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Genetically monomorphic invasive populations of the 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, USA, and in the Rio de la Plata between Uruguay and Argentina. This study surveyed variation in two mitochondrial gene regions to investigate the invasion pathways of *R. venosa*, identify likely sources for introduced populations, and evaluate current hypotheses of potential transportation vectors. Sequence data were obtained for the mitochondrial cytochrome *c* oxidase I and NADH dehydrogenase subunit 2 gene regions of 178 individuals from eight native locations and 106 individuals from 12 introduced locations. Collections from within the native range displayed very high levels of genetic variation while collections from all introduced populations showed a complete lack of genetic diversity; a single haplotype was common to all introduced individuals. This finding is consistent with the hypothesis that *R. venosa* was initially introduced into the Black Sea, and this Black Sea population then served as a source for the other secondary invasions by various introduction vectors including ballast water transport. Although non-native *R. venosa* populations currently appear to be thriving in their new environments, the lack of genetic variability raises questions regarding the evolutionary persistence of these populations.**

Keywords: COI, invasions, mitochondrial genes, ND2, *Rapana venosa*, veined rapa whelk

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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; Wilcove *et al*. 1998; Simberloff & Von Holle 1999; Walton *et al*. 2002). 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 on 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 in two ways. First, molecular markers can be used to determine the occurrence and frequency of alleles within native populations, and the degree of genetic population structure within the native range (Wares *et al*. 2005; Muirhead *et al*. 2008). This information can then be compared to the occurrence

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and frequency of alleles in the introduced populations to determine which native population or populations could have served as sources for the invasion. Second, 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 & 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 in conjunction with 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, some recent studies of marine invasive species have reported an increased genetic diversity in introduced populations, likely resulting from multiple introductions to the area from distinct, genetically differentiated native source populations (Ting & Geller 2000; Bachelet *et al*. 2004; Voisin *et al*. 2005; Kelly *et al*. 2006; Simon-Bouhet *et al*. 2006). Other studies have documented a decrease in genetic diversity in introduced populations, but the observed decreases were not large (Woodruff *et al*. 1986; Marsden *et al*. 1996; Bagley & Geller 2000; Cristescu *et al*. 2001; Martel *et al*. 2004; Astanei *et al*. 2005; Provan *et al*. 2005; Städler *et al*. 2005; Azzurro *et al*. 2006; Kelly *et al*. 2006; May *et al*. 2006). In many of these studies, ballast water, aquaculture, or intentional introductions are reported as the most likely transportation vectors. These vectors often allow for a large number of organisms to be transported to a new region, either in single or multiple introductions, resulting in successful invasions that do not exhibit decreased genetic variation (Roman & Darling 2007). 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,* an organism that is believed to have been introduced around the world by both shellfish introductions and ballast water transport.

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 (Lai & Pan 1980 and Tsi *et al*. 1983, as cited in ICES 2004). 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 & Voultsiadou-Koukoura 1990, as cited in ICES 2004) and Adriatic seas (Ghisotti 1971, 1974; Mel 1976; Cucaz 1983; Rinaldi 1985, as cited in Bombace *et al*. 1994; ICES 2004). Additionally, new populations of *R. venosa* were discovered in the Bay of Quiberon, France in 1997 (ICES 2004), in Chesapeake Bay, USA in 1998 (Harding & Mann 1999), in the Rio de la Plata between Uruguay and Argentina in 2000 (Pastorino *et al*. 2000), and off the coast of the Netherlands in 2005 (Nieweg *et al*. 2005).

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. *R. venosa* are dioecious (ICES 2004) and adult females lay large mats of egg cases from April through September (Chung *et al*. 1993, as cited in Harding *et al*. 2002; 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 produce over 10 different egg mats per year (Ware 2002). The larvae hatch as planktonic veligers and spend 3 to 6 weeks in the water column (Harding & Mann 2003). The larvae then settle to benthic hard substrates and metamorphose into juveniles. Individuals become sexually mature at about 1 year (Harding & Mann 2003) and can live up to 15 years (Mann *et al*. 2006).

The first introduction of *R. 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). *R. venosa* subsequently spread, likely by natural range expansion of planktonic larvae, throughout the Aegean and Adriatic seas (ICES 2004). 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 & 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.

