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Contents lists available at SciVerse ScienceDirect

Journal of Great Lakes Research

journal homepage: www.elsevier.com/locate/jglr

New data on mitochondrial diversity and origin of *Hemimysis anomala* in the Laurentian Great Lakes

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ARTICLE INFO

Article history:

Received 5 February 2011

Accepted 15 October 2011

Available online 10 December 2011

Communicated by: O. Johannsson

Keywords:

Mysid
Invasive species
Range expansion
North America
Ponto-Caspian

ABSTRACT

The most recent Ponto-Caspian species to invade the Laurentian Great Lakes is the crustacean *Hemimysis anomala*, first reported in 2006. A previous study described three haplotype groups (A, B, C) of *H. anomala* in native and invaded areas within Europe, but only one haplotype (A1) in a sample from Lake Michigan. Our study expands these results to additional populations in the Great Lakes basin, and evaluates relationships among North American and European populations. A 549-bp fragment of the mitochondrial cytochrome oxidase I (COI) gene was analyzed from populations of *H. anomala* in Lakes Ontario, Erie, Huron, and the St. Lawrence River. Two different haplotypes, A1 and B1, were observed in the sampled populations of *H. anomala* and in a previous analysis from *H. anomala* in Oneida Lake (New York). Our results, in contrast with a previous study, detect an additional haplotype in North America.

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Introduction

Since the 1800s, one of the biggest threats to freshwater ecosystems has been the introduction of nonindigenous species (NIS) via human activity (Mills et al., 1993). The most recognized vector for NIS into the Laurentian Great Lakes is through discharged ballast water from transoceanic ships (Holeck et al., 2004). Ballast water can harbor planktonic organisms capable of tolerating salinity changes associated with ballast water exchange, which has contributed to the more than 170 NIS established in the Laurentian Great Lakes (Grigorovich et al., 2003; Roman, 2006; Ellis and MacIsaac, 2009). Over 75% of the NIS recorded in the Great Lakes are endemic to the Ponto-Caspian region (Black, Caspian and Azov Seas; Ricciardi and MacIsaac, 2000). Some of the most widely recognized Ponto-Caspian invaders are the molluscs *Dreissena polymorpha* (Hebert et al., 1989) and *D. bugensis* (May and Marsden, 1992), fish such as round goby (*Neogobius melanostomus*; Jude et al., 1991) and ruffe

(*Gymnocephalus cernuus*; Stepien et al., 1998), the cladoceran *Cercopagis pengoi* (MacIsaac et al., 1999; Ojaveer et al., 2001; Panov et al., 2007), and the amphipod *Echinogammarus ischnus* (Witt et al., 1997). Understanding the pathways by which an organism enters a new environment, and where that organism originated from, can aid in strengthening current regulations to prevent future invasions.

The most recent Ponto-Caspian species to invade the Laurentian Great Lakes is the crustacean *Hemimysis anomala* G. O. Sars, 1907. *H. anomala* is native to coastal areas and river deltas in the Black Sea, Sea of Azov, and northern and eastern Caspian Sea (Wittmann, 2007). In the 1960s *H. anomala* was intentionally introduced into the Kaunas reservoir in Lithuania to improve fish stocks (Ketelaars et al., 1999). This introduced population is the likely source of the species' spread to the Baltic Sea with subsequent expansion to the Rhine Delta. A second possible invasