp, except in *H. rubra* where the length is 935 bp. It should be noted, however, that the non-coding sequences adjacent to COIII are not always the longest non-coding sequence in the mitochondrial genome of the organisms.

As previously mentioned, the mitochondrial gene arrangement in molluscs is extremely variable. Evidence of rearrangements among molluscan classes and within the class Gastropoda has been demonstrated multiple times (e.g. Bandyopadhyay *et al.* 2006, Kurabayashi and Ueshima 2000, Wilding *et al.* 1999, Yamazaki *et al.* 1997). Within the suborder Neogastropoda, there are three other published mitochondrial genomes, those of *Ilyanassa obsoleta* (Simison *et al.* 2006), *Lophiotoma cerithiformis* (Bandyopadhyay *et al.* 2006), and *Conus textile* (Bandyopadhyay *et al.* 2007). This study presents a fourth sequence to this suborder, that of *Rapana venosa.* A comparison between these four

genomes shows that all protein coding and rRNA gene regions, and the tRNAs, are in the same order and are encoded on the same DNA strands. Based on the annotation of the gene sequences published in GenBank (National Center for Biotechnology Information, Bethesda, MD) there appear to be differences in the location of the two $tRNA^{Leu}$ genes; $tRNA^{Leu}$ order is conserved in *R. venosa* and *L. cerithiformis* but not in *I. obsoleta* and *C. textile*. Sequence comparison between these regions, however, clearly identifies the two distinct $tRNA^{Leu}$ genes, and the location of these tRNAs in *I. obsoleta* and *C. textile* are simply incorrectly annotated in GenBank. These results showing identical mitochondrial gene orders in *R. venosa, I. obsoleta, L. cerithiformis,* and *C. textile* provide evidence that, at least in Neogastropoda, gene order appears to be conserved at the suborder level.

When the neogastropod mitochondrial genome sequences were compared to the partial mitochondrial sequence, from COI to Cyt b, of *Littorina saxatilis* (Wilding *et al.* 1999), a gastropod within the same order as the neogastropods but belonging to the suborder Littorinimorpha, gene order was again found to be conserved. This similarity supports the idea that gene order within gastropods may also be conserved within the order Hypsogastropoda. This conserved gene order, however, is not found in all Hypsogastropoda. Rawlings *et al.* (2001) found that within species of the genus *Dendropoma*, vermetid gastropods within the same suborder as *L. saxatilis*, the ND6 gene position is rearranged.

While the gene order of the four neogastropods *Ilyanassa obsoleta, Lophiotoma cerithiformis, Conus textile,* and *Rapana venosa* show conservation, the mitochondrial sequences vary in the amount of non-coding nucleotide sequence and the size of the

largest length of non-coding sequence. The total amount of non-coding sequence is largest in *C. textile*, 476 bp, and smallest in *R. venosa*, 217 bp. The largest non-coding regions in *R. venosa, I. obsoleta, L. cerithiformis*, and *C. textile* are 55 bp, 74 bp, 139 bp, and 165 bp, respectively.

Nucleotide Variability Within Mitochondrial Gene Regions of the Neogastropods

In the last year, the sequences of the complete mitochondrial genomes of neogastropods, *Ilyanassa obsoleta* (Simison *et al.* 2006), *Lophiotoma cerithiformis* (Bandyopadhyay *et al.* 2006), and *Conus textile* (Bandyopadhyay *et al.* 2007), were published. These genomes, in addition to the *Rapana venosa* genome presented in this study, allow for a comparison between each protein coding gene region to determine which gene regions would be best suited for future intraspecific and interspecific studies. A comparison between the coding regions of these four species found mean nucleotide diversity (π) over these gene regions to range from 0.181 to 0.550 (Table 4). The ND4 gene, followed by ND2 and ND6, showed the most variation (π = 0.550, 0.540, and 0.322, respectively), while COI, followed by COII and COIII, showed the lowest (π = 0.181, π = 0.189, and π = 0.193, respectively). Table 5 lists each gene region by increasing genetic variability.

Analysis of mitochondrial gene diversity in mammals also found COI to be the least variable, followed by Cyt b, COIII, and COII (Saccone *et al.* 1999) (Table 5). The most diverse gene regions in mammals, however, differ from those found in this study. In mammals, ND6, ATP8, and 12S are the most variable compared to ND4, ND2, and ND6 in neogastropods. In particular, ND4, the most variable gene region in the

neogastropods, is only the eighth most variable region in mammals. Thus, some genes show similar levels of diversity in mammals and gastropods (e.g. COI), while others do not (e.g. ND4, ATP8). In addition, *Rapana venosa* mitochondrial sequence data from 178 samples show a similar pattern of genetic diversity as presented here for the neogastropods. The ND2 gene yields 82 distinct *R. venosa* haplotypes and a intraspecific mean nucleotide diversity of 0.006, while the COI region displays only 51 haplotypes and a mean nucleotide diversity of 0.005 (Data presented in Chapter 2). Thus, in this intraspecific comparison the mean nucleotide diversities were much lower within each gene region than in the interspecific comparisons (ND2 $\pi = 0.006$ vs. 0.540, COI $\pi =$ 0.005 vs. 0.181, intraspecific vs. interspecific), but the ND2 gene again shows more diversity than the COI gene.

In conclusion, the mitochondrial genome of *Rapana venosa* adds to the growing number of published mitochondrial sequences within the Phylum Mollusca. This genome is consistent in size and identical in gene order with the three other published genomes within the suborder Neogastropoda. This consistency provides evidence that although molluscs, and gastropods specifically, show major mitochondrial gene rearrangement at higher taxonomic levels, at lower taxonomical levels gene order is conserved. In addition, mitochondrial genes of the neogastropods show a varying amount of genetic diversity. High levels of variation are found in the ND4, ND2, and ND6 genes. This high variability makes these genes ideal candidate regions for use in intraspecific population studies of neogastropod species that lack a large non-coding region.

Table 1: List of primers used in PCR reactions. Primers developed in this study were based on other gastropod mitochondrial gene sequences and *Rapana venosa* mitochondrial gene sequences.

Primer Name	**Primer Sequence (5' to 3')**	**Source**
HCO2198	TAAACTTTCAGGGTGACCAAAAAATCA	Folmer *et al.* 1994
LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer *et al.* 1994
TCOI end 2R	GATCATCTCCTAACAACG	This study
CO2 cons-F	TGCTCTGAAATTTGTGGGGCAAATC	Maynard *et al.* 2005
CO2 out F	AAACTTTATGGAATGAGTCG	This study
CO2 out RA	CTAGTAAATACAGTAAACG	This study
CO2-16S F	TTCTTCGTGCCCTAACACCG	This study
CO2-16S R	TCTAATCCCAGTTTCCCTCAAGG	This study
TATP8 out F	TGACTCAAGGCTGACAACC	This study
ATP6 end F	CTATTACACTCTCGGTTCG	This study
16S out FA	TATACCGTCGTCGTCAGG	This study
16S out F2	GACTGAGGAACAAGAAAAGC	This study
16S in R	ACCTTGAAAAAAGAACTAGC	This study
16SR	AACATCGAGGTCACAAACC	This study
16S out R	CTAAACCATGATGCAAAAGG	This study
ND1 in F2	AATGGATATTTCTGTAATTTCG	This study
NADH1 F2	GCTTGCGCCGGTCTGAACTC	Maynard *et al.* 2005
ND6 out R	AAGGGTTGGGATATAAATGG	This study
M cyt b out R	CTCCATTAGCATGAAGTGC	This study
M cyt b F	TCTAATCTCTCAGTTTGATGAA	Collins *et al.* 1996
M cyt b R	TGATCGAAAATAGCATAGGCAA	Collins *et al.* 1996
M cyt b out F	AGAGATGGTGAAAAAGTTC	This study
ND4L in F	GTATAAACATTTACTAAGAATCC	This study
IO ND4 F2	CCGTAGCAGCTTCTTTACC	This study
ND4 out F	CCAGCATGGTGGAAGTCC	This study
ND5 out R	AACCAAGTAAATCGTTTAAGG	This study
ND5 midin R	AGAGAACAGAAAGAAAGTCG	This study
ND5 in R	TATATGTAAGCTATCTGAGC	This study
CO3 F	ATTTAGTTGATCCTAGGCCTTGACC	Milbury 2003
CO3 R	ACTCAAACCACATCTACAAAATG	Milbury 2003
CO3 out R	TGAAATCAACCAGCTAATCC	This study
TCO3 out R	ATTATACCCCAACGAAGTCC	This study
CO3 out F	TTGTCTTGTTCGTGTCTGG	This study
TND3 R3	CCCAAAACAAAGTTAGCAGCCC	This study

TND3F	GCATCTTCCTGCCACTAAGAGAATG	This study
TND3 F3A	CCTGTGTTGTGATAGGTCTTGGATG	This study
TND2 em R	GATCATACAAACAATGGAAGCCG	This study
TND2 3R	CAAAAAGGAGAGCATTTCCCCTTC	This study
TND2 in F	TGAGTAAAACAGGGCTGC	This study
TND2 em F	TTGGAGTAAAACAGGGCTGCTAAC	This study
TND2 3F	AAGGACTTCAGAAAGAGAGTCGGC	This study
TND2 out R	CTTCACTTAACGCTTTAAAGC	This study

Table 2: List of annealing temperatures used with each primer pair.

Primer 1	Primer 2	Annealing Temp (°C)
TND2 in F	TND2 em R	65
TND2 em F	HCO2198	65
COII cons-F	16SR	61
TND3F	TCOI end 2R	45.6
COII cons-F	16S out R	61
ATP6 end F	16S in R	57.3
CO3 F	CO3 R	45.6
TATP8 out F	TND3 R3	48.2
CO2 out F	16S out R	57.3
CO3 out F	TND2 out R	57.3
CO3 out F	TCOI end 2R	61
CO3 out F	TND3 R3	65
CO3 out F	TND2 3R	65
ATP6 end F	16S in R	57.3
LCO1490	CO2 out RA	45.6
TND3 F3A	CO2 out RA	52
TND23F	CO2 out RA	52
16S out FA	NADH1 F2	65
CO2-16S F	CO2-16S R	65
M cyt b F	M cyt b R	52
16S out F2	M cyt b R	48.2
16S out F	M cyt b R	57.3
M cyt b out F	TCO3 out R	57.3
16S out F	M cyt b out R	48.2
16S out FA	M cyt b out R	61
M cyt b out F	CO3 out R	57.3
ND4L in F	ND5 in R	52
ND4L in F	ND5 midin R	57.3
ND1 in F2	ND6 out R	57.3
IO ND4 F2	ND5 in R	52
IO ND4 F2	ND5 midin R	61
ND4 out F	ND5 out R	57.3
IO ND4 F2	ND5 out R	57.3
ND4 out F	ND5 midin R	61

Table 3: Annotated *Rapana venosa* mitochondrial genome. Feature beginning and ending positions in parentheses indicates feature is coded on reverse strand.

Feature	Beginning Base Pair Location	Ending Base Pair Location	Nucleotide Length	Amino Acid Length	Start Codon	Stop Codon	Anticodon	Number of intervening nt before next feature
COI	1	1533	1533	510	ATG	TAG		25
COII	1559	2245	687	228	ATG	TAA		0
tAsp	2244	2312	69				GUC	1
ATP8	2314	2472	159	52	ATG	TAG		5
ATP6	2478	3173	696	231	ATG	TAA		36
tMet	(3210)	(3277)	68				CAU	1
tTyr	(3279)	(3346)	68				GUA	1
tCys	(3348)	(3410)	63				GCA	0
tTrp	(3411)	(3476)	66				UCA	-3
tGln	(3474)	(3540)	67				UUG	10
tGly	(3551)	(3617)	67				UCC	-1
tGlu	(3617)	(3683)	67				UUC	0
s-rRNA	3684	4655	972					0
tVal	4656	4723	68				UAC	0
l-rRNA	4724	6081	1358					23
tLeu	6082	6151	70				UAG	0
tLeu	6152	6220	69				UAA	0
ND1	6221	7162	942	313	ATG	TAA		7
tPro	7170	7237	68				UGG	1
ND6	7239	7739	501	166	ATG	TAA		6
Cyt b	7746	8885	1140	379	ATG	TAA		6
tSer	8892	8958	67				UGA	2
tThr	(8959)	(9025)	67				UGU	9
ND4L	9035	9331	297	98	ATG	TAG		-7
ND4	9319	10695	1377	458	ATA	TAA		1
tHis	10697	10762	66				GUG	0
ND5	10763	12472	1710	569	ATG	TAG		-1
tPhe	12472	12539	68				GAA	55
COIII	12595	13374	780	259	ATG	TAG		-1
tLys	13397	13465	69				UUU	3
tAla	13469	13535	67				UGC	14
tArg	13550	13618	69				UCG	3
tAsn	13622	13689	68				GUU	17
tIle	13708	13774	67				GAU	2
ND3	13777	14130	354	117	ATG	TAA		12
tSer	14143	14210	68				GCU	0
ND2	14211	15272	1062	353	ATG	TAA		

Figure 1: Illustration of *Rapana venosa* mitochondrial genome. tRNAs are represented by the standard single letter amino acid abbreviations for which they code.

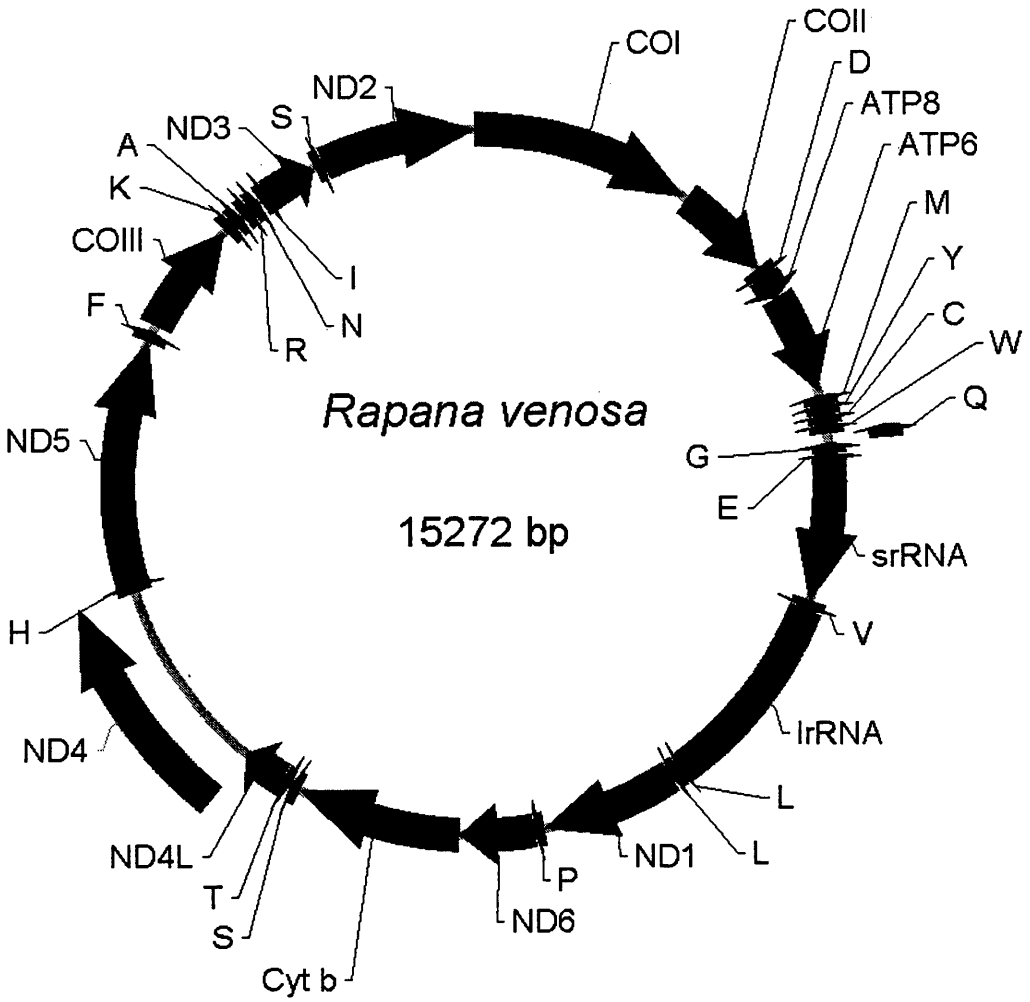


Table 4: Comparison of levels of genetic variation within each mitochondrial gene region among four neogastropod species.

	COI	COII	COIII	cyt b	ATP6
COI length analyzed	1548	687	780	1140	696
polymorphic sites (S)	460	222	244	419	265
# observed transitions	303	158	175	298	180
# observed transversions	239	102	128	221	151
# of substitutions	543	260	303	519	331
# of indels	15	0	0	0	0
mean number of pairwise differences (k)	279.667 +/- 153.204	129.667 +/- 71.223	150.667 +/- 82.700	258.167 +/- 141.453	163.000 +/- 89.441
nucleotide diversity (π)	0.181 +/- 0.118	0.189 +/- 0.124	0.193 +/- 0.127	0.226 +/- 0.148	0.234 +/- 0.153

ND1	ND4L	16S	ND3	ND5	ATP8	12S
942	297	1352	354	1722	162	984
352	117	601	150	790	77	468
261	80	303	115	559	53	252
201	63	350	81	456	39	244
462	143	653	196	1015	92	496
0	0	77	0	18	3	117
221.667 +/- 121.505	70.167 +/- 38.703	363.833 +/- 199.204	95.833 +/- 52.732	500.833 +/- 274.080	47.667 +/- 26.406	296.333 +/- 162.313
0.235 +/- 0.154	0.236 +/- 0.156	0.269 +/- 0.176	0.271 +/- 0.178	0.291 +/- 0.190	0.294 +/- 0.195	0.301 +/- 0.197

ND6	ND2	ND4
501	1095	1383
263	930	1109
191	479	599
157	651	830
348	1130	1429
0	94	60
166.333 +/- 91.263	591.833 +/- 323.814	760.667 +/- 416.088
0.332 +/- 0.218	0.540 +/- 0.353	0.550 +/- 0.359

Table 5: List of mitochondrial gene regions by increasing genetic variability as seen in neogastropods and mammals (Saccone *et al.* 1999).

In Neogastropods	In Mammals
COI	COI
COII	Cyt b
COIII	COIII
Cyt b	COIII
ATP6	ND1
ND1	ND3
ND4L	16S
16S	ND4
ND3	ATP6
ND5	ND5
ATP8	ND4L
12S	ND2
ND6	12S
ND2	ATP8
ND4	ND6

CHAPTER II:

POPULATIONS OF GENETIC MONOMORPHISM: A STUDY OF RAPA WHELK, (*RAPANA VENOSA*) INVASIONS

INTRODUCTION

Non-native species in both marine and terrestrial environments often cause grave damage to native ecosystems; on a global scale, they are considered the second greatest threat to native biodiversity after habitat loss (UNEP 2005). Invasive species have also been deemed responsible for changes to ecological interactions, species abundance, fisheries, and for facilitating invasions of other non-native species (e.g. Carlton 1996, Vitousek *et al.* 1996, Walton *et al.* 2002, Wilcove *et al.* 1998, Simberloff and Von Holle 1999). Many of these changes result in economic loss. Pimentel *et al.* (2005) estimate that non-native species cost approximately $120 billion per year in damages and control.

For several reasons, determining the invasion pathways of introduced species has been recognized as an important step in understanding and controlling the spread of invasive species. First, identifying the source of an invasive population may help determine which transport vectors are most important in carrying organisms to new areas and, therefore, need more regulation (Carlton 2001). Second, existing knowledge of an organism's biology and ecology in a source location can provide clues as to how to better control the damage and predict the spread of the organism in its new location (Bond *et al.* 2002). Finally, determining the invasion pathway can lead to a deeper understanding of the criteria and mechanisms needed for a successful invasion (Vermeij 1996).

Molecular genetic techniques provide a means to follow the invasion pathways of non-native species. When a species introduction occurs, typically a small subset of

individuals from the native population arrives in the receptor location. These individuals likely do not represent the full range of genetic diversity maintained within the source population, an event referred to as the founder effect (Conner and Hartl 2004). If a new subset of these individuals is transported to yet another receptor location, there should be a secondary reduction of genetic diversity in the newest population. By following this decrease in genetic variation and the genetic signature of each population, one can deduce the pathway of the invasions.

Contrary to the expectation that introduced populations should show a significant decrease in genetic diversity, many recent marine invasion studies have reported an increase in genetic diversity in introduced populations likely resulting from multiple introductions to the area from distinct, genetically differentiated native populations (Kelly *et al.* 2006, Voisin *et al.* 2005, Simon-Bouhet *et al.* 2006, Bachelet *et al.* 2004, Ting and Geller 2000). Other studies have documented a decrease in genetic diversity in introduced populations, but the observed decreases were not large (Azzurro *et al.* 2006, Kelly *et al.* 2006, May *et al.* 2006, Astanei *et al.* 2005, Provan *et al.* 2005, Städler *et al.* 2005, Martel *et al.* 2004, Bagley and Geller 2000, Marsden *et al.* 1996, Woodruff *et al.* 1986, Cristescu *et al.* 2001). Thus, current studies of marine invasions have not demonstrated the expected large decrease in genetic diversity from native sources to introduced populations. Additionally, there appears to be no consistency in the amount of genetic variation found in introduced marine populations and each invasion needs to be considered separately. In this study, molecular markers were used to assess the genetic diversity and composition of native and introduced populations of the invasive marine whelk, *Rapana venosa*.

Rapana venosa Valenciennes 1846 (Gastropoda, Muricidae), the Asian veined rapa whelk, is a predatory marine gastropod whose native range extends from the Sea of Japan through the Bohai and Yellow seas and into the East China Sea (Tsi *et al.* 1983 and Lai and Pan 1980, as cited in ICES 2004; Figure 2). In the 1940s, *R. venosa* were discovered in the Black Sea (Drapkin 1963). From this initial introduction, rapa whelks have spread to the Aegean (Koutsoubas and Voultsiadou-Koukoura 1990, as cited in ICES 2004) and Adriatic seas (Ghistotti 1971, 1974, Mel 1976, Cucaz 1983, Rinaldi 1985, as cited in ICES 2004; Bombace *et al.* 1994). Additionally, new populations of *R. venosa* were discovered in Chesapeake Bay, U.S.A. in 1998 (Harding and Mann 1999), in the Bay of Quiberon, France, in 1997 (ICES 2004), in the Rio del Plata between Uruguay and Argentina in 2000 (Pastorino *et al.* 2000), and off the coast of The Netherlands in 2005 (Nieweg *et al.* 2005).

The first introduction of *Rapana venosa* into the Black Sea was probably caused by either egg cases being transported to the region in association with introduced oysters or as fouling organisms on boat hulls (ICES 2004). *Rapana venosa* subsequently spread, likely by natural range expansion of planktonic larvae, throughout the Aegean and Adriatic seas (ICES 2004). In contrast, the introduced populations of *R. venosa* in France, The Netherlands, Chesapeake Bay, and Rio de la Plata probably did not come directly from the gastropod's native range, but rather may represent secondary invasions with animals from the Black Sea/Mediterranean Sea area serving as the source populations. It has been hypothesized that the Quiberon Bay, France population may have originated by human-mediated introduction of rapa whelks by aquaculturists or shell collectors (ICES 2004). The Netherlands population may have originated by natural

range expansion from the Quiberon Bay population or through human-mediated transport by aquaculture, hull fouling, or ballast water transport (ICES 2004). The Chesapeake Bay population likely originated with planktonic larvae from the Black Sea/Mediterranean Sea area transported west via ballast water (Mann and Harding 2000). Additionally, ballast water is the suspected vector for the Rio de la Plata population, with larvae transported to the area from Chesapeake Bay (R. Mann, personal communication), although transport to the area from the native range or the Black Sea/Mediterranean Sea area cannot be excluded.

Rapana venosa pose significant ecological and economic threats to introduced areas. First, within the Black Sea, rapa whelks have been cited as the major cause of the decline in abundance of several native mollusc species (Zolotarev 1996). Based on predation studies and the potential for a range expansion within Chesapeake Bay, Harding and Mann (1999) suggested that rapa whelks could similarly devastate Chesapeake Bay populations of hard clams (*Mercenaria mercenaria*), a commercially valuable species. Second, rapa whelks pose a threat to the ecological balance of the ecosystems into which they have been introduced due to the lack of natural predators in these areas and their tremendous reproductive output (Harding and Mann 1999). Finally, *R. venosa* appear to have large temperature and salinity tolerances, which may allow them to spread throughout a broad geographic area surrounding their initial point of invasion. Harding and Mann (2003) have suggested that rapa whelks may be capable of extending their range from Chesapeake Bay north to Cape Cod and south to Charleston, SC, thus, threatening the majority of the eastern U.S. coastline.

Rapana venosa are highly fecund and their eggs hatch as planktonic veliger larvae that can be carried in ballast water, characteristics that make them effective invasive species. Adult *R. venosa* are dioecious (ICES 2004) and females lay large mats of egg cases from April through September (Chung *et al.* 1993, as cited in ICES 2004, Harding *et al.* 2002). Each egg case contains approximately 100 to 3000 eggs (Ware 2002), and a female can lay up to 500 egg cases in each mat (Harding *et al.* 2002). Additionally, females may produce over 10 different egg mats per year (Ware 2002). The larvae hatch as planktonic veligers and spend three to six weeks in the water column (Harding and Mann 2003). The larvae then settle to benthic hard substrates and metamorphose into juveniles. Individuals become sexually mature at about 1 year (Harding and Mann 2003) and can live up to 15 years (Mann *et al.* 2006).

Due to the threats that *Rapana venosa* pose to invaded ecosystems, it is important to identify the source of the invasions. In doing so, it may be possible to identify how *R. venosa* is being introduced into new areas and focus can be placed on implementing regulations and controls on these vectors to reduce the number of new invasions. Previous studies have successfully used mitochondrial sequence data to trace the invasion pathways and identify source populations in a variety of marine introductions: marine snails *Ocinebrellus inornatus* (Martel *et al.* 2004) and *Cyclope neritea* (Simon-Bouhet *et al.* 2006, Bachelet *et al.* 2004), Chinese mitten crabs *Eriocheir sinensis* (Hänfling *et al.* 2002), brown algae *Undaria pinnatifida* (Voisin *et al.* 2005), zebra mussels *Dreissena polymorpha* (May *et al.* 2006), amphipods *Gammarus tigrinus* (Kelly *et al.* 2006), cladocerans *Cercopagis pengoi* (Cristescu *et al.* 2001), and rabbitfish *Siganus luridus* (Azzurro *et al.* 2006). In this study, mitochondrial gene regions of cytochrome c oxidase

I (COI) and NADH dehydrogenase subunit 2 (ND2) were surveyed to assess the genetic composition of *Rapana venosa* within its native and introduced ranges. Genetic data were analyzed for evidence of a genetic bottleneck in the introduced populations and to deduce a likely invasion pathway of these animals. Finally, current hypotheses of invasion transport vectors were compared with the genetic data to assess their plausibility.

MATERIALS AND METHODS

Rapana venosa samples were collected from eight locations within their native range (Figure 3) and from 12 introduced populations (Figures 4-5). While the majority of samples were obtained between 2004 and 2006, the samples from the Korean locations were collected in 1999 and samples from the Adriatic Sea and Chesapeake Bay were collected during both time periods. Sample sizes by location and year ranged from one to 30 individuals (Table 6). Samples consisted of a small amount of foot tissue taken from individual rapa whelks and then preserved in either DMSO storage buffer (20% dimethyl sulfoxyde, 0.25 M EDTA, saturated NaCl, pH 8.0, Seutin *et al.* 1991) or 95% ethanol until processing.

Total genomic DNA was extracted from the foot tissue samples using the DNeasy Tissue Kit (QIAGEN, Inc., Valencia, CA) according to the manufacturer's protocol. The COI and ND2 gene regions were amplified separately in 25 µl PCR reactions containing the following: 2.5 µl 10X PCR buffer plus 15 mM $MgCl_2$, 0.5 µl 10 mM dNTP, 0.25 µl 100 pmol/µl forward primer, 0.25 µl 100 pmol/µl reverse primer, 0.5 µl 10 mg/ml BSA, 0.125 µl 5 units/µl *Taq* polymerase, 20.625 µl sterile water, and 0.25 µl template *Rapana venosa* total DNA. COI was amplified using universal COI primers (Folmer *et al.* 1994):

HCO2198: 5'– taaacttcagggtgaccaaaaaatca –3'

LCO1490: 5'– ggtcaacaaatcataaagatattgg –3'

ND2 was amplified using primers specific to the rapa whelk sequence:

ND2F3: 5'– caaaaaggagagcatttccccttc –3'

ND2R3: 5'– aaggacttcagaaagagagtcggc –3'

All primers were provided by Invitrogen Corporation (Carlsbad, CA).

The COI gene region amplification was conducted under the following PCR conditions: 94°C for 5 minutes; 35 cycles of 94°C for 1 minute, 54°C for 1 minute, and 72°C for 2 minutes; 72°C for 7 minutes and a 4°C hold. Conditions for the ND2 gene region were 94°C for 5 minutes; 35 cycles of 94°C for 1 minute, 65°C for 1 minute and 72°C for 2 minutes; 72°C for 7 minutes and a 4°C hold. All PCR reactions were carried out on a MJ Research Corporation PTC-200 Peltier thermal cycler (Watertown, MA). Five µl of the amplified PCR products were electrophoresed on a 1% agarose gel containing ethidium bromide and visualized on a UV transilluminator.

PCR products were purified either with the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol or with ExoSAP reagents (USB Corporation, Cleveland, OH) by adding 0.32 µl 10 units/µl exonuclease 1, 0.8 µl 1 unit/µl SAP, and 1.6 µl 10x SAP buffer to 20 µl PCR product and incubated at 37°C for 45 minutes, followed by 15 minutes at 80°C. The concentration of each PCR product cleaned with the QIAquick PCR Purification Kit was quantified on a BioMate™ 3 Series Spectrophotometer (ThermoSpectronic, Rochester, NY). The concentration of each PCR product cleaned using ExoSAP reagents was estimated by visualization on a 1% agarose gel.

Cleaned PCR products were prepared for sequencing using the ABI Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) at a 1/8 dilution according to the manufacturer's protocol and sequenced on an ABI 31300x*l* genetic analyzer (Applied Biosystems, Foster City, CA). Standard chromatographic format (SCF) curves of each forward and reverse sequence were created using Sequencing

Analysis v. 5.1.1 software (Applied Biosystems, Warrington, UK). The SCF curves of forward and reverse reactions were used to form a consensus sequence for each individual and bases were edited using Sequencher 4.2.2 (Gene Codes Corp., Ann Arbor, MI). Consensus sequences were aligned using the CLUSTALW algorithum (Thompson *et al.* 1994) in MacVector 7.2 (Oxford Molecular Ltd, Madison, WI). Sequences displaying a unique haplotype were submitted to GenBank.

The amount of genetic variation within each population was assessed using the Arlequin 3.01 software package (Excoffier *et al.* 2005) to calculate several diversity indices including number of polymorphic sites (S), number of transitions (ti), number of transversions (tv), number of haplotypes (N_h), haplotype diversity (h), mean number of pairwise differences (k), and mean nucleotide sequence diversity (π) (Nei 1987). Each haplotype was translated into an amino acid sequence using MacVector 7.2 and a multiple sequence alignment was created to assess the number of non-synonymous nucleotide substitutions.

Genetic relatedness of the native and introduced populations was evaluated by testing Φ_{st} (Excoffier *et al.* 1992) values in an analysis of molecular variance (AMOVA) using Arlequin 3.01 (Excoffier *et al.* 2005). In addition, Φ_{st} values were compared in pairwise tests between each sampled population. The α values were set at 0.05 with no corrections for multiple comparisons. Additionally, the relationships between the populations were investigated by creating a minimum spanning network in Arlequin 3.01. Distances between haplotypes were computed using pairwise differences.

RESULTS

A 636 base pair (bp) segment of the mitochondrial COI gene region and a 668 bp segment of the ND2 gene region were both successfully amplified and sequenced from a total of 284 individuals. Samples analyzed for the COI region yielded 51 unique haplotypes with 56 polymorphic sites consisting of 51 transitions, 6 transversions, and no insertions or deletions (indels). Six nucleotide changes occurred at the first position codon site, three at the second position, and 49 at the third position. The nucleotide changes resulted in four non-synonymous amino acid changes. The mean number of pairwise differences between all native COI haplotypes was 3.123. The mean haplotype diversity for all native samples was 0.884, but despite this high level of haplotype diversity, mean nucleotide diversity was only 0.005.