By locating potential sources of the invasions, 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 (e.g. Cristescu *et al*. 2001; Hänfling *et al*. 2002; Bachelet *et al*. 2004; Martel *et al*. 2004; Voisin *et al*. 2005;

Table 1 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	No. of samples	Combined regional and temporal samples
Bohai Sea, Laizhou Bay, Weifang, Shandong, China	LB	2006	30	30
North 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
Tongyeong, 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
North Adriatic Sea, Goro, Italy	AG	2004	5	
North Adriatic Sea, Cesenatico, Italy	AC	2004	5	
North Adriatic Sea, Fano, Italy	AF	2004	5	
North Adriatic Sea, Goro, Italy	AG99	1999	5	
North Adriatic Sea, Porto Garibaldi, Italy	APG99	1999	5	
North Adriatic Sea, Cesenatico, Italy	AC99	1999	5	
North 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, USA	CBJR	2005	10	
Chesapeake Bay, Oceanview, Virginia, USA	CBOR	2005	11	
Chesapeake Bay, Tangier Sound, Virginia, USA	CBTR	2005	1	
Chesapeake Bay, Virginia, USA	CB99	1999–2000	10	32

Azzurro *et al*. 2006; Kelly *et al*. 2006; May *et al*. 2006; Simon-Bouhet *et al*. 2006). In this study, the mitochondrial cytochrome *c* oxidase I (COI) and NADH dehydrogenase subunit 2 (ND2) gene regions were surveyed to assess the amount of population structure within the native and introduced populations of *R. venosa* and to look for the existence of a genetic bottleneck within the introduced populations. Finally, the genetic patterns were evaluated to identify possible source populations and evaluate current hypotheses of invasion transport vectors.

Materials and methods

Rapana venosa samples were collected from eight locations within their native range and from 12 introduced populations (Fig. 1). The majority of samples were obtained between 2004 and 2006, although 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. Collections ranged from 10 to 32 individuals (Table 1) and 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 a DNeasy Tissue Kit (QIAGEN) according to the manufacturer's protocol. The COI and ND2 gene regions were amplified separately in 25 μL polymerase chain reactions (PCRs) containing the following: 2.5 μL 10× PCR buffer plus 15 mM $MgCl_2$, 0.5 μL 10 mM dNTP, 0.25 μL 100 pM/μL forward primer, 0.25 μL 100 pM/μL reverse primer, 0.5 μL 10 mg/mL bovine serum albumin, 0.125 μL 5 U/μL *Taq* polymerase, 20.625 μL sterile water, and 0.25 μL template *R. 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.

The COI gene region amplification was conducted under the following PCR conditions: 94 °C for 5 min; 35 cycles of 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 2 min; 72 °C for 7 min and a 4 °C hold. Conditions for the ND2 gene region were 94 °C for 5 min; 35 cycles of 94 °C for 1 min, 65 °C for 1 min and 72 °C for 2 min; 72 °C for 7 min and a 4 °C hold. All PCR amplifications were carried out on an MJ Research Corporation PTC-200 Peltier thermal cycler. Five