Samples analyzed at the ND2 gene region diplayed 82 haplotypes with 80 polymorphic sites consisting of 74 transitions, 10 transversions, and no indels. At the first codon position 21 nucleotide changes were observed, five changes occurred at the second position, and 51 at the third. These nucleotide changes resulted in 34 non-synonymous amino acid changes. The mean number of pairwise differences between all native ND2 haplotypes was 3.858. As seen in COI, mean haplotype diversity in the native samples was high, 0.956, but mean nucleotide diversity was low, 0.006. When data from both mitochondrial gene regions were combined, there was a total of 110 haplotypes with 136 polymorphic sites consisting of 125 transitions, 16 transversions, and no indels (Table 7, Appendix 2). Examination of the relationship among halpotyes

indicates that the haplotypes display a star-like pattern (star phylogeny); all haplotypes radiate out from one central haplotype (Figure 6).

Due to the fact that the mitochondrial genome does not undergo recombination, the COI and ND2 genes are a single genetic locus and further genetic diversity analyses were conducted using the combined COI/ND2 data. Results from this combined data set showed that collections from the native range displayed high genetic diversity. The combined native samples had an overall haplotype diversity of 0.981 with the Xiangshan Bay collection showing the highest haplotype diversity (1.000) and the Japan collection the lowest (0.946) (Table 7). The mean number of pairwise differences between individuals within native collections was 6.988, ranging from 5.430 in Laishou Bay to 8.695 in Xiangshan Bay. The mean nucleotide sequence diversity ranged from 0.004 in Laishou Bay to 0.007 in Xiangshan Bay, with an overall value in the native range collections of 0.005.

This high diversity seen in all the native collections was in stark contrast to the lack of variation seen in the introduced collections (Table 7). All individuals from introduced locations (n = 106) shared the same combined COI/ND2 haplotype and thus, showed no haplotype diversity. This same haplotype was found in four rapa whelks from native collections: three individuals from Japan and one individual from Cheju-do, Korea. A minimum spanning network of the combined COI/ND2 haplotypes clearly shows the high diversity within native collections and the extreme low diversity within the introduced collections (Figure 6).

Since there was no genetic variation within the introduced areas, samples taken from the same sea or close geographic location and samples taken at different times from

the same area were pooled, resulting in four groups of introduced samples: Black Sea (n = 33), Adriatic Sea (n = 31), France and The Netherlands (n = 10), and Chesapeake Bay (n = 32). Statistical analyses of population structure were done using these modified groupings.

Based on the combined COI/ND2 data, the Φ_{st} value between the native collections (LB, YAN, QD, XS, J, KC, KI, KT) and introduced collections (T, A, FN, CB) was 0.573 ($p<0.001$), showing very strong genetic structure between the two groups (Table 8). Variation among individuals within a collection accounted for 40.70% ($p<0.001$) of the observed variance and 1.99% ($p<0.001$) of the variance was attributable to differences among collections.

As would be expected due to the presence of only one haplotype in the introduced collections, all pairwise comparisons between native range collections and introduced collections showed large and significant divergence (Φ_{st} values ranged from 0.037, $p<0.001$ to 0.769, $p<0.001$) and introduced populations could not be differentiated (Φ_{st} = 0.000, p=0.991, for all comparisons) (Table 9). Levels of divergence, however, varied among collections from the native range. Samples from Japan were significantly different from all other native range samples; pairwise Φ_{st} values ranged from 0.072 ($p=0.009$) to 0.115 ($p<0.001$). The collection from Inch'on, Korea displayed differences from Laizhou Bay, China (Φ_{st} = 0.054, p=0.018) and Yantai, China (Φ_{st} = 0.067, p=0.018). Finally, there was significant population differentiation between the Yantai, China collection and the Tongyeong, Korea collection (Φ_{st} = 0.030, p = 0.036).

DISCUSSION

The goals of this study were to evaluate the genetic variation in native and introduced collections of *Rapana venosa* and to use the genetic data to identify potential source populations, deduce the pathway of the rapa whelk invasions, and evaluate current hypotheses of possible invasion vectors.

Nucleotide sequence data from the mitochondrial COI and ND2 gene regions revealed the presence of a high level of genetic variability within native collections of *Rapana venosa* and no genetic variability within any of the introduced collections. Mean haplotype diversities within all native range populations were high in both gene regions examined ($h = 0.830 - 0.926$) and comparable with levels of diversity found in native populations of the aquatic snail *Potamopyrgus antipodarum*, using mitochondrial 16S rRNA gene sequences ($h = 0.661 - 0.836$) (Städler *et al.* 2005). Nucleotide diversity was low in *R. venosa* populations, but was consistent with levels of nucleotide diversity seen in other gastropod mitochondrial DNA studies ($\pi = 0.001 - 0.010$, Simon-Bouhet *et al.* 2006 and $\pi = 0.001 - 0.003$, Martel *et al.* 2004). The low mean nucleotide diversity in *R. venosa* resulted from the fact that the haplotypes differed from each other by only a few nucleotide changes (Figure 6).

Genetic theory predicts that when a new population is founded by a subset of individuals from a source population, the genetic diversity in this new area should be lower than in the source population (Nei *et al.* 1975). In the *Rapana venosa* invasions, not only was there a decrease in genetic diversity in the introduced areas, but this decrease was dramatic, resulting in a monomorphic mitochondrial DNA COI/ND2

haplotype in all introduced collections. As would be expected, the presence of only one haplotype in all introduced collections resulted in large and significant genetic differentiation between introduced and native collections and no genetic differentiation between the introduced populations.

The genetic bottleneck seen in this study is much more dramatic than in previously documented marine invasions. Prior studies of marine introductions have found decreases in genetic variation in invasive populations of a variety of plants and animals. However, in these cases, the observed decreases were often not large, either because the introduced population still displayed high levels of diversity (Azzurro *et al.* 2006, Astanei *et al.* 2005, Martel *et al.* 2004, Bagley and Geller 2000, Marsden *et al.* 1996, Woodruff *et al.* 1986) or because the amount of variation in the native populations was also low (Kelly *et al.* 2006, May *et al.* 2006, Provan *et al.* 2005, Städler *et al.* 2005, Cristescu *et al.* 2001). For example, a study of *Eriocheir sinensis*, the Chinese mitten crab, which surveyed COI sequence data, found a small decrease in genetic diversity from the native Chinese populations ($N_h = 5$; mean haplotype diversity = 0.75; n = 22) to introduced European populations ($N_h = 5$; mean haplotype diversity = 0.61; n = 76) (Hänfling *et al.* 2002). The combined number of haplotypes observed in these two populations was low, $N_h = 7$, despite a total sample size of 98. A further decrease in genetic variation occurred, resulting in the presence of only one haplotype in introduced California populations (n = 23). However, this decrease to one haplotype was not large when compared to that seen in *R. venosa*: the number of *Eriocheir sinensis* haplotypes decreased from only seven total haplotypes to one. In *Rapana venosa* populations, the

number of haplotypes decreased from 110 in the native populations (n = 178) to one in the introduced populations (n = 106).

Comparisons of Φ_{st} values among several populations within the native range of *Rapana vensoa* revealed significant population structuring. Genetic differentiation between native populations of benthic marine molluscs is not uncommon. For example, *Littorina plena*, a benthic gastropod with planktonic veliger larvae, have shown low but significant population structure over a distance of less than 20 km (Kyle and Boulding 2000). The planktonic larvae of *L. plena* have a long duration time (seven to ten weeks), yet show population structure over a very small distance

The genetic divergence seen between the Japan population of *Rapana venosa* and all other native populations is not surprising. This population is located on the eastern side of Japan in Mikawa Bay. In order to have gene flow between this location and any of the other native populations, adults or planktonic larvae would have to travel southwest down the coast of Japan and either across the width of the East China Sea to Xiangshan Bay or around the southern end of Japan to Cheju-do, Korea. For an adult rapa whelk to travel from Mikawa Bay, Japan to Cheju-do, Korea, the animal would have to cover approximately 900 miles (~1450 km). The distance from Mikawa Bay, Japan to Xiangshan Bay, China is close to 1,000 miles (~1600 km). While little is known about how far adult rapa whelks can travel, adult *Strombus gigas*, a large marine gastropod, ranging from 13 to 16 cm in length, have home ranges of 2.5 to 5.0 km (Heese 1979). Therefore, it is probably safe to assume that distances of 1500 km are too far to be covered by a benthic snail such as *R. venosa*. It is equally unlikely that planktonic rapa larvae are transported from the Japan population to any of the other sampled native

locations because the prevailing ocean currents off the eastern coast of Japan would tend to carry larvae to the northeast through the Kuroshio current, not southwest toward the other sampled native collections (Figure 7).

The cause of the observed population structure seen between the other native collections is not as clear. Pairwise Φ_{st} comparisons found collections from Inch'on, Korea to be significantly differentiated from collections take from Laizhou Bay, China and Yantai, China. Since a local gyre carries water from the Yellow Sea north along the western coast of Korea into the Bohai Sea and then back out into the Yellow Sea along the eastern coast of China, one would expect planktonic *Rapana venosa* larvae to be freely transported between all the Korean and Chinese locations sampled (Figure 7). The Inch'on, Korea collection, however, despite its location on this local gyre, showed genetic differentiation from the Bohai Sea collection in Laishou Bay, China and the North Yellow Sea collection in Yantai, China, both of which are geographically close to Inch'on, Korea.

In addition, the Tongyeong, Korea collection was significantly different from the Yantai, China collection. This location is more geographically removed from the northern Chinese samples and is not directly influenced by the local gyre. If geographic separation was the sole cause of the exhibited genetic differentiation with the Yantai collection, it would be expected that the Tongyeong collection would also display significant genetic differences from the other northern Chinese collections and from the Inch'on, Korea collection, which it does not. Humans, however, have been transporting oysters for aquaculture purposes to various locations throughout the native range of *R. venosa* for many years (e.g. Zhou and Allen 2003). If any rapa whelks were transported

along with these oysters, as likely happened in the Black Sea, gene flow between native rapa whelk populations would have been increased, promoting genetic homogeneity due to non-natural events.

The genetic composition of the introduced *Rapana venosa* populations proved to be consistent with current hypotheses regarding the source populations and the methods of movement of these animals around the world. As mentioned previously, the original introduced population of *R. venosa* may have been transported into the Black Sea with a culture of introduced Japanese oysters, *Crassostrea gigas* (ICES 2004). Results indicate that the introduced haplotype was shared only with native collections from Japan (three individuals) and Cheju-do, Korea (one individual) (Figure 6). As the introduced haplotype was seen most often in the Japan samples, this study provides support to the hypothesis that the Black Sea introduced population originated with samples from Japan, perhaps in association with an intentional oyster introduction. The introduced haplotype was also seen at a low frequency in Cheju-do, Korean; thus, the possibility could not be eliminated that the invasion did not originate from this site or from another site that also shares this haplotype at a low frequency.

Since the Black Sea population contains only one haplotype, this population may have begun by one single female or an egg case laid by one single female or by multiple animals all possessing this same haplotype. The probability that this population occurred from multiple invasions is equal to the probability of two individuals from the native range chosen at random possessing the same haplotype. Since there were a total of four individuals in the native range that displayed the introduced range haplotype, in a total of 178 native range individuals, the chances of picking two is the squared probability of

picking this introduced range haplotype or $(4/178)^2 = 0.0005$ or 0.05%. Using this calculation, in which it is assumed that an introduction is equally likely from all areas of the native range, the chances that the Black Sea population started from multiple introductions is unlikely, $p = 0.0005$. Given the hypothesis that the initial introduction into the Black Sea originated from Japan and the fact that the introduced haplotype was most often found in the Japan collection, it may not be accurate to assume that all of the native range populations were equally likely to be the source population of the Black Sea introduction. Thus, it may be more accurate to conduct the same calculation for the chance of multiple introduction using the Japanese collection as the only potential source population. In this case, the chance of multiple introductions is equal to the squared probability of picking the introduced range haplotype out of the Japan collection or $(3/24)^2 = 0.0156$ or 1.56%. Again, the likelihood of multiple invasions into the Black Sea is seen to be very low and the Black Sea population likely started from a single introduction rather than multiple introductions.

The lack of any variation in the combined COI/ND2 gene regions of introduced samples suggests that the Adriatic Sea, France, The Netherlands, and Chesapeake Bay rapa populations originated from the introduced monomorphic Black Sea population (either directly or indirectly), rather than from the highly diverse native range. The chances that an introduction occurred from the native range directly into these other introduced populations is equal to the frequency of the haplotype in the native range. Since there were a total of four individuals in the native range that displayed the introduced range haplotype, in a total of 178 native range individuals, the chances of choosing this introduced range haplotype is $(4/178) = 0.023$. The chance of this

haplotype being introduced directly from the native range is much less likely than the chance of it being introduced from the Black Sea population, where its frequency is 1.00. Thus, the other introduced populations presumably all originated, either directly or indirectly, for the Black Sea population.

The Adriatic Sea population could easily have been established by natural range expansion of *Rapana venosa* through the spread of its planktonic larvae from populations in the Black Sea (ICES 2004). The Quiberon Bay, France population may have been established by rapa whelks brought to the area in clam (*Tapes philippinarum*) culture bags from the Adriatic Sea or by importation by shell collectors (ICES 2004). These possible vectors are consistent with the results of this study that show that both the Adriatic collection and Quiberon Bay, France collection likely originated from animals in the Black Sea/Mediterranean Sea area. Transportation for The Netherlands population has been speculated to be through ballast water, hull fouling, aquaculture introductions, or through natural range expansion from the Quiberon Bay, France population (Nieweg *et al.* 2005). All of these vectors, if originating from the Black Sea/Mediterranean Sea area, are also possible.

The genetic evidence that the Chesapeake Bay rapa whelk population originated from the Black Sea/Mediterranean area supports the hypothesis that ballast water carrying *Rapana venosa* veliger larvae was the likely introduction vector into the area. For more than 40 years, a strong coal trade has existed between Chesapeake Bay and the eastern Mediterranean (Mann and Harding 2000). Ships leave Hampton Roads, Virginia and Baltimore, Maryland carrying coal to the Mediterranean, where it is unloaded. The shipping vessels then fill their ballast tanks with water from the Mediterranean and carry

this ballast water back to Chesapeake Bay. It is estimated that 40% of the ballast water discharged in Chesapeake Bay originates from the Mediterranean (G. Ruiz, personal communication, as cited in Mann and Harding 2000). Thus, *R. venosa* planktonic larvae could easily be carried within this large amount of ballast water that is continuously brought into Chesapeake Bay. However, how many times *R. venosa* larvae have arrived in Chesapeake Bay is unknown; due to the lack of genetic variation with Chesapeake Bay and Black Sea/Mediterranean Sea collections, the number of independent invasions into the area could not be evaluated.

The lack of genetic variability of *Rapana venosa* in the Black Sea population raises important questions about the invasion risk of this animal. Since the likelihood of multiple introductions into this area is extremely small (0.050% or 1.56%), it is probable that the initial introduction consisted of one adult female or one egg mat laid by one female. It has been observed that female rapa whelks can lay viable eggs for at least 5 years after their last mating event (J.M. Harding, personal communication). Additionally, a single egg mat, laid by one female, can contain as many as 15 million eggs (Ware 2002, Harding *et al.* 2002). Thus, one female or one egg mat could have produced a large number of individuals.

While it is clear that the Black Sea population originated from one maternal lineage, the mitochondrial sequence data reveal no information regarding the amount of variation the introduced animals have inherited from their paternal lineage(s). To date there have been no studies testing for multiple paternity in eggs laid by a single *Rapana venosa* female, nor is there any information regarding whether the females' sperm storage ability allows her to store sperm from single or multiple males. Thus, while all the

introduced *R. venosa* individuals represent the same maternal lineage, there may be more variation in their paternal lineage. Nuclear DNA markers that target non-coding regions (e.g. AFLPs, microsatellites) are bi-parentally inherited and may provide insight into the amount of paternal variation present. Additionally, nuclear non-coding markers may allow for a more detailed description of the relationships between the introduced populations and the number of times invasions have occurred in each area.