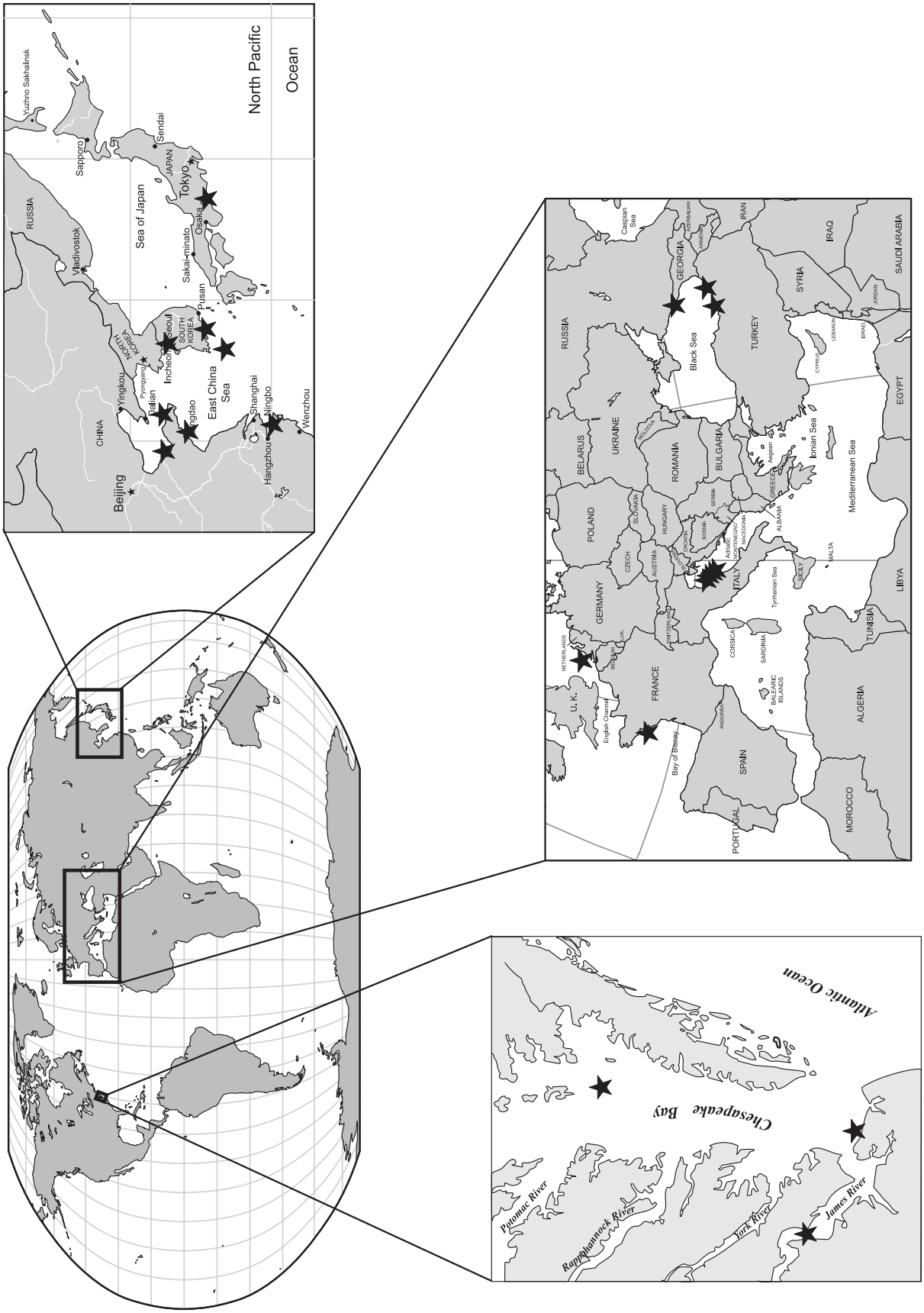

Fig. 1 Collection locations of *Rapana venosa* samples.

microlitres of the amplified PCR products were electrophoresed on a 1% agarose gel containing ethidium bromide and visualized on an UV-light transilluminator to verify the success of the amplification.

PCR products were purified either with the QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's protocol or with ExoSAP reagents (USB Corporation) by adding 0.32 μL 10 U/μL exonuclease I, 0.8 μL 1 unit/μL shrimp alkaline phosphatase (SAP), and 1.6 μL 10× SAP buffer to 20 μL PCR product and incubating at 37 °C for 45 min, followed by 15 min 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). 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) at a one-eighth dilution according to the manufacturer's protocol and sequenced on an ABI 3130x*I* genetic analyzer (Applied Biosystems). Standard chromatographic format (SCF) curves of each forward and reverse sequence were created using Sequencing Analysis version 5.1.1 software (Applied Biosystems). 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). Consensus sequences were aligned using the ClustalW algorithm (Thompson *et al*. 1994) in MacVector 7.2 (Oxford Molecular Ltd). Sequences displaying a unique haplotype were submitted to GenBank (Accession nos EU250077–EU250209).

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, number of transitions, number of transversions, number of haplotypes, haplotype diversity (*h*), mean number of pairwise differences, 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 using ClustalW algorithm (Thompson *et al*. 1994) was created to assess the number of nonsynonymous nucleotide substitutions.

Genetic structure within the native range (both among and within regional native collections) and between the native and introduced collections of *R. venosa* were evaluated by an analysis of molecular variance (AMOVA) of Φ_{ST} values (Excoffier *et al*. 1992) 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 correction for multiple comparisons. The evolutionary relationships among the populations were investigated by creating a minimum spanning network in Network 4.500 (Fluxus 2008) using the post-processing maximum parsimony calculation (Polzin & Daneschmand 2003). Finally, each native population was assessed for genetic evidence of population growth by conducting a mismatch distribution analysis (Rogers & Harpending 1992; Harpending 1994; pairwise difference molecular distance, 1000 bootstrap replicates) and calculating both Fu's F_S neutrality test (Fu 1997; 1000 simulated samples) and Harpending's raggedness index (Harpending 1994) using Arlequin 3.01.