In conclusion, the genetic variation seen in the native and introduced collections of *Rapana venosa* illustrates the fact that a large genetic bottleneck occurred when individuals from the native range were introduced into the Black Sea. A decrease this dramatic, from 110 native haplotypes to one introduced haplotype, has never before been documented for a marine invasion. Previous, marine invasions have shown either a much smaller decrease in genetic variation in introduced populations or have shown an increase in genetic variability in new populations. Clearly, there is no commonality in the amount of genetic variation possessed by successful invasive species. Thus, the amount of genetic variation in an introduced population cannot be assumed and needs to be evaluated with each new introduction. In addition, while the introduced populations of *R. venosa* appear to be healthy, with such low genetic diversity, the long-term stability of these populations may be questionable, especially in the face of environmental change.

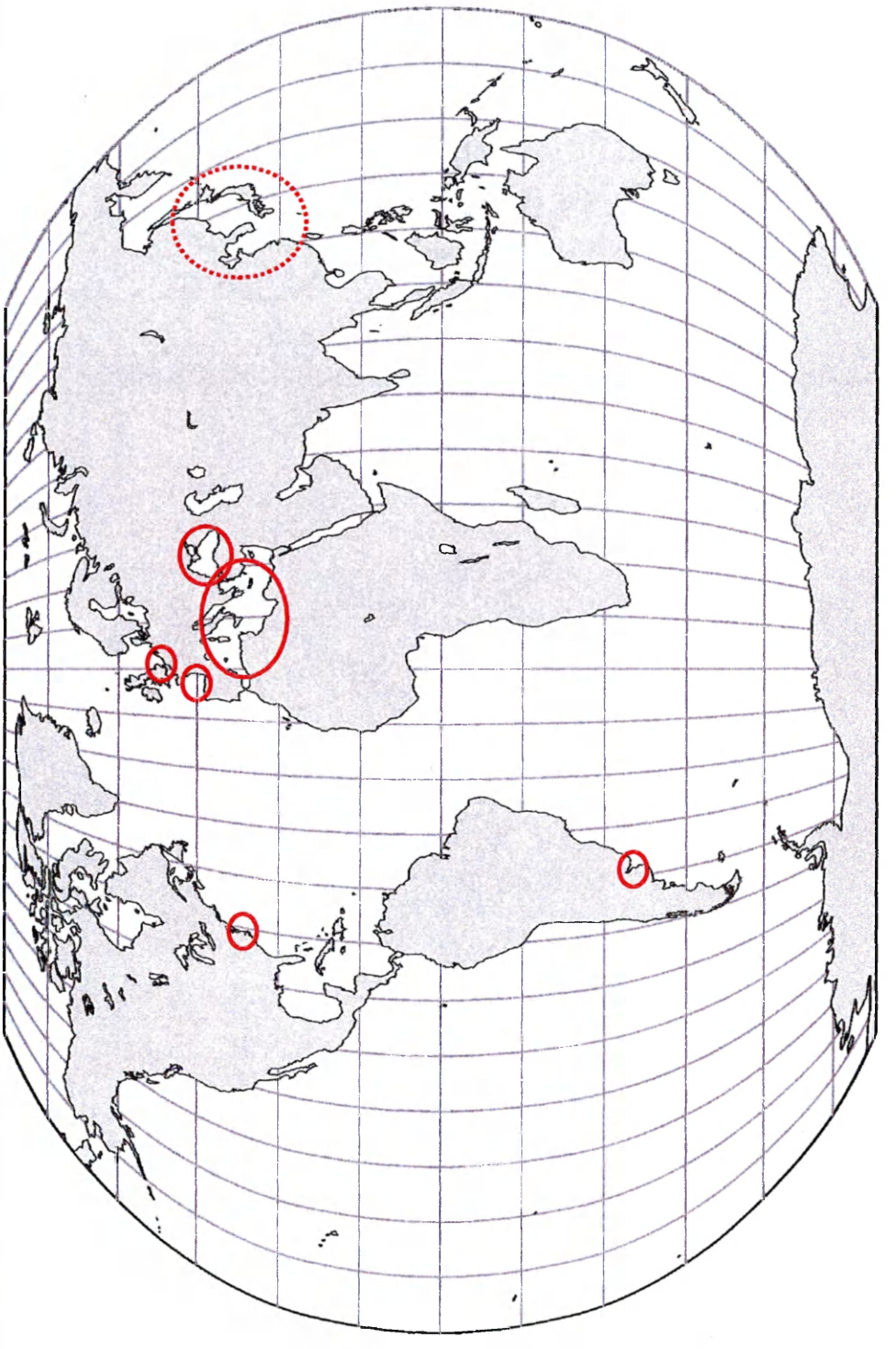


Figure 2: Map of current locations of *Rapana venosa* populations. Native populations are marked with a broken circle.

Figure 3: Locations of *Rapana venosa* samples taken within its native range. Collections were obtained from Laizhou Bay, China; Yantai, China; Qingdao, China; Xiangshan Bay, China; Mikawa Bay, Japan; Cheju-do, Korea; Inch'on, Korea; and Tongyeong, Korea.

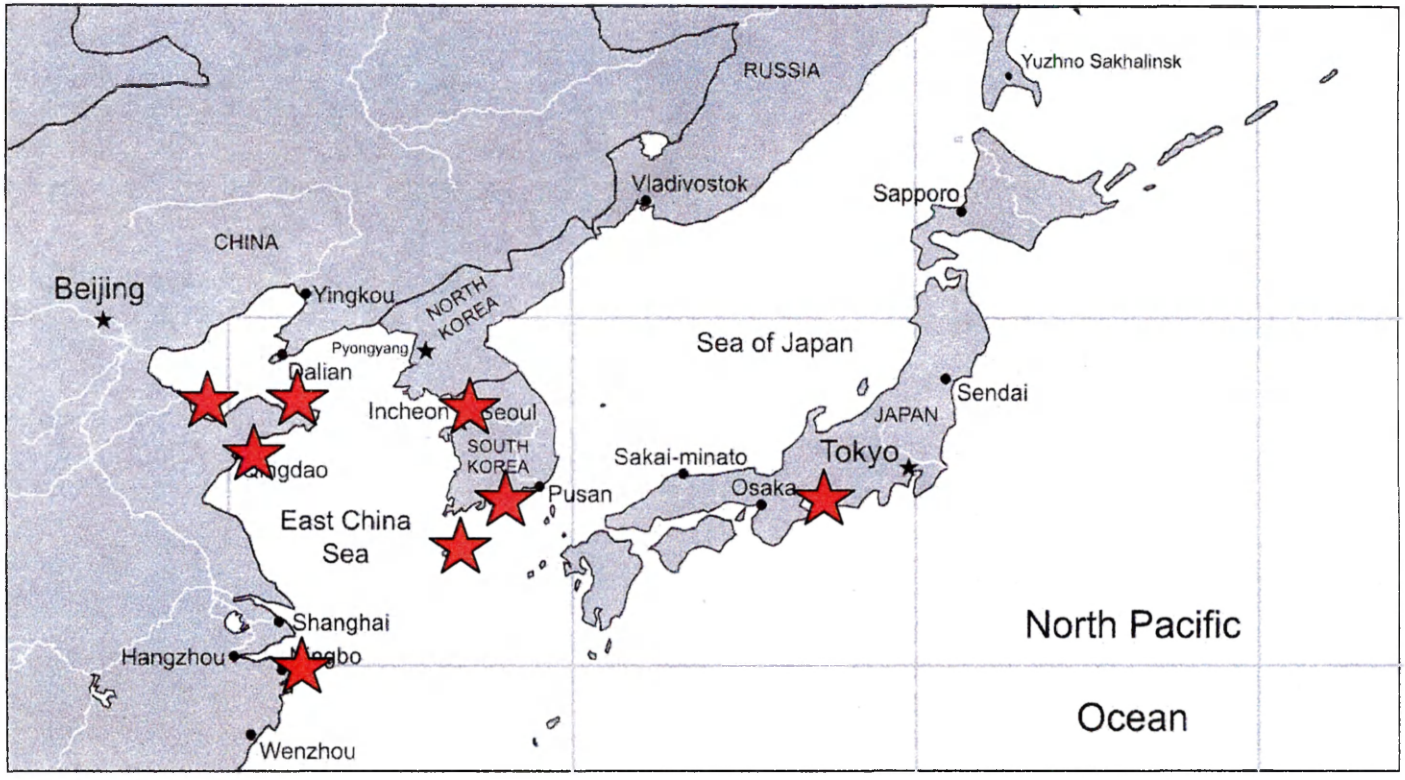


Figure 4: Locations of *Rapana venosa* samples taken from introduced populations in the Black Sea, the Adriatic Sea, Quiberon Bay, France, and off the coast of The Netherlands.

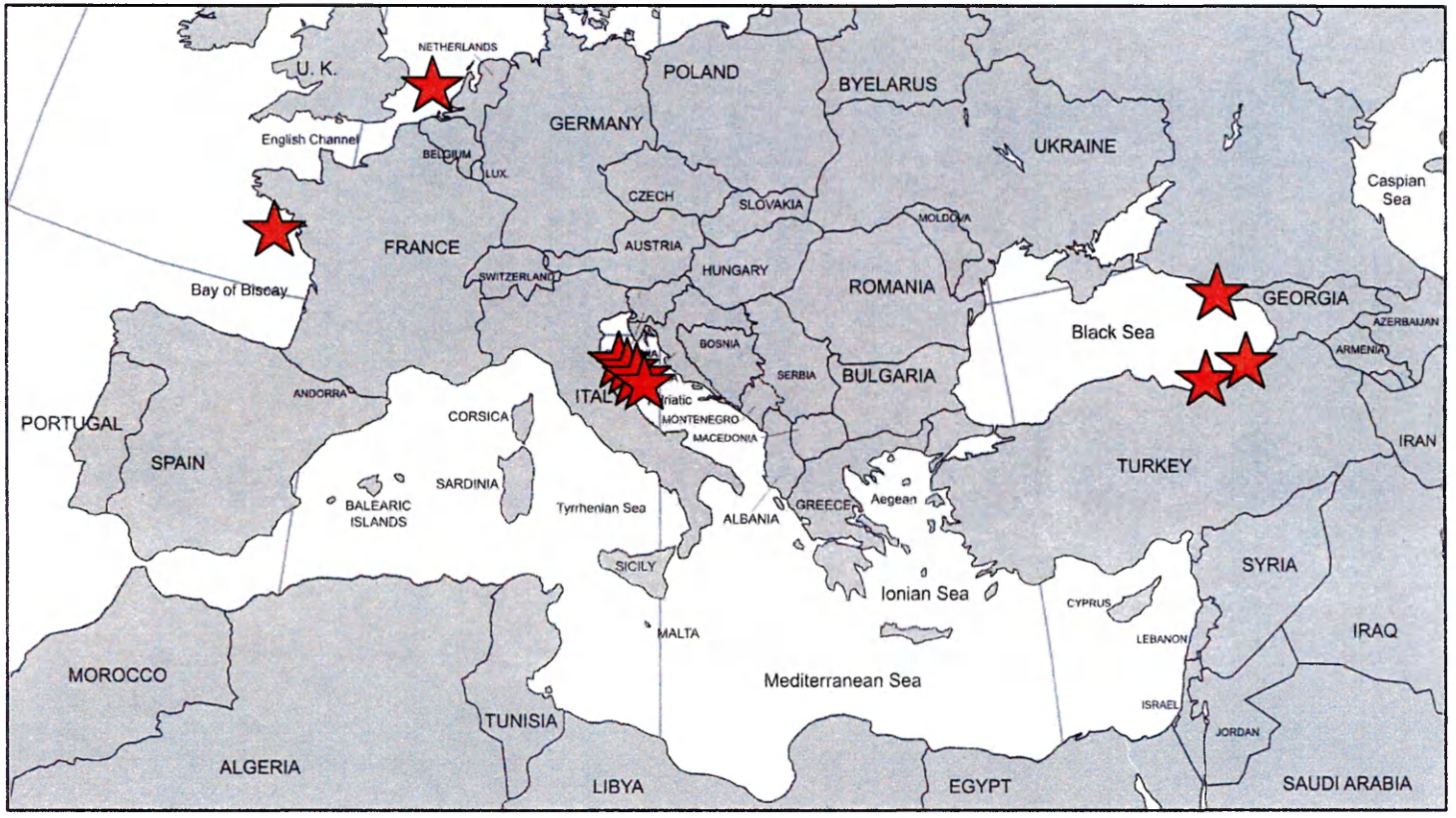


Figure 5: Locations of *Rapana venosa* samples taken within Chesapeake Bay. Samples collected in 2005 were from three location areas: the James River, the Ocean View area, and Tangier Island. Exact locations of samples collected from 1999-2000 were not recorded.

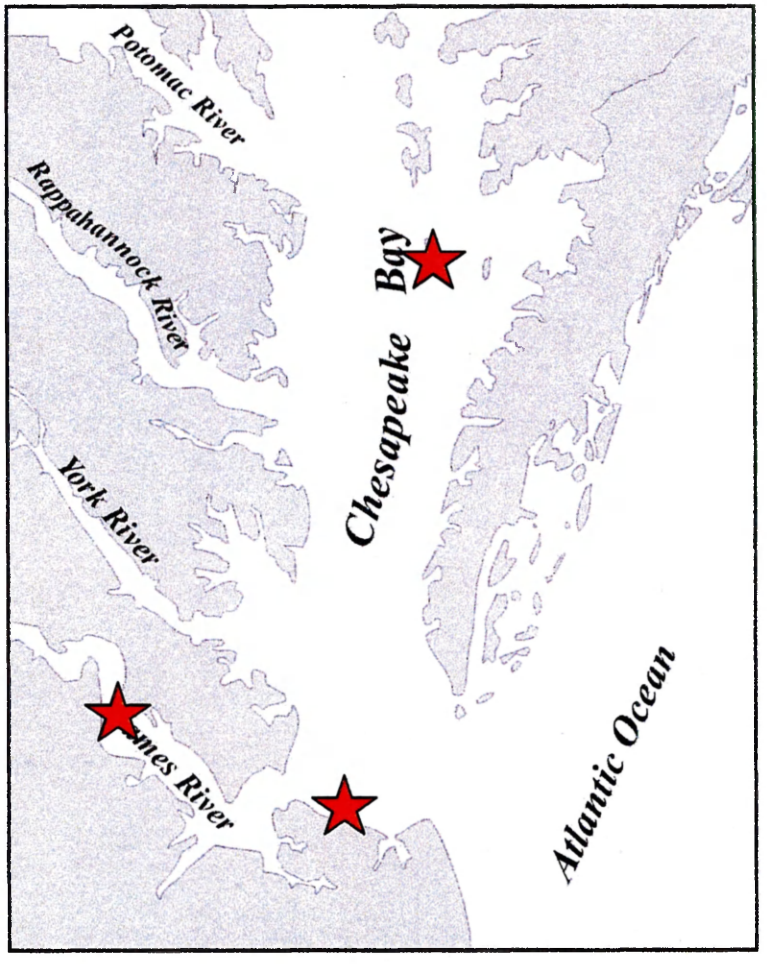


Table 6: Information on *Rapana venosa* samples including the collection location, the sample abbreviation, the time period over which the samples were collected, and the number of samples sequenced from each location.