Results

A 636-base pair (bp) segment of the mitochondrial COI gene region and a 656-bp segment of the ND2 gene region were amplified and sequenced for 284 individuals. Samples analyzed for the COI region yielded 51 unique haplotypes with 56 polymorphic sites consisting of 51 transitions, six transversions, and no insertions or deletions (indels). All nucleotide changes resulted in synonymous amino acid changes. Samples analyzed at the ND2 gene region displayed 82 haplotypes with 80 polymorphic sites consisting of 74 transitions, 10 transversions, and no indels. These nucleotide changes resulted in 35 nonsynonymous amino acid changes. As the COI and ND2 gene regions are part of a single genetic locus, data for these two gene regions were combined for further analyses, resulting in a total of 110 haplotypes with 136 polymorphic sites consisting of 125 transitions, 16 transversions, and no indels (Table 2). Examination of the evolutionary relationships among haplotypes revealed a star-like pattern with haplotypes radiating out from one central haplotype (Fig. 2). Collections of *Rapana venosa* from the native range displayed high genetic variation, with an overall haplotype diversity of 0.981. The Xiangshan Bay collection showed the highest haplotype diversity (1.000) and the Japan collection the lowest (0.946) (Table 2). The mean number of pairwise differences between individuals within native collections was 6.988 (π = 0.0055), ranging from 5.430 (π = 0.0041) in Laizhou Bay to 8.695 (π = 0.0067) in Xiangshan Bay.

The high diversity seen in all the native collections was in stark contrast to the lack of variation observed in the introduced collections (Table 2). All individuals from introduced locations (n = 106) shared the same combined COI/ND2 haplotype, and thus, all introduced collections were monomorphic. This same haplotype was found in four rapa whelks from native collections: three individuals from Japan and one individual from Cheju-do, Korea.

The mismatch distribution analysis found that in all eight native collections, simulated mismatch values were not significantly different from the observed values under the sudden expansion model. Additionally, Harpending's raggedness index was consistent with the sudden expansion model for all populations (Table 3). These tests indicate that all native collections showed genetic evidence of population

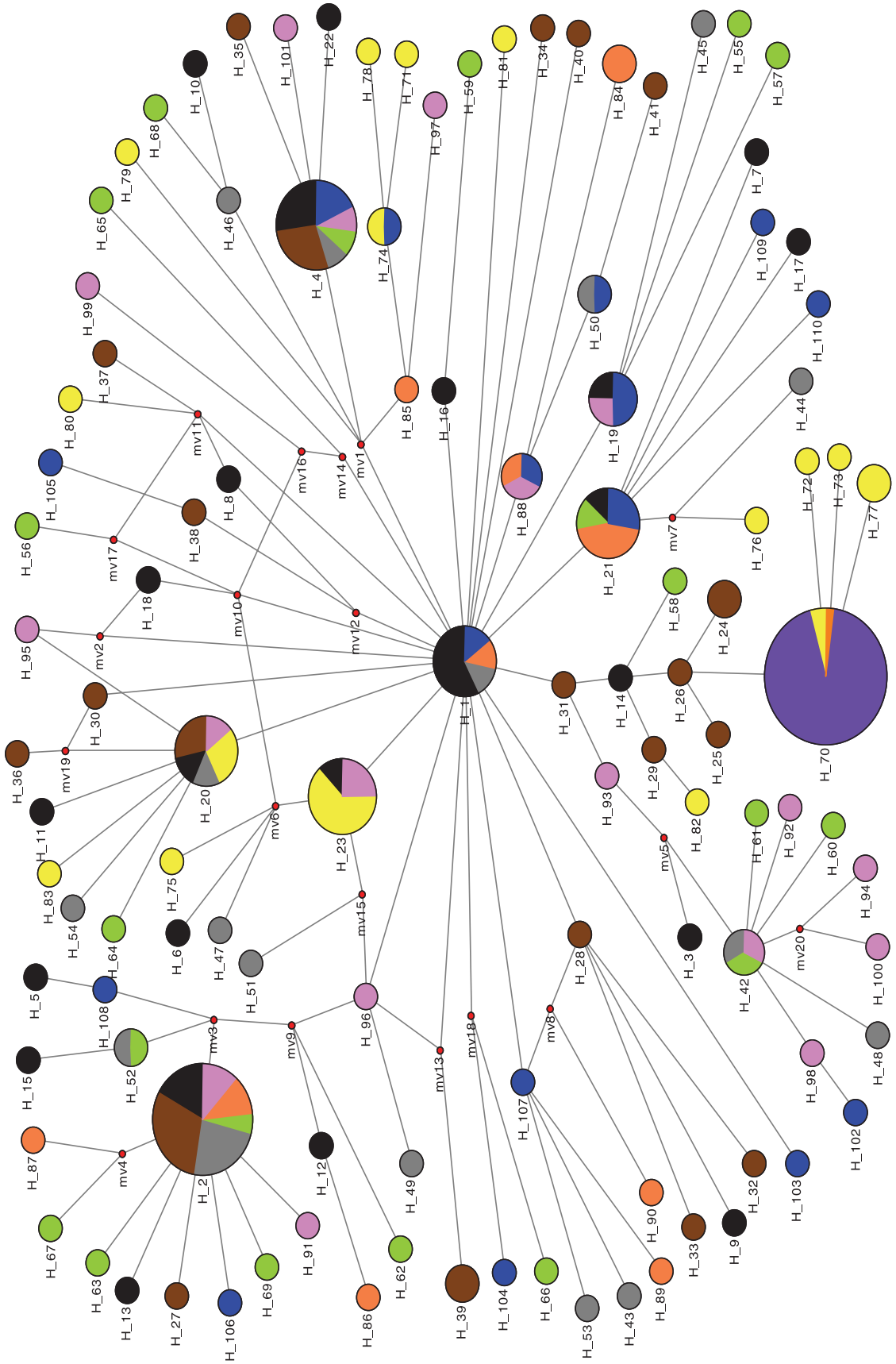

Fig. 2 Minimum spanning network of combined COI and ND2 haplotypes. Collections are colour-coded as follows: Laizhou Bay, China, black; Yantai, China, brown; Qingdao, China, grey; Xiangshan Bay, China, green; Mikawa Bay, Japan, yellow; Cheju-do, Korea, orange; Inch'on, Korea, pink; Tongyeong, Korea, blue; all introduced collections, purple.

Table 2 Population genetics statistics for each *Rapana venosa* collection calculated from the combined COI/ND2 sequence data

Location	*N*	No. of haplotypes	No. of transitions	No. of transversions	No. of polymorphic sites	Haplotpye diversity ± SE	Mean no. of pairwise differences ± SE
Laizhou Bay, China	30	23	41	5	46	0.972 ± 0.018	5.430 ± 2.690
Yantai, China	30	21	37	5	41	0.963 ± 0.021	6.262 ± 3.058
Qingdao, China	20	17	36	3	39	0.968 ± 0.033	6.974 ± 3.421
Xiangshan Bay, China	20	20	47	3	50	1.000 ± 0.016	8.695 ± 4.190
Mikawa Bay, Japan	24	16	31	5	35	0.946 ± 0.031	6.558 ± 3.211
Cheju-do, Korea	15	11	29	1	30	0.952 ± 0.040	6.524 ± 3.268
Inch'on, Korea	20	18	38	1	39	0.990 ± 0.019	8.147 ± 3.946
Tongyeong, Korea	19	16	42	1	43	0.983 ± 0.022	6.304 ± 3.129
Black Sea, Turkey	33	1	0	0	0	0.000 ± 0.000	0.000 ± 0.000
Adriatic Sea, Italy	31	1	0	0	0	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
Chesapeake Bay, Virginia, USA	32	1	0	0	0	0.000 ± 0.000	0.000 ± 0.000
Combined native populations	178	110	125	16	136	0.981 ± 0.004	6.988 ± 3.299

Table 3 Results of population expansion tests on the eight native collections of *Rapana venosa*

Native collection	Sudden expansion model *P* value	Harpending's raggedness index	*P* value	Fu's F_S	*P* value
Laizhou Bay, China	0.975	0.008	0.948	–14.255	< 0.001
Yantai, China	0.563	0.017	0.492	–9.041	< 0.001
Qingdao, China	0.416	0.023	0.400	–7.767	0.002
Xiangshan Bay, China	0.341	0.026	0.230	–13.658	< 0.001
Japan	0.493	0.034	0.341	–4.392	0.046
Cheju-do, Korea	0.576	0.031	0.463	–2.488	0.111
Inch'on, Korea	0.614	0.014	0.681	–8.642	0.002
Tongyeong, Korea	0.904	0.010	0.942	–7.450	0.002