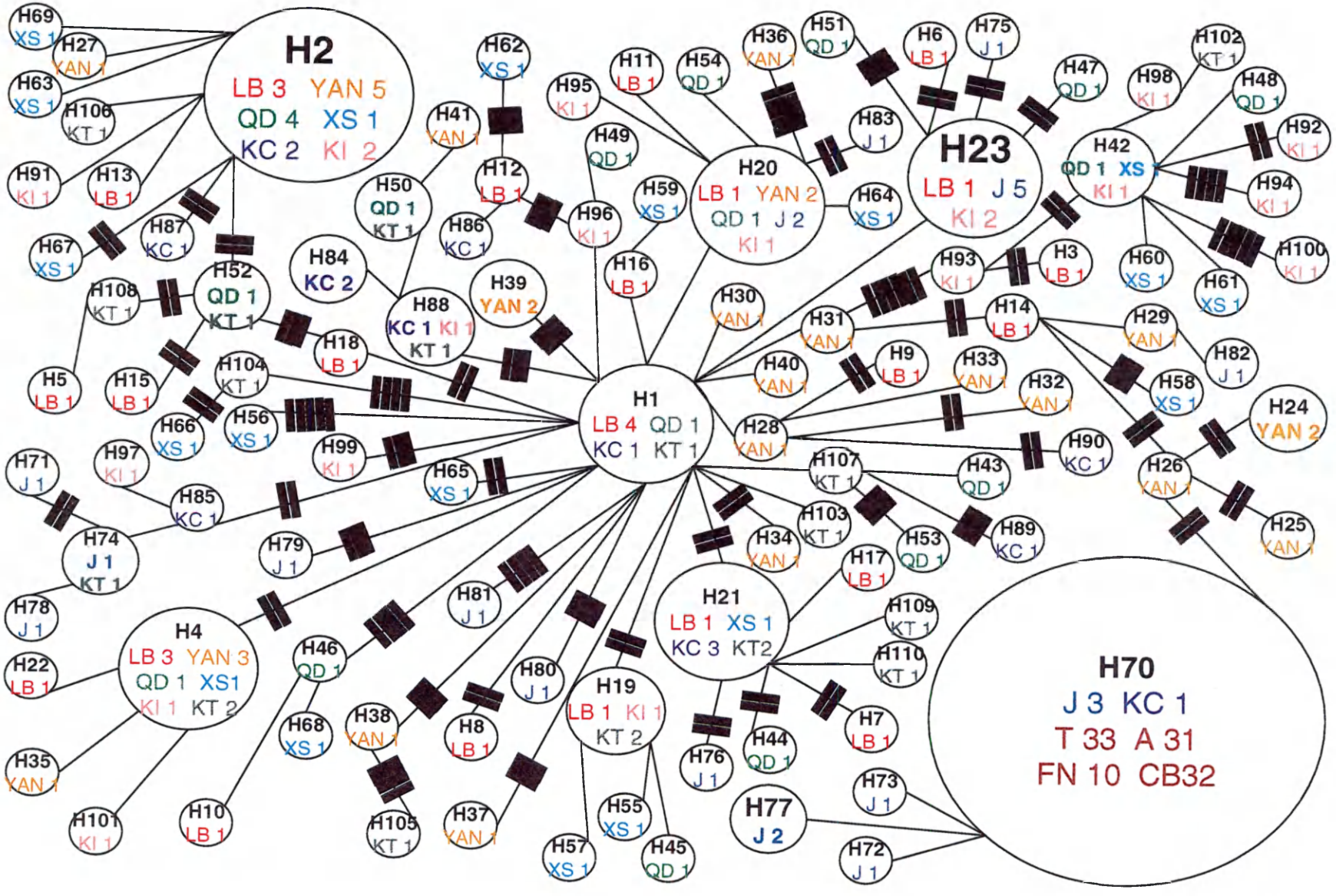
Sample Location	Sample Abbreviation	Date Sampled	Number of Samples	Combined Regional and Temporal Samples
Bohai Sea, Laizhou Bay Weifang, Shandong China	LB	2006	30	30
N. Yellow Sea near Yantai, China	YAN	2006	30	30
Yellow Sea Qingdao, China	QD	2005	20	20
East China Sea Xiangshan Bay Zhejiang Province	XS	2005	20	20
Mikawa Bay Eastern Japan	J	2006	24	24
Cheju-do, Korea	KC	1999	15	15
Inch'on, Korea	KI	1999	20	20
Tongyeng, Korea	KT	1999	19	19
Black Sea Rize, Turkey	TR	2005	11	
Black Sea Tuapse, Russia	TA	2006	2	
Black Sea Trabzon, Turkey	T	1999	20	33
N. Adriatic Sea Goro, Italy	AG	2004	5	
N. Adriatic Sea Cesenatico, Italy	AC	2004	5	
N. Adriatic Sea Fano, Italy	AF	2004	5	
N. Adriatic Sea Goro, Italy	AG99	1999	5	
N. Adriatic Sea Porto Garibaldi, Italy	APG99	1999	5	

N. Adriatic Sea Cesenatico, Italy	AC99	1999	5	
N. Adriatic Sea Fano, Italy	AF99	1999	1	31
Quiberon Bay South Brittany, France	F	1998-2005	9	
Scheveningen The Netherlands	N	2005	1	10
Chesapeake Bay James River Virginia, U.S.A.	CBJR	2005	10	
Chesapeake Bay Oceanview Virginia, U.S.A.	CBOR	2005	11	
Chesapeake Bay Tangier Sound Virginia, U.S.A.	CBTR	2005	1	
Chesapeake Bay Virginia, U.S.A.	CB99	1999-2000	10	32

Table 7: Population genetics statistics for each *Rapana venosa* collection calculated from the combined COI/ND2 sequence data.

Location	*n*	No. Haplotypes (N_h)	No. Transitions.	No. Transversions	No. Polymorphic Sites (S)	Haplotpye Diversity (*h*) ± SE	Nucleotide Diversity (p) ± SE	Mean No. Pairwise Differences (*k*) ± SE
Laizhou Bay, China	30	23	41	5	46	0.972 ± 0.018	0.004 ± 0.002	5.430 ± 2.690
Yantai, China	30	21	37	5	41	0.963 ± 0.021	0.005 ± 0.003	6.262 ± 3.058
Qingdao, China	20	17	36	3	39	0.968 ± 0.033	0.005 ± 0.003	6.974 ± 3.421
Xiangshan Bay, China	20	20	47	3	50	1.000 ± 0.016	0.007 ± 0.004	8.695 ± 4.190
Mikawa Bay, Japan	24	16	31	5	35	0.946 ± 0.031	0.005 ± 0.003	6.558 ± 3.211
Cheju-do, Korea	15	11	29	1	30	0.952 ± 0.040	0.005 ± 0.003	6.524 ± 3.268
Inch'on, Korea	20	18	38	1	39	0.990 ± 0.019	0.006 ± 0.003	8.147 ± 3.946
Tongyeong, Korea	19	16	42	1	43	0.983 ± 0.022	0.005 ± 0.003	6.304 ± 3.129
Black Sea, Turkey	33	1	0	0	0	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Adriatic Sea, Italy	31	1	0	0	0	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
France and The Netherlands	10	1	0	0	0	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Chesapeake Bay, Virginia, USA	32	1	0	0	0	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Combined Native Populations	178	110	125	16	136	0.981 ± 0.004	0.005 ± 0.003	6.988 ± 3.299
Overall	284	110	125	16	136	0.843 ± 0.022	0.006 ± 0.003	7.195 ± 3.382

Figure 6: Minimum spanning network of combined COI and ND2 haplotypes. Unless otherwise indicated by black bars, haplotypes are separated by one nucleotide base change. For simplicity, alternate connections are not shown


H2
LB 3 YAN 5
QD 4 XS 1
KC 2 KI 2
H1
LB 4 QD 1
KC 1 KT 1
H23
LB 1 J 5
KI 2
H20
LB 1 YAN 2
QD 1 J 2
KI 1
H4
LB 3 YAN 3
QD 1 XS1
KI 1 KT 2
H21
LB 1 XS 1
KC 3 KT2
H19
LB 1 KI 1
KT 2
H70
J 3 KC 1
T 33 A 31
FN 10 CB32
H77
J 2
H42
QD 1 XS
KI 1
H52
QD 1
KT 1
H88
KC 1 KI 1
KT 1
H84
KC 2
H50
QD 1
KT 1
H74
J 1
KT 1
H69 XS 1
H27 YAN 1
H63 XS 1
H106 KT 1
H91 KI 1
H13 LB 1
H67 XS 1
H87 KC 1
H108 KT 1
H5 LB 1
H15 LB 1
H66 XS 1
H71 J 1
H97 KI 1
H78 J 1
H22 LB 1
H35 YAN 1
H101 KI 1
H10 LB 1
H68 XS 1
H46 QD 1
H38 YAN 1
H105 KT 1
H37 YAN 1
H8 LB 1
H80 J 1
H81 J 1
H79 J 1
H85 KC 1
H104 KT 1
H56 XS 1
H99 KI 1
H65 XS 1
H18 LB 1
H62 XS 1
H41 YAN 1
H12 LB 1
H86 KC 1
H39 YAN 2
H16 LB 1
H96 KI 1
H95 KI 1
H11 LB 1
H54 QD 1
H49 QD 1
H59 XS 1
H36 YAN 1
H51 QD 1
H6 LB 1
H75 J 1
H83 J 1
H64 XS 1
H47 QD 1
H98 KI 1
H102 KT 1
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H100 KI 1
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H61 XS 1
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H14 LB 1
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H82 J 1
H58 XS 1
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H26 YAN 1
H25 YAN 1
H90 KC 1
H43 QD 1
H89 KC 1
H107 KT 1
H103 KT 1
H53 QD 1
H34 YAN 1
H17 LB 1
H109 KT 1
H110 KT 1
H7 LB 1
H44 QD 1
H76 J 1
H73 J 1
H72 J 1
H55 XS 1
H57 XS 1
H45 QD 1
H30 YAN 1
H31 YAN 1
H40 YAN 1
H9 LB 1
H33 YAN 1
H32 YAN 1
H28 YAN 1

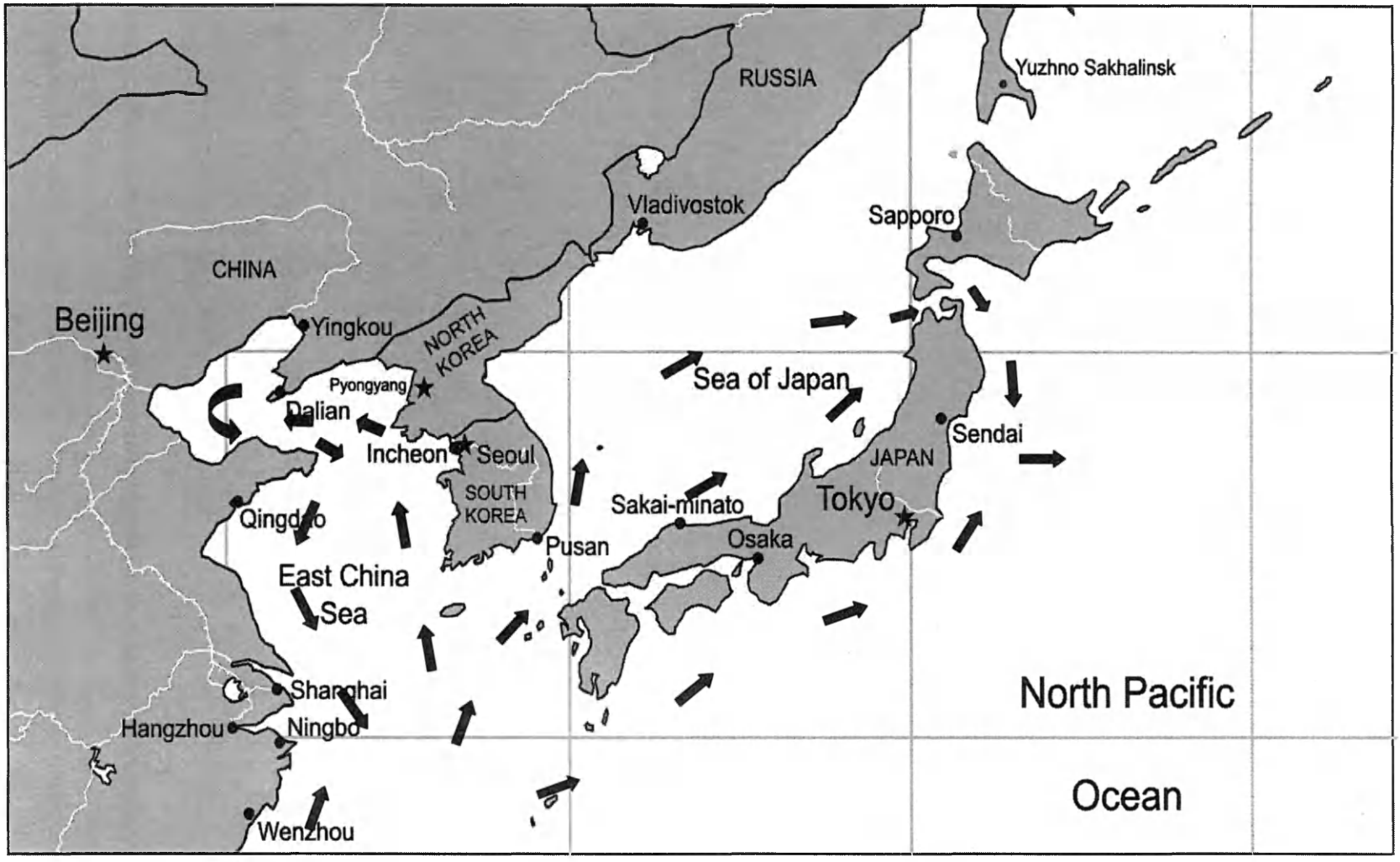
Table 8: AMOVA results of Φ comparison between native collections (LB, YAN, QD, XS, J, KC, KI, KT) and introduced collections (T, A, FN, CB).

	Variance	Percentage of Variation	Φ Statistic	Φ Statistic Value	p value
Among Groups	2.96988	57.31	Φ_{CT}	0.573	0.000
Among Populations Within Groups	0.10311	1.99	Φ_{SC}	0.047	0.000
Within Populations	2.10884	40.7	Φ_{ST}	0.593	0.000

Table 9: Φ_{st} Values for Pairwise Comparisons. Φ_{st} values appear below the diagonal; p values are given above the diagonal and significant p values are in bold.

	LB	YAN	QD	XS	J	KC	KI	KT	T	A	FN	CB
LB	*	0.360	0.739	0.387	**0.009**	0.342	**0.018**	0.405	**<0.001**	**<0.001**	**<0.001**	**<0.001**
YAN	0.003	*	0.135	0.117	**0.009**	0.189	**0.018**	**0.036**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
QD	-0.013	0.013	*	0.973	**<0.001**	0.550	0.333	0.234	**<0.001**	**<0.001**	**<0.001**	**<0.001**
XS	0.000	0.021	-0.031	*	**<0.001**	0.207	0.595	0.234	**<0.001**	**<0.001**	**<0.001**	**<0.001**
J	0.110	0.072	0.111	0.096	*	**<0.001**	**<0.001**	**0.009**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
KC	0.003	0.015	-0.008	0.011	0.103	*	0.063	0.568	**<0.001**	**<0.001**	**<0.001**	**<0.001**
KI	0.054	0.070	0.007	-0.009	0.115	0.066	*	**0.045**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
KT	0.003	0.030	0.010	0.015	0.096	-0.006	0.043	*	**<0.001**	**<0.001**	**<0.001**	**<0.001**
T	0.728	0.661	0.727	0.665	0.555	0.769	0.688	0.756	*	0.991	0.991	0.991
A	0.721	0.654	0.719	0.656	0.546	0.760	0.680	0.748	0.000	*	0.991	0.991
FN	0.618	0.539	0.585	0.509	0.404	0.616	0.537	0.618	0.000	0.000	*	0.991
CB	0.724	0.657	0.723	0.661	0.551	0.765	0.684	0.752	0.000	0.000	0.000	*

Figure 7: Generalized diagram of prevailing currents in the native range of *Rapana venosa* (adapted from Perkins 2001).



CONCLUSION

The entire mitochondrial genome of *Rapana venosa* was sequenced and the order of the mitochondrial genes determined. The *R. venosa* mitochondrial genome was found to be 15,272 bp in length and to contain the 13 protein coding genes, 2 ribosomal RNA (rRNA) coding regions, and 22 transfer RNAs (tRNA) usually present within metazoan mitochondrial genomes. Gene regions of the *R. venosa* mitochondrial genome displayed similarity to those of other neogastropods, *Lophiotoma cerithiformis, Ilyanassa obsoleta*, and *Conus textile,* and the mitochondrial gene order of these four gastropods was found to be identical. The conservation seen between these genomes presents evidence that, although gastropod genomes at high taxonomical levels often show numerous gene rearrangements, at lower taxonomical levels, such as within the suborder Neogastropoda, gene order is conserved. A comparison between each of the mitochondrial gene regions within the neogastropods found the ND4, ND2, and ND6 genes to display the most genetic variation while the COI, COII, and COIII genes displayed the least. The identification of levels of genetic variation within the mitochondrial genes of the neogastropods provides much needed information for researchers choosing highly variable mitochondrial gene regions for use in intraspecfic population studies as well as for researchers choosing highly conserved gene regions for interspecific phylogeny studies.