Table 4 AMOVA results of comparison between native collections from China (LB, YAN, QD, XS), Korea (KC, KI, KT) and Japan (J); sample locations and abbreviation codes are given in Table 1

Sample	d.f.	Sums of squares	Variance components	Percentage of variance
Differences among the three native areas	2	22.61	0.13	3.76*
Differences among each location within a native area	5	22.26	0.05	1.38**
Differences among individuals within each location	170	573.60	0.37	94.86***

*$P < 0.05$ **$P < 0.005$ ***$P < 0.001$.

expansion. Results of the Fu's F_S test were also consistent with demographic population expansion, with the exceptions of the Japan and Cheju-do, Korea collections.

An AMOVA used to evaluate the population structure of the native range collections found low but significant genetic structure between the China, Korea, and Japan populations of *R. venosa*; the differences between these areas accounted for 3.76% of the total variance ($P = 0.026$; Table 4). 1.38% ($P = 0.003$) of the variation was accounted for by differences among locations within each area (i.e. between LB, YAN, QD, and XS in China and between KC, KI, and KT in Korea) and 94.86% ($P < 0.001$) of the variation was attributable to differences among individuals within each location.

Since no genetic variation was observed within the introduced areas, samples taken from all introduced populations

Table 5 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. Sample locations and abbreviation codes are given in Table 1

Sample	LB	YAN	QD	XS	J	KC	KI	KT	T/A/FN/CB
LB	—	0.297	0.739	0.423	**0.000**	0.351	**0.000**	0.279	**0.000**
YAN	0.003	—	0.153	0.144	**0.000**	0.180	**0.009**	0.054	**0.000**
QD	–0.013	0.013	—	0.981	**0.000**	0.550	0.297	0.216	**0.000**
XS	0.000	0.021	–0.031	—	**0.000**	0.234	0.450	0.180	**0.000**
J	0.110	0.072	0.111	0.096	—	**0.027**	**0.000**	**0.000**	**0.000**
KC	0.003	0.015	–0.008	0.011	0.103	—	**0.045**	0.468	**0.000**
KI	0.054	0.070	0.007	–0.009	0.115	0.066	—	0.063	**0.000**
KT	0.003	0.030	0.010	0.015	0.096	–0.006	0.043	—	**0.000**
T/A/FN/CB	0.855	0.813	0.868	0.831	0.748	0.897	0.845	0.885	—

were pooled, resulting in one group of introduced samples ($N = 106$). Statistical analyses of population structure were done using this modified grouping. An AMOVA test comparing the native and introduced populations found that 55.80% ($P < 0.001$) of the observed variation resulted from differences between the native group (LB, YAN, QD, XS, J, KC, KI, KT) and introduced group (T, A, FN, CB), showing very strong genetic structure between the two collections. 3.79% ($P < 0.001$) of the variance was attributable to differences among each location within a group (i.e. differences between LB, YAN, QD, XS, J, KC, KI, and KT and differences between T, A, FN, and CB). However, since all the genotypes in the introduced areas were the same, this variation is attributable to the variation found among the locations within the native collection. Variation among individuals within a location accounted for 40.41% ($P < 0.001$) of the observed variance.

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 0.748, $P < 0.001$, to 0.897, $P < 0.001$; Table 5). 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 ($P < 0.001$) to 0.115 ($P < 0.001$). The collection from Inch'on, Korea displayed differences from Laizhou Bay, China ($\Phi_{ST} = 0.054$, $P < 0.001$), Yantai, China ($\Phi_{ST} = 0.070$, $P = 0.009$), and Cheju-do, Korea ($\Phi_{ST} = 0.066$, $P = 0.043$).

Discussion

The goals of this study were to evaluate the genetic structure and variation in the native and introduced populations 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 *R. venosa*. 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. This low mean nucleotide diversity combined with the star-like phylogeny seen in the minimum spanning network (Fig. 2) provides evidence that *R. venosa* has undergone a population expansion in the native range (Avise 2000). The mismatch distribution analysis, Harpending's raggedness index, and the Fu's F_S tests also indicated evidence of population expansion in most of the native collections. The minimum spanning network suggests that haplotype H1 is the most ancestral haplotype. Given that this haplotype occurs in two of the China and two of the Korean collections, it appears that *R. venosa* may have originated in Chinese and Korean waters and then eventually spread throughout the Sea of Japan.