While the *Rapana venosa* mitochondrial genome does not exhibit a specific and sizeable non-coding control region as seen in vertebrates, it does contain small regions of non-coding sequence. Further analysis of these areas, including searches for potential hairpin structures and palandromic repeats, may help to determine their function and assess whether these regions play a role in controlling the replication and transcription of the mitochondrial genome. Potential hairpin structures have been found in small non-coding regions of the opisthobranches *Roboastra europaea* (Grande *et al.* 2002) and *Pupa strigosa* (Kurabayashi and Ueshima 2000), and palandromic repeats have been found in *Pupa strigosa,* the pulmonate *Albinaria coerulea* (Hatzoglou *et al.* 1995), and the neogastropod *Lophiotoma cerithiformis* (Bandyopadhyay *et al.* 2006).

The phylogeny of gastropods has been an ongoing debate since the 1970s (Colgan *et al.* 2000, Colgan *et al.* 2003). While recent studies have suggested that mitochondrial gene order may help to elucidate these phylogenetic relationships (e.g Knudsen *et al.* 2006), few molluscan mitochondrial genomes have been sequenced and therefore gene order has not yet been used for a large phylogenetic study. Comparisons have been done, however, between the gene orders of the pulmonates (land snails) and opisthobranchs (sea slugs) (Knudsen *et al.* 2006, Grande *et al.* 2002, Kurabayashi and Ueshima 2000). These studies have found that gene order supports morphological and molecular phylogenetic based hypotheses of the monophylogeny of the opisthobranchs and the close relationship between the pulmonates and opisthobranchs, a relationship that in early phylogenetic work was thought to be distant (Trueman and Clarke 1985). Future genetics studies should focus on sequencing whole mitochondrial genomes in order to determine

gastropod and molluscan gene orders, characters that may be useful in evaluating phylogenetic relationships.

Analysis of the mitochondrial COI and ND2 gene sequence data for a range of native and introduced collections of *Rapana venosa* revealed high levels of genetic diversity in native populations. However, all introduced collections exhibited the same mitochondrial haplotype, resulting in no genetic variation. This dramatic decrease in genetic variation is larger than any previously documented for a marine invasion.

One of the goals for identifying source populations of invasions was to identify likely transportation vectors. The genetic composition of *Rapana venosa* within the Chesapeake Bay population is consistent with the hypothesis that ballast water is the probable carrier of rapa whelks to the area. While other dispersal mechanisms have been identified for exotic introductions, ballast water has been deemed the number one vector involved in the transport of marine organisms into new, non-native environments (Carlton 2001, Carlton 1996, Geller and Carlton 1993, Carlton 1985). It is estimated that between 3.5 billion tons (Endresen *et al.* 2004) and 10 billion tons (IMO 1999) of transported ballast water are released globally each year. Additionally, many ballast water sampling studies have shown that ballast water and associated sediments contain a variety of plants and animals comprising many taxa (Gollasch *et al.* 2000a, 2000b, Olenin *et al.* 2000, Williams *et al.* 1988, Carlton 1985, Wonham *et al.*2001, Geller and Carlton 1993, McCarthy and Crowder 2000, Medcof 1975). The results of this study support the idea that ballast water is causing great damage to ecosystems worldwide, and the results add to the growing amount of research that indicate the importance of control measures for ballast water discharge in potentially susceptible receiving environments.

The observed differences in genetic composition of the native and introduced populations of *Rapana venosa* provide evidence that the initial introduction into the Black Sea occurred when one adult female or an egg mat laid by one female was transported into the area; there is at most a 1.56% chance that this introduction occurred as the result of two independent introductions. However, because the mitochondrial genome is inherited maternally, the current study could not provide insight into the amount of variation seen in the paternal lineage of the animals in introduced areas. It is possible that the one female introduced into the Black Sea could have been inseminated by multiple males or that the one egg mat could have contained eggs fertilized by multiple males. A bi-parentally inherited nuclear molecular marker could be used to assess the amount of paternal genetic variation in the introduced populations of *R. venosa*. In addition, if variation were found in a nuclear marker, the invasion pathways between the different introduced populations could be more clearly described. For example, paternal variation may allow us to deduce whether *R. venosa* has been introduced into Chesapeake Bay from the Black Sea/Mediterranean area once or multiple times.

Further molecular work with a nuclear marker could also shed more light onto the reproductive biology of *Rapana venosa.* Based on the observation that females in captivity can lay viable eggs for at least five years after their last mating event (J.M. Harding, personal communication), it appears that females can store sperm. However, it is not known if females can store sperm from a single male or multiple males. Nuclear markers could be used to assess the genetic composition of *R. venosa* eggs to test for multiple paternity within a single egg case or egg mat.

The major genetic bottleneck found in the introduced populations of *Rapana venosa* raises important questions about the ability of these populations to survive in the long term. Nei *et al.* (1975) showed that population bottlenecks cause a loss in the number of alleles present in a population. Since this loss of alleles is not affected much by the rate at which the population grows after the bottleneck, the loss of alleles could cause the population to be unable to quickly adapt to environmental changes.

Rapana venosa populations appear to have overcome any immediate negative fitness consequences resulting from this loss of alleles and have developed growing, reproducing populations with extremely low genetic diversity. Other studies have also documented invasive species with decreased genetic variability surviving and maintaining healthy populations in their new environments (e.g. Ellegren *et al.* 1993, Saltonstall 2002). In addition, Tsutsui *et al.* (2000) found that not only do invasive Argentine ant, *Linepithema humile*, populations with low genetic variation survive in introduced areas, but the lack of genetic variation enables these animals to form supercolonies that do not exist in the native range of these animals. Thus, these geneticly deficient populations are, in the short term, more successful than the more variant native animals.

The low genetic variability of introduced *Rapana venosa* populations, however, may limit the populations' ability to adapt to future long-term environmental change. Very few long-term studies have been conducted on invasive populations and little is known about how low genetic variability will affect long-term success (Stayer *et al.* 2006). Studies focusing on inbreeding depression in natural populations, however, have found a decrease in viability after a population bottleneck. For example, Saccheri *et al.*

(1998) found butterfly populations in southwest Finland with lower levels of genetic heterozygosity to display higher local extinction rates. These populations also exhibited lower larval survival, shorter adult longevity, and lower egg-hatching rates than more diverse butterfly populations. In addition, Mead (1979) found that invasive populations can do well until the individuals face other stresses in addition to that of simply adapting to an new environment. In a study of the giant African snail *Achatina fulica*, introduced populations initially were very successful, but when exposed to disease they underwent large population decreases. Mead concluded that the negative fitness effects of the population bottlenecks experienced by introduced *A. fulica* populations only manifested themselves when the populations were under multiple stresses. Finally, Simberloff and Gibbons (2004) have noted the frequent occurrence of spontaneous population crashes of established introduced species. While some of these crashes can be attributed to a specific cause (e.g. competition, limited resources, parasitism, and adaptation of native species), others have no known cause. Could these crashes be a result of low genetic variability in the introduced populations, resulting in a lack in the populations' ability to evolve and adapt to changes in the new environment? With these questions unanswered and evidence of decreased fitness in populations of low genetic diversity, the long-term success of *R. venosa* introduced populations may be uncertain. One way to assess the ability of the introduced populations of *R. venosa* to adapt to environmental changes would be to look for differences in environmental tolerances between native and introduced populations. If the more genetically diverse animals show a significant increase in their ability to handle environmental stress, they may also be more able to adapt in the future to longer term ecological changes.

APPENDICES

Appendix 1: The nucleotide sequence of the mitochondrial genome of *Rapana venosa.*

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   1 ATGCGTTGAT TATTTTCGAC AAATCATAAA GACATTGGTA CATTATATAT TTTATTCGGA
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 121 CCTGGGGCGT TGTTAGGAGA TGATCAGTTA TACAATGTTA TTGTTACTGC ACATGCTTTT
 181 GTAATAATTT TTTTCTTAGT TATGCCAATA ATAATTGGTG GATTTGGAAA TTGATTAGTT
 241 CCTTTAATAC TAGGAGCTCC TGATATAGCT TTCCCTCGAT TAAACAATAT AAGTTTCTGA
 301 NTACTNCCTC CAGCTCTGTT ATTACTTCTT TCTTCAGCTG CTGTTGAAAG AGGGGTTGGG
 361 ACAGGATGAA CTGTTTATCC TCCTCTAGCA GGGAATTTNG CTCATGCTGG TGGTTCTGTA
 421 GATCTTGCAA TTTTTTCTTT ACATTTAGCA GGTGTTTCTT CTATTTTAGG AGCTGTTAAT
 481 TTTATTACTA CAATTATTAA TATACGTTGA CGTGGTATAC AATTTGAACG GCTTCCATTG
 541 TTTGTATGAT CAGTTAARAT TACAGCAATT CTTCTMTTAC TATCTTTACC TGTTTTAGCA
 601 GGTGCTATTA CAATATTATT AACGGACCGG AACTTTAATA CGGCATTTTT TGACCCAGCA
 661 GGAGGTGGGG ATCCTATTTT GTACCAACAC TTGTTTTGAT TTTTTGGACA TCCAGAAGTA
 721 TATATTTTGA TTCTTCCTGG TTTTGGAATA ATTTCTCATA TTGTAAGTCA TTATTCTGCT
 781 AAGAAAGAAA CTTTTGGTAC TTTAGGTATA ATTTATGCAA TATTAGCTAT TGGGGTTTHA
 841 GGTTATATTG TCTGAGCTCA TCATATATTT ACTGTAGGAA TGGATGTGGA TACACGTGCT
 901 TATTTTACAG CAGCTACTAT AATTATTGCT GTACCTACAG GAATTAAAGT ATTTAGTTGA
 961 CTAGCTACAA TTCATGGAGC TAAAATTAAA TACGAAACAC CTATGCTATG AGCTTTAGGT
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1081 GATATTATGC TTCATGATAC ATATTATGTT GTTGMTCATT TCCATTATGT ATTATCTATA
1141 GGAGCGGTTT TTGCTTTGTT CGGGGCTTTT AAVTACTGAT TTCCTTTACT TAGNGGAGTA
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1981 AATGGTGCTT CCAACTCAAA CAGATATTCG AGTATTAGTA ACTTCTGCAG ATGTAATTCA
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2101 ACTAGGATTT TTTATTAAGT ACCCAGGTGT GTTTTACGGT CAATGTTCCG AGATTTGTGG
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3781 TGGCTCTGAC TTTAAACATA ATAAAGAACT AAAAAATACA CATGGTTTTT ATAGAAAGAG
3841 GCGAGGAGAA GAAATATTAC TGTTGAATTA AAAAAATTAA CGGCTGGTTT TTCTTCAAGA
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4201 TATTCTTGGA TATAAAAAAT ATATGTATTA CCAGAGTACT ATGGATTGAT TACATTTAAA
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4621 AACATAGCAG GGGTAATGGA AATTGTCCCT AAACTAAAAA CATAGTATAA CTACAATACA
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4801 ACCTTATAAA GTAACAAGTA CTGAGAAGGA AAATAATAAT TAAATAATAG AAAGAATAAA
4861 TTTCTGTACC TTTTGCATCA TGGTTTAGCT AGATTTAATT TTCTTAAGAA ATTTCTCGAA
4921 ATCTAATGGG CTATTTTCAA CCAGTATTAT TAGATACTAA AAACTAGTKG TAGCAAAAAT
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5341 AATTATAAAT TAAATAAAGT ATTTTTATTA TAAATTGATG AAAGGAACTC GGCAAAACTA
5401 AATTCCGCCT GTTTATCAAA AACATGGCTC CTCGTTTTTT TACTATGGGG AGTCGGACCT
5461 GCTCAGTGAA TACATTTTTA ACAGCCGCGG TACTCTGACC GTGCAAAGGT AGCATAATCA
5521 TTTGCCTTAT AATTGAAGGC TAGTATGAAT GGTTTGACGA GAATTAAGCT GTCTCTTTTC
5581 AACTAAATAT AATTTTATTT ATAGGTGAAG AAGCCTATAT TCTATTGAAA GACAAGAAGA
5641 CCCTATCGAG CTTAAAAGAA TTTAATAAAT TTATCATATA TTTATAAAAA AGTAAAATTA
5701 TTAAAGATTT TGGTTGGGGC GACTGAGGAA CAAGAAAAGC TTCCTTTATT TTCATTAAAA
5761 CTTACAAGTA TTGATCCAAT AAATTTGATT AAAGAAATTA GTTACCGTAG GGATAACAGC
5821 ATAATCCTCT TTGAGAGCCC TTATCGAAAA GGGGGTTTGT GACCTCGATG TTGGACCAGA
5881 ATATCCAGAA GATGCAGCCG TCTTCAATGG TTGGTCTGTT CGACCATTAA AATTCTACGT
5941 GATCTGAGTT CAGACCGGCG TGAGCCAGGT CAGTTTCTAT CTTCAATTTT TATAATGGCT
6001 TTAGTACGAA AGGACCAAGC TATTGCAAAA TAATTTGTAA TATTTGATTA AATATAAGGA
6061 GTGATTAAAT TTCTAATAAA CATCAAATTA SCAAAKAWTA ATGCAATTGA CTTRGGAWCA
6121 WTAGACATAK GTGAAACCCC TTTATTTGAT AATAAAGATG GCAAAAAAAG TGCATTAGGT
6181 TTAAGCCCTA AATATGAAGA TTAAATTTCT TCTCTTTATA ATGNATATTT CTGTAATTTC
6241 GTGTTTATGT TCCTATNTCT GCGTTTTATT GGCTGTCGCT NTTTTTACTC TTTTAGAACG
6301 AAAAGGACTT AGTTATATAC AGCTACAAAA AGGTCCTAAT AAAGTTGGGA TAATAGGACT
6361 TCCTCAGCCT ATTGCAGACG CAGCCNAACT TTTAACTAAA GAGATTGCTA AGCCAACAAT