An AMOVA test among the China, Korea and Japan collections of *R. venosa* revealed significant genetic structuring as did pairwise comparisons of Φ_{ST} values among several collections within these three areas of the native range. Genetic differentiation between native populations of benthic marine molluscs is not uncommon. For example, *Littorina plena*, a benthic marine gastropod with planktonic veliger larvae, have shown low but significant population structure over a distance of less than 20 km (Kyle & Boulding 2000). The planktonic larvae of *L. plena* have a long duration time (7–10 weeks), yet population structure exists over very small distances.

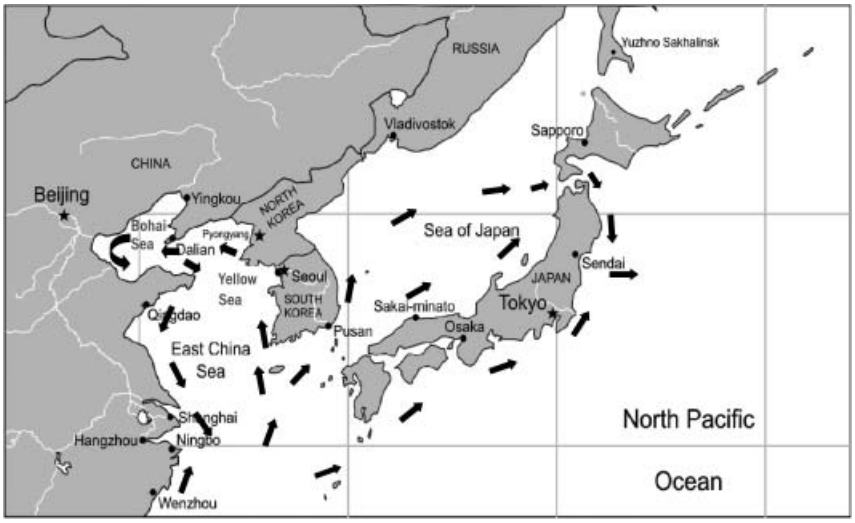

Fig. 3 Generalized diagram of prevailing currents in the native range of *Rapana venosa* (adapted from Perkins 2001).

The genetic divergence seen between the Japan collection of *R. venosa* and all other native collections is not surprising. This collection site 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 locations, 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 1150 km (~700 miles). The distance from Mikawa Bay, Japan, to Xiangshan Bay, China, is close to 1600 km (~1000 miles). 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 km (Hesse 1979). Therefore, it is probably safe to assume that distances of 1500 km are too far to be covered by a benthic snail. 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 (Fig. 3).

The cause of the population structuring observed between the other native collections is not as clear. Pairwise Φ_{ST} comparisons found collections from Inch'on, Korea to be differentiated from collections taken from Laizhou Bay, China, Yantai, China, and Cheju-do, Korea. 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 *R. venosa* larvae to be freely transported between all the Korean and Chinese locations sampled (Fig. 3). The Inch'on, Korea collection, despite its location on this local gyre, showed genetic differentiation from the Bohai Sea collection in Laizhou Bay, China, the North Yellow Sea collection in Yantai, China, and the East China Sea collection in Cheju-do, Korea, all of which are geographically close to Inch'on, Korea.

Humans have been transporting oysters for aquaculture purposes to various locations throughout the native range of *R. venosa* for many years (e.g. Zhou & Allen 2003). If any rapa whelks were transported along with these oysters, gene flow between native rapa whelk populations would have been increased, promoting genetic homogeneity due to non-natural events. Thus, the genetic relationships observed between the native populations may not represent natural genetic exchange.

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). However, many recent studies have found invasive populations to have high levels of genetic diversity (Roman & Darling 2007). In the marine environment, this increased diversity may result from the vectors involved in transporting organisms to new areas. Marine invasions are often caused by ballast water transport or repeated intentional introductions of shellfish (e.g. Marsden *et al*. 1996; Cristescu *et al*. 2001; Bachelet *et al*. 2004; Astanei *et al*. 2005; Provan *et al*. 2005; Voisin *et al*. 2005; Kelly *et al*. 2006; May *et al*. 2006; Simon-Bouhet *et al*. 2006). These vectors allow for the transport of large numbers of individuals and often multiple introductions into the same area, resulting in new populations that do not exhibit low levels of genetic diversity.