6421 AGCAAATTAT TCTCCCTATT TCTTAGCTCC TATTTTTAGT TTTATCTTGG CTCTGTTACT
6481 TTGACAGTTA TATCCTAGCT TATATTCTTT AGGTTATTTT AAATGGGGAA TCTTATTTTT
6541 TTTATGTGTA TCAGGTATAA ATGTTTACGG TACTTTGTTA GCAGGTTGAG CAAGTAACTC
6601 TAAATATGCT TTATTAGGAA GTCTTCGGGC AATTGCTCAA ACTATTTCAT ATGAAATTAG
6661 AATGGCTTTA ATTCTTCTTT TTCCTCTTTT CTTAGTTGGA ACCTTTAGTT TTATTGAAGT
6721 TAAAGAATCG CAAGAAATTA TTTGATTAAG ATTTTTAATA ATTCCTGTCT CTCTTATTTG
6781 ATTTGTGACT TGTGTGGCAG AAACAAATCG AGCTCCTTTT GACTTTGCTG AAGGAGAATC
6841 GGAGTTAGTC TCTGGGTTTA ATATTGAATA TGGCTCAGCT GGATTTGCTT TAATTTTTTT
6901 AGCTGAATAT GCAAATATTC TAGTAATAAG GCTTTTTTCT GCCTTGCTTT TCTTTGGCGG
6961 GAGATCAATT TTTTTTACTG ATAGGGATAT TGTATTCATA ATGAAAGTTT TATTCTTTGC
7021 ATTTTTATTT ATTTGAGTTC GTGGGAGATA TCCTCGATTT CGCTATGACT TATTAATAGG
7081 ATTAACCTGA AAAGGTTTTC TCCCAGCATC ACTTTCTTTT CTTCTAATAA TTGCAATGTT
7141 AGCTTCTTGT ATTTATTATT AATCAAAACC GAAAATGTAG TTTAATTAAA ATTTTAGCAT
7201 TGGAAGCTAA AGATTAGGTA TCAATCCTAC ATTTCGAAAT GACTGCCTTA ATTATCTTCA
7261 GTATAGCTTT TTCTAGATTT CTTATATTAC CATTTATATC CCAACCCTTG AGTTTAGGAT
7321 TAGTTGTTAT GGTTTCAACA CTATTTATAT GTGTTGCTAG GGCAATTACT TTATCATCGT
7381 GATATGGATA TATCTTATTT TTAATCTATG TTGGAGGCTT GTTAGTAATA TTTGCCTATG
7441 TTGCTGCTCT CTCTCCCAAT GTCTTATTTG GTAGGGGAGC TCCTTTAATC TTTTTCTTCA
7501 TATCTTTCTT TTTCTTTTTA GTTCTTATAT TTAATTTTAA TCTTGTTGAT CTTCCTTTTT
7561 TATCATATAA TATAGAATCT AGAAAATTTA GTTTCCTAAA AACATATGGA TCTGAGATAG
7621 TTTCCCCACA AATGATTTCT ATTCTTATTG GTTTAGCTAT TATTTTATTA ATTAACTTAA
7681 TTGTTGTTGT AAAAATTTGT TATTACACTC ATACTTCATT ACGTCCATTT AGAACCTAAG
7741 TTACTATGCG AAGACCTATC CGAAAGGTTC ACCCAGTCTT AAAAGTTGTG AACGGAGCTT
7801 TTGTAGATCT CCCTGCTCCA TCTAATCTGT CAGTTTGATG AAATTTTGGA TCTCTTTTAG
7861 GACTCTGTTT GGTAATTCAA ATTGCTACTG GACTGTTTCT TGCAATGCAT TATACGGCTC
7921 ACGTAGATCT AGCATTTAGT TCTGTAGTGC ATATTAGGCG AGATGTCACT TATGGTTGAC
7981 TTCTTCGAGC ACTTCATGCT AATGGAGCCT CTTGATTTTT CATTTGTTTA TATTTTCATA
8041 TTGCTCGTGG TATATACTAC GGATCATATC TTTATTTGCA CGTTTGAAAC GTTGGAGTAA
8101 TTCTTTTATT TCTAATTATA GGAACAGCAT TTTTAGGATA TGTTCTTCCA TGAGGGCAAA
8161 TATCTTTTTG AGGAGCAACT GTAATTACAA ATTTACTCTC AGCAGTTCCA TATGTTGGTA
8221 AAATGTTAGT AGAATGAGTT TGAGGAGGGT TTGCAGTTGA TAATGCAACT CTTACACGAT
8281 TCTTCGCTCT TCATTTTCTT TTACCATTTG CTGTTGCAGG CTTAGCAATC TTACATATGC
8341 TATTCCTTCA TGAAACAGGC TCTAACAATC CATTAGGATT AAATAGAGAT GGTGAAAAAG
8401 TTCCATTTCA TTCCTACTAC ACTTTTAAAG ATTTAGTCGG TTTTTTAGTA GTTATAACAC
8461 TTTTAACAAT ATTAGCTTTG TTTTCACCTC AATTATTAAC AGATCCTGAA AACTTTATTC
8521 CAGCTAATCC TCTTGTCACC CCAGTACACA TTCAACCAGA GTGATACTTT CTTTTTGCAT
8581 ATGCTATTCT TCGATCTATT CCTAATAAAC TAGGAGGGGT TTTAGGATTA GCKGGATSWK
8641 TTKWWATTTT GTTTATTCTA CCCTTTACAC ATCAAGGAAA ATTTCGTTCT TCTGCATTTT
8701 ACCCACTTAA TCAAATTTTA TTCTGAACTT TTATTGGAAT TTTTTTTGTA TTAACCTGAA
8761 TTGGTAGATG TCCTGTAGAA GCCCCATATG AGCAAATTGG ACAAGTTTTT ACAGGGTTAT
8821 ATTTTATATA CTTTGCTATT AATCCTTTAA TTCAAAAGCT ATGAGATAAT ATTTTAGATT
8881 ATTAAGACTT CAGTTATTTC CTGGGGACAG TTTGTCTTGA AAACAAACTT AAAGTGTTCG
8941 ATTCACTTGA TAACTTTAAG CAATAAGAAC ATCTGTATTC ACCTTTGACT TACAAGACCA
9001 ATGCTCTAAT TTAAGCTATT ATTGCCTATA TGAAATGAGT ACATTTTATT TAACATTATT
9061 AAGTATATTT GGAATTTTAA TAAGATTTCT TACTCTTTCT TTACAGTATA AACATTTACT
9121 AAGAATCCTT CTAAGCTTAG AAGCTATTAC TATAAGACTT TTTATCATAA TATTCTCTAT
9181 ATCCAATAAT ATTATATTAA GAGGAGAAAC TTCTCTTATC CTTATTACTA TAGGGGCATG
9241 TGAAGCADGG TTAGGTCTTG CTATCTTAGT TGCAATTATT CGAAGAGAAG GTAATGACTA
9301 CGTCTCAAGG TTTTCTACAT ATAACTGCTA GGTYTAGTCT TAATAGGTTT CTCATTTATA
9361 CTATTACCTA AATTAAATTC TTGATACTTA AAAATATGAT CTCTTGCTTT AGGTAGCATG
9421 ATTTCTTTAA TTCACCTGTT TACTCCATTC TTTTCTTACK AAAGTATTAA TTTGATATTA
9481 GCTTCTGATT CTTTATCTAK TATTCTAATT TCTCTAACTT TATGAATTTC TCTTATAATA
9541 ATACTAGCGA GACAAAATAR AGTTAAAATT AATAATAATA ATTATTCTCT TTTTTCTTCT
9601 TTCTTATTAT TATTGAATCT AATTCTGATT ATTACTTTCT TATTGTCAAG AAGTTTATTA
9661 TTTTATTTCA TATTTGAAGC ATCTTTAATC CCAACTTTAC TTTTGATTTT AGGATGAGGC

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 9721 TATCAACCAG AACGTCTACA AGCTGGGATA TACATAATAA TTTATACAGT TGCAGCTTCT
 9781 TTACCTTTAC TTTTAACTAT TTTATGAGCA TCTCAAGAAC TTTTTACTAG AAAAATACTT
 9841 TTAGGTAATT TGTTACGTAA CTCAGTTGTT AGTACTGGAA GAATCTGAAC TTGAAATATT
 9901 TTGGTTATTC TAATCTTTAG GGCTTTTCTT GTAAAACTTC CAATATTTAT AGTTCACTTA
 9961 TGACTTCCCA AGGCCCATGT TGAAGCTCCT GTTGCCGGAT CAATAGTACT TGCAGCCATT
10021 TTATTAAAAT TAGGTGGTTA TGGAATTTTA CGATTCTATC AATACCTAAA TTTCTTTCCT
10081 TTAGAAAATC TAATTATCAT TTATTCACTA GCAATTTGAG GTGGAGTTTT GACTAGAATT
10141 ATTTGTTTTC GACAAATTGA CTTGAAATCT TTAATTGCCT ACTCCTCCAT TGGACATATA
10201 TCTTTAATAT TAGCAGGTGT ATTCTCAAAC TCTTCATGAG GGTGAGCTGG AGCTCTAGTT
10261 TTAATATTAT CTCATGGTTT TTGTTCATCT GCTCTTTTTG CTTTAGCAAA TTATACATAT
10321 GAAAAATCCC ACACTCGAAG TCTATTTTTG AGAAAAGGAA TGCTTATGCT CCTTCCTATA
10381 CTAAGTATAT GATGGTTCTT TTTTTGTATT ATGAATATAG CTGCGCCTCC AAGAATTAAC
10441 TTACTTGGAG AAATTTTGAT TTTTCCTTCT GTAATTTTTA GTTCTACTTA TTACTTTATT
10501 CCACTAGGTT TAATAAGTTT TTTAGCTGCC TTATATAGTA TATATCTTTT TACTTCTATC
10561 CAGCANGGTG GAAGTCCTAA GTTTATAAAA CCGTTTAATC AATTTAAACC AGCAGGTTTT
10621 CTTTTACTTT TTCTACATTG AATTCCAGGA AATTTTTTAA TTTTTAAGAG AGAGCTTCTA
10681 TTTATATGGA TTTAAGGTCA AGTTAGTTTA ATTAAAACAT CAGCTTGTGG ATTTGAAAAT
10741 GAAAGACAAC TCTCACTTGA CCATGTTTAC AAAATTAAAA TCTTCCTCTA TTAGTTCACT
10801 TTTTTTATTA GCTTATAGTA CTATATTAAT TCCAGTAACT ATAATATTTA TCTTTAAGGA
10861 AACAACAATT ATTCTAGAAT GAAGTATTAT TCAAGTTAGG TCTTGTATAA TAACCTTAAT
10921 TCTTATTTTG GACCCTATTA GACTTAGTTT TAGTAATGTA GTATGTCTAA TTTCAGGTTG
10981 TGTTATGTTA TTTTCATCTA GTTATATATC TCATGACCCG TTCCTTAAAC GATTTACTTG
11041 GTTAGTAATA CTCTTTGTTC TATCAATAAA TTTATTAGTA TTCATTCCAA GCTTACCTGC
11101 ACTTCTATTA GGTTGAGATG GTCTCGGGAT TGTTTCTTTC GTCCTTGTTA TTTACTACCA
11161 AAATATAAAG TCATTAGGTG CTGGTATATT AACAGTTTTA GCTAATCGAA TTGGTGATGT
11221 TATAATTTTA ATTTCTATCG GTCTTCTAGT TTTACAAGGC CATTGAATAA TTATTTCAAT
11281 CTGAGATTTT TACCTAAGAG CTTGAGTAGC CGTAACCATT ACCTTAGCTG CCATAACTAA
11341 AAGAGCTCAA ATTCCTTTTT CAAGATGACT TCCGGCAGCC ATAGCTGCCC CAACCCCAGT
11401 TTCAGCTCTT GTTCATTCTT CAACTCTAGT TACTGCAGGA GTATTTTTAA TTATCCGATT
11461 TTTTCCTTTT TTAAGATCAA TTTCTGGATT TAATACTTTT CTATTGTTTA TTTCTGTATT
11521 AACTCTTTTA ATAGCCGGTA TTGGAGCAAA CTATGAAAAT GACTTAAAAA AAGTAATCGC
11581 TTTATCCACT TTAAGACAAC TAGGTGTAAT AATAATAAGA CTCGGGATAG GTATGCCTTA
11641 TCTTGCTTTA TTTCATCTTT ATACTCATGC CTTATTTAAG GCCCTTTTAT TCCTCTGTGC
11701 CGGAATAATT ATTCACAATA GATCAAATAC ACAGGATATT CGGCACATAG GACTTTTATT
11761 TTCTCAAGCA CCTCTTACAG TGGGCTGTAT AAATGTTGCA AACTTAGCTT TATGTGGAGC
11821 CCCCTTTCTA AGGGGATTTT ACTCTAAAGA TCTTATTTTA GAATTCTCTT TATATAGCCC
11881 TACCAACCTT CTTATGATTT TATTAATTTT TTTAGCTACA GGAATAACAG CCGCTTATTC
11941 TCTCCGACTT TCTTTCTGTT CTCTTTGAGG TTCAATAAAA AATAGTCCAT ATCACGCAAA
12001 GCAAGAAGCA GATCCTTACG TAAACTGAGC AACTACCACT CTTACATTAG CAGCCATCAC
12061 AGCGGGTTTA TATTTTCAGA ATATTTTTTT AACATTTTCT CCAACCCCAT TTATTCTCCC
12121 AACATTGCAT AAAATATTGA CAATAACAGT TATTTTGCTA GGACTTCTAA CAGCATCTAT
12181 CTTATGAGAT GCAAATCACA CTACTGTCAA AATAAACAAA ATTAAGTTTT TCTTCTCTAC
12241 AATGTGATTT TTAGCTCCTA TTTCAGCTCA GCCAATAACT AAGTTTTCTA TACTTATAGG
12301 AACAAACATG ATAAAATCAA TTGATATGGG ATGACTAGAA ATTTTAGGCG GACAAGGATC
12361 GGCTGTTATT ACAAGCAATT TCTCAGTTCT AAATCAAAAA CTACAAATTA AAACCTTTAA
12421 TTTTTTCATT GTTCTAATAT TATTTGCATT GATAATATTT ATTCAAATTT AGCTCAGATA
12481 GCTTACATAT AGAGCATAGC ACTGAAGATG CTAGGGTGAC AATTACTGTC TCAAAGCAAG
12541 CCAGCGCTCA CCACGAGAGC GCTGGCTTGC TAGTAACCAA TAACAAATAA TTATATGGGA
12601 CGGAATCCAT TTCATTTAGT AGAATYTAGK CCTTGACCTT TAACAGGGTC AATAGGAGCA
12661 TTATTTTTAA CTTCTGGATT AGCTGGTTGA TTTCATGGTT ATGGCTATAT TACTATAGTT
12721 CTAGGATTGG TTTTAATTGC TATAACTATA GTTCAATGGT GACGGGATGT AATTCGGGAA
12781 GCTACATTTC AAGGATATCA TACAATTCAA GTTTCTAAAG GACTTCGTTG GGGTATAATT
12841 TTATTTATTG TATCAGAAGT TTGTTTCTTT TTTGCTTTCT TTTGAGCATA TTTTCATAGA
12901 AGACTTGCGC CAAGTCCTGA ACTTGGCTCT TGCTGACCTC CTAGGGGAAT TGTTCCATTA
12961 AATCCATTTG AAGTTCCGTT ACTTAATACT GGGGTTTTAT TAGCTTCTGG TGTAACTGTA
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13021 ACATGGGCTC ATCATAGTTT AATGGAAGGG GATAATCCCA GTGGACTTCA AGGATTAGTT
13081 GCAACAGTAA TTTTAGGCGT TTATTTTACT TTTTTACAGG GAGGTGAATA TTATGAAGCC
13141 TCATTTACTA TTGCTGATGG AGTTTATGGA TCTAGTTTTT TTGTGGCTAC AGGATTTCAT
13201 GGTTTACATG TGCTTATTGG AAGAACTTTT CTCTTGGTTT GTCTTGTTCG TGTCTGGCTA
13261 CAGCATTTTT CTACAGGACA TCATTTTGGG TTCGAGGCTG CTGCATGATA CTGACATTTT
13321 GTAGATGTTG TATGACTTTT TTTATACCTT TCTATTTACT GATGGGGCTG TTAAAGTTAT
13381 AATTAATTTT TATTTATTCT TAGGTGACTG AGTTAAATAA GTGTTAGATT TTTAATCTAA
13441 AAACAGCAGA TTTTGCTCCT AAGAGCCAGA CTAGGTACTT TAAAATAAAA GATCTGATTT
13501 GCATTTAGAA AATAAGTATT TATACTTCTT GGTCAATTTT TAATTTAAGG TATAAAAAGC
13561 GAGAAATTTG CATATGGTTT CGGCCCATAC CTTGAGGGTG TAAGTCCTTC TTTATATTTA
13621 TTAAATGAAA GCCAAAGTAG AGGCACCTAC CTGTTAACTA GGAGAATGTA ATAAAATTTA
13681 CTCATTTAGC TTTAAAGCGT TAAGTGAAGT ATTACGCCGG ATGAACGGAA ATCATTGATG
13741 TTGATTAACA TGTGGCTAAA AGTCTCTGAT ACTAGAATGT TAGGTAGATT ACTAAGTAGA
13801 TTTATTGCTA TTGTTTTATC CTGTGTTGTG ATAGGTCTTG GATGGGTGTT AGCTAAACGA
13861 GCTATTTCAG ATCGTGAAAA GAGCTCTCCT TTTGAGTGTG GATTTGATCC TATTAAATCT
13921 GCACGGCTTC CTTTTTCTTT GCGATTTTTC TTGCTTGCTA TTATTTTTTT AATTTTTGAT
13981 GTAGAAATTG TTCTTTTATT CCCTATTTTA ATTAGAATGA CAAGAAGATT TTCTTTACCT
14041 GTAGTAATTA GTTTATTTGT ATTTTTAATA ATTTTAATTG TCGGGTTGTT TCACGAGTGA
14101 AACGAAGGGT CATTAGACTG AGCTCAATAA ATTCAAAAAT TTAGAAAAAA CTTGGAGTAA
14161 AACAGGGCTG CTAACTTTGT TTTGGGTAAT TCGATTTTAT CCTTTTTCTT ATGTTTTCTG
14221 TTCTGCCTTT TAGTTATATA TTTATAATAG TTATAGTAAT GGGGACTCTT CTTTCTGTCT
14281 CTTCTTTTCA TTGATTAAGA ATTTGAGCGG GTTTAGAAAT TAATTTAATT GGATTTTTAC
14341 CTTTATTAGT CTATCAAAAA AGGACTTCAG AAAGAGAGTC GGCTGTAAAA TATTTTATTG
14401 TTCAAGCACT AGGATCCAGG ATGCTTATGT TTGGAAGACT TATATCATTT AACATATCTT
14461 TCACATGAGA TTTATATGTA AATGGAATAT CACATTCTGT TGGGTTATTA ATTATTTTAA
14521 GAGGACTATG TATAAAACTT GGTTTATTTC CCTTTCATTA TTGATTACCT AGGGTGATAG
14581 CAGGTTTGCC TTGAATTACT TGTTTGCTTT TGGCTACTTG ACAAAAGTTT GCTCCTCTTT
14641 TCCTTTTTTT ATGTTTATTA GAACTAAGAG AATCTTATAT CTTAATCTTA TCTTTATGTA
14701 TTATTAGAGC AGGCTCTAGA CTTGTAGGTG GAATTGGAGG GATGAATCAA ACACAAATTC
14761 GGGCGTTATT AGCTTATTCT TCTATTGGTC ATTTAGGTTG AATAACTTTC GCTTTATTGC
14821 ATAGAGAATG ATCTATAAAA TTTTATTTAC TCATTTATGT GCTTGTATCN GTATTTATAT
14881 TTGTTAGATT ATGGGGAGCA GATATAAGAA CAATAAAAGA TATCAGGAGA TTAAAAAACT
14941 TTAGTTTTGT GCAGATAAGA GTTATACTTT TCTTATTATC TTTAGGTGGG TTACCTCCTT
15001 TATTAGGCTT TGTTTCTAAA TGATTGGTAA TTCTAGTAAG AAGGGGAAAT GCTCTCCTTT
15061 TTGTTTTATT TTTCCTTATT TTAGGTTCTT TAATAAGTTT ATTTTATTAT TTAAGTTTAT
15121 TTTTTTCTAT TTTTCTAAGG AACTTAAAAG AGGGGGAAAT TAGAAGTTTA ATGTTTAAAG
15181 GAAAAGAAAG TAGTTTTTTA TCCTTGGCTG TTTTATTTAA CATGGCCGGA GGTATTATAA
15241 TTGCTTTTAG AAACTTATTT TATATACTTT AA

Appendix 2: Table of polymorphic sites based on combined COI/ND2 data. Vertical numbers at the top of the haplotype list indicate the position number of the sites in the combined COI/ND2 sequence.

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Haplotype
[                1 1111111111 2222222223 3333333344 4444445555 5667777777 7777788888 ]
[       3455566890 1123445779 1123489995 6667888800 1125693478 9190001255 6888901123 ]
[       2403925651 0984685047 2546873690 2781056917 3652403557 8373476525 9045094876 ]
H1      GCAGGCTTAA GTTTCACACA ATATATCTTT TACAAGTGGT GAATTAGATA GAAATGACCG TGCCAACCGA
H2      .......... .......... .......... .........C .......... .......... ....G..T..
H3      .......... .....G.... ....G..... .G...A.... .......G.. .......... ..........
H4      .......... .......... .......... .......... .......... .......... .....G....
H5      .......... .......... .......... .........C .........T .......... ....G..T..
H6      .T........ .......... .......... .......... ....G..... .......... ..........
H7      .......... .......... .......... .......... ........C. .......... ..T.......
H8      .......... .......... .C........ .......... .......... .......... ..........
H9      .......... .......... .......... .......... .......... .......... ........A.
H10     .......... T......... .......... .......... .......... .......... ......G...
H11     .......... .......... .......... .......... .......... .......... ..........
H12     .......... .......... .......... ...G.....C .......... .......... ....G..T..
H13     .......... .......... ........C. .........C .......... .......... ....G..T..
H14     .......... T......... .....C.... .....A.... .......... .......... ..........
H15     .......... .......... .......... .........C .......... .......... ....G..T..
H16     .......... .......... .......... .......... .......... .......... ..........
H17     .......... .......... .......... .......... ........C. .......... C.........
H18     .......... .......... .......... .......... .......... .......... ..........
H19     .......... .......... ...C...... .......... .......... ......G... ..........
H20     .......... .......... .......... .......... .......... .......... ..........
H21     .......... .......... .......... .......... ........C. .......... ..........
H22     .......... .......... .......... .......... .......... .......... .....G....
H23     .......... .......... .......... .......... ....G..... .......... ..........
H24     .......... T.C....... .....C.... ....GA.... .......... .......... .....G....
H25     .......... T.C....... .....C.... ....GA.... .......... ........A. ..........
H26     .......... T.C....... .....C.... ....GA.... .......... .......... ..........
H27     .......... .......... .......... .........C .......... .......... ....G..T..
H28     .......... .......... .......... .......... .......... .......... ..........
H29     .......... T......... .....C.... .....A.... .......... .......... ..........
H30     .......... .......... .......... ..T....... .......... .......... ..........
H31     .......... .......... .......... .....A.... .......... .......... ..........
H32     .......... .......... .......... .......... .......... .......... ..........
H33     .......... .......... .......... .......... .......... .......... ..........
H34     .......... .......... .......... .......... .......... ..G....... ..........
H35     .......... .......... .......... .......... .......... ...G..... .....G....
H36     .......... ......T... ......T... ..T....... .......... .......... ..........
H37     ........G. .......... .......... .......... .......... .......... ...T......
H38     .......... ...C...... .C........ ......C... .......... .......... ..........
H39     .......... .......... ......T... .......... .....G.... .......... .......G..
H40     ..G....... .......... .......... .......... .......... .......... ..........
H41     .......... .......... .......... .......... .......... .........A ..........
H42     .......... .....G.... ....G..... .G...A.... .......G.. .......... ..........
H43     .......... .......... .......... C......... .......... .......... ..........
H44     .......... ....T..... .......... .....A.... ........C. .......... ..........
H45     .......... .......... ...C...... .......... .......... ......G... ..........
H46     .......... T......... .......... .......... .......... .......... ..........
H47     .....T.... .......... .......... .......... ....G..... .......... ..........
H48     .......... .....G.... ....G..... .G...A.... .......G.. .......... ..........
H49     .......... .......... .......... .......... .......... .......... .A.....T..
H50     .......... .......... .......... .......... .......... .........A ..........
H51     .......... .......... .......... .......... ....G..... .......... ....G..T..
H52     .......... .......... .......... .........C .......... .......... ....G..T..
H53     .......... .........G .......... .......... .......... .........A ..........
H54     .......... .......... .......... .......... .......... .......... ........C.
H55     .......... .C........ ...C...... .......... .......... ......G... ..........
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H56       .......... .......... .......... .......... ...C...... .......... ..........
H57       .......... T......... ...C...... .......... .......... ......G... ..........
H58       .......... T......... .....C.... .....A.... .......... .......... ..........
H59       .......... .......... .......... .......... .......... .......... ..........
H60       .......... .....G.... ....G..... .G...A.... .......G.. .......... ..........
H61       .......... .....G.... ....G..... .G...A.... .......G.. A......... ..........
H62       .......... .......... .......... .........C .......... ....C..... ....G..T..
H63       .......... .......... .......... .........C .......... .......... ....G..T..
H64       .......... .......... .......... .......... ......A... .......... ..........
H65       .......... .......... .......... .......... .......... .......... .........G
H66       .......... ......AG.. .......... .......... .......... .......... ..........
H67       .......... .......... .......... .........C .......... .......T.. ....G..T..
H68       .......... T......... .......... .......... .......... .......... .A........
H69       .......... .......... .......... ......C..C .......... .......... ....G..T..
H70       .......... T.C....... .....C.... ....GA.... .......... .......... ..........
H71       .........G .......... .......... .......... .......... .......... ..........
H72       .......... T.C....... .....C.... ....GA.... .......... .......... ..........
H73       .......... T.C....... .....C.... ....GA.... .......... .G........ ..........
H74       .........G .......... .......... .......... .......... .......... ..........
H75       .......... .......... ...C...... .......... ....G..... .......... ..........
H76       .......... ....T..... .......... .......... .........C. .......... ..........
H77       ....A..... T.C....... .....C.... ....GA.... .......... .......... ..........
H78       .........G .......... .......... .......... .......... .....A.... ..........
H79       .......... .......... .......... ........A. .......... .......... ..........
H80       .......... .......... .......... .......... .G........ .......... ..........
H81       ......CC.. .......... .......... .......... .......... .......... ..........
H82       .......... T......... .....C.... .....A.... .......... .......... ..........
H83       .......... ........T. .......... .......... A......... .......... ..........
H84       .......... .......... .......C.. .......... .......... .........A ..........
H85       .........G .......... .......... .......... .......... .......... ..........
H86       .......... .......... .......... ...G.....C .......... .......... ....G..T..
H87       .......... .......... .......... .........C .......... .......T.. ....G..T..
H88       .......... .......... .......... .......... .......... .........A ..........
H89       ...A...... .......... .......... .......... .......... .......... ...T......
H90       .......... .......... .......... .......A.. .......... .......... ..........
H91       .......... .......... .......... .........C .......... .......... ....G..T..
H92       A......... .....G.... ....G..... .G...A.A.. .......G.. .......... ..........
H93       .......... .....G.... ....G..... .G...A.... .......G.. .......... ..........
H94       .......... .....G.... G...G..... .....A..A. .......G.. .......... ..........
H95       .......... .......... .......... .......... .......... .......... ..........
H96       .......... .......... .......... .......... .......... .......... .......T..
H97       .........G .......... .......... .......... .......... A......... ..........
H98       .......... .....G.... ....G..... .G...A.... .......G.. .......... ..........
H99       .......... .......... .......... .......... .......... .......... .........G
H100      .......... .....G.... ....G....C .....A.... .......G.. .......... ..........
H101      .......... .......... .......... .......... .......... .......... .....G....
H102      .......... .....G.... ....G..... .G...A.... .......G.. .......... ..........
H103      .......... .......... .......... .......... .......... .......... ..........
H104      .......... .......G.. .......... .......... .......... .......... ..........
H105      .......... ...C...... .CT....... ......C... ..G....... .......... ..........
H106      .......... .......... .......... .........C .......... .......... ....G..T..
H107      .......... .......... .......... .......... .......... .......... ..........
H108      .......... .......... .......... .........C .......... .......... ....G..T..
H109      .......... .......... .......... .......... ........C. .......... ..........
H110      .......... .......... .......... .......... ........C. .......... ..........

[                              1111111111 1111111111 1111111111 111]
[         8888888999 9999999999 0000000000 1111111111 1111222222 222]
[         5566779123 4455577889 2226777999 1235577777 8889001126 677]
[         4709453498 6735989068 1244236179 5703714579 0160257932 501]
H1        TACGTTACTC TTTCTATAAG ATAAGCACTC TGAGTAACCA CAGTATGATG TGT
H2        .......... .......... .....T.... .......... .......... ...
H3        .......... .C........ .......T.. .......... ........C. ...
H4        .......... .......... .......... .......... .......... ...
H5        .......... .........A .......... .......... .......... ...
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H6      .......... .......... .......... .......... .......... ...
H7      .G........ .......... ........C. .......... .......... ...
H8      .......... .......... .......... .........G .......... ...
H9      .......... .......... .C........ .......... .......... ...
H10     ...A...... .......... .......... .......... ......A... ...
H11     ......G... .......... .......... .......... .......... ...
H12     .......... .......... .......... .......... .......... ...
H13     .......... .......... .....T.... .......... .......... ...
H14     .......... .......... .......... .......... .......... ...
H15     C........T .......... .......... .......... T......... ...
H16     .......... .......... .......... .......... .....C.... ...
H17     .G........ .......... .......... .......... .......... ...
H18     .........T .......... .......... .......... .......... ...
H19     .......... .......... .......... .......... .......... ...
H20     .......... .......... .......... .......... .......... ...
H21     .G........ .......... .......... .......... .......... ...
H22     .......... ..C....... .......... .......... .......... ...
H23     .......... .......... .......... .......... .......... ...
H24     .......... .......... .......... .......... .......... ...
H25     .......... .......... .C........ .......... .......... ...
H26     .......... .......... .......... .......... .......... ...
H27     .......... .......... .....T.... .......... .......... ...
H28     .......... .......... .C........ .......... .......... ...
H29     .......... ........G. .......... .......... .......... ...
H30     .......... .......... .......... .......... .......... ...
H31     .......... .......... .......... .......... .......... ...
H32     .......... .......... GC........ ...A...... .......... ...
H33     .......... .......... .CG....... .......... .......... ...
H34     .......... .......... .......... .......... .......... C..
H35     .......... .......... .......... .......... .......... ...
H36     .......... .......... ....T..... .....G.... .......... ...
H37     .......... .......... .......... .........G .......... ...
H38     .......... .......... .......... .......... .......... ...
H39     .......... .......... .......... .......... .......... ...
H40     .......... .......... .......... .......... .......... ...
H41     ..T....... .......... .........T .A........ .......... ...
H42     ........C. .......... .......T.. .......... ........C. ...
H43     .......... .......... .......... C......... .......... ...
H44     .G........ .......... .......... .......... .......... ...
H45     .......... .......... .......... .......... .G........ ...
H46     ...A...... .......... .......... .......... ......A... ...
H47     .......... .......... .......... .......... .......... ...
H48     ........C. .......... ..G....T.. .......... ........C. ...
H49     .......... .......... .......... .......... .......... ...
H50     ..T....... .......... .......... .A........ .......... ...
H51     .......... .......... .....T.... .......... .......... ...
H52     .........T .......... .......... .......... .......... ...
H53     ...A...... .......... .......... C......... .......... ...
H54     .......... .......... .......... .......... .......... ...
H55     .......... .......... .......... .......... .......... ...
H56     .......... .......... .......... ........TG .......... ...
H57     .......... .......... .......... .......... .......... ...
H58     .......... C......... .......T.. .......... ......A... ...
H59     .......... .......... .......... ...A...... .....C.... ...
H60     ........C. .......... .......T.. C......... ........C. ...
H61     ........C. .......... .......T.. .......... ........C. ...
H62     .......... ......G... .......... .......... .......... ...
H63     .......... .......... .....T.... .......... ...C...... ...
H64     .......... .......... .......... .......... .......... ...
H65     .......... .......... ....A..... .......... .......... ...
H66     .......... .......... .......... .A........ .........A ...
H67     .......... .......... .....T.... ..G....... .......... ...
H68     ...A...... .......... .......... .......... ......A... ...
H69     .......... .......... .....T.... .......... .......... ...
H70     .......... ....C..... .......T.. .......... .......... ...
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H71     .....C.... .......... .......... .......... .......... ...
H72     .......... ....C..... .......T.. .......... .......... ..G
H73     .......... ....C..... .......T.. .......... .......... ...
H74     .....C.... .......... .......... .......... .......... ...
H75     .......... .......... .......... .......... .......... ...
H76     .G........ .......... .......... .......... ......A... ...
H77     .......... ....C..... .......T.. .......... .......... ...
H78     .....C.... .......... .......... .......... .......... ...
H79     .......... .......... .......... .......... ....G..... ...
H80     .......... .......... ...G...... .........G .......... ...
H81     .......... ........G. .......... .......... .......... ...
H82     .......... ........G. .......... ....C..... .......... ...
H83     .......... .......... .......... .......... .......... ...
H84     ..T....... .......... .......... .......... .......... ...
H85     .......... .......... .......... .......... .......... ...
H86     .......... .......... .......... .......... .......... .A.
H87     .......... .......... .....T.... .......... .......G.. ...
H88     ..T....... .......... .......... .......... .......... ...
H89     .......... .......... .......... C......T.. .......... ...
H90     .......... .......... .C........ C......... .......... ...
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H92     ........C. .......... .......T.. .......... ........C. ...
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H94     ........C. .......... .......T.. .......... .......... ...
H95     .........T .......... .......... .......... .......... ...
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H106    .......... .......... .....T.... .......... .......... ...
H107    .......... .......... .......... C......... .......... ...
H108    .......... .........A .......... .......... .......... ...
H109    .G........ .......... G......... .......... .......... ...
H110    .G........ .......... .......... .......... ..A....... ...
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