The genetic bottleneck observed in introduced populations of *R. venosa* in this study is dramatic; the number of combined COI/ND2 haplotypes decreased from 110 in the native collections ($N = 178$) to one in the introduced collections

($N = 106$). Due to the fact that only one of the native populations likely served as a source for the Black Sea, it may be more accurate to compare the decrease in haplotypes between the introduced locations and the two native populations in which the invasive haplotype was found. The Japan collection ($N = 24$) displayed a total of 16 unique COI/ND2 haplotypes and the Cheju-do, Korea collection ($N = 15$) displayed a total of 11. Most studies of marine introductions that have reported decreases in genetic variation in invasive populations have found decreases that were not as large as those observed in *R. venosa*, either because the introduced population still displayed high levels of diversity (Woodruff *et al*. 1986; Marsden *et al*. 1996; Bagley & Geller 2000; Martel *et al*. 2004; Astanei *et al*. 2005; Azzurro *et al*. 2006) or because the amount of variation in the native populations was also low (Cristescu *et al*. 2001; Provan *et al*. 2005; Städler *et al*. 2005; May *et al*. 2006). However, a decrease in haplotypic diversity similar to that seen here was reported by Kelly *et al*. (2006) for introduced populations of the amphipod *Gammarus tigrinus*. In that study, collections from two native populations, the Delaware estuary ($N = 30$) and the Elizabeth estuary ($N = 40$), contained nine and 19 haplotypes, respectively. Both of these populations may have served as sources for introduced populations. Collections from the introduced populations displayed one to four haplotypes; however, the sample size in these introduced collections ranged from only two to 10 individuals.

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 *R. venosa* haplotype was shared only with native collections from Japan (three out of 24 sequenced individuals) and Cheju-do, Korea (one out of 15 individuals). As the introduced haplotype was seen most often in the Japan samples, this study is consistent with the hypothesis that the introduced Black Sea population originated with samples from Japan, perhaps in association with an intentional oyster introduction. However, given the relatively high haplotype diversity in all native populations and the small sample sizes in some native populations, we cannot eliminate the possibility that the Black Sea invasion originated from the Cheju-do, Korea region, where the introduced haplotype was seen, or from another native population, where the haplotype may occur at a low frequency and was not sampled.

In this study, all individuals taken from three different locations within the Black Sea ($N = 33$) possessed the same haplotype. From these collections, it would appear that the Black Sea population may only contain rapa whelks possessing this haplotype, although it is possible other haplotypes exist in the population and were not represented in our collections. If the Black Sea population is monomorphic, it could have been founded by multiple animals all possessing this same haplotype, by a single female, or an egg case laid by a single female. While it is generally believed that successful invasions often involve multiple introductions, the introduced haplotype was seen fairly infrequently in the native range (in only four individuals), and thus, it is unlikely that multiple individuals with the same haplotype were transported to the Black Sea. Given the huge reproductive output of rapa whelks, it seems possible that the Black Sea population may instead have been started by the introduction of a single female or egg case. 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 (Harding *et al*. 2002; Ware 2002). Thus, *R. venosa* pose a great risk to new areas; only one individual needs to be transported for an invasion to be possible.

The lack of any variation in the combined COI/ND2 gene regions of introduced collections suggests that the Adriatic Sea, France, the Netherlands, and Chesapeake Bay rapa populations originated, either directly or indirectly, from the introduced monomorphic Black Sea population, rather than from the highly diverse native range. The Adriatic Sea population could have been established by natural range expansion of *R. venosa* through the dispersal 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 Sea area supports the hypothesis that ballast water carrying *R. 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 & 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 & Harding 2000). Thus, *R. venosa* planktonic larvae could easily be carried within this large amount of ballast water that is continuously discharged into Chesapeake Bay.

While our data are consistent with the hypotheses that the Black Sea population originated from one maternal lineage and that subsequent introduced populations originated from the Black Sea, the lack of genetic variation observed in all introduced populations prevents us from conclusively validating the source of any introduced population or precisely determining the number of independent invasions into each area. The use of nuclear DNA markers would provide insights into the amount of paternal contributions in introduced populations, as well as additional information on the relationships among introduced populations and the number of times invasions into each location have occurred.

In conclusion, the genetic variation seen in the native and introduced collections of *R. 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 not been documented for a marine invasion. The Black Sea population, which most likely acted as a source population for all other introductions, appears to be the result of the successful establishment of one female or one egg case into the area. 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 disease or environmental change (Sakai *et al*. 2001).

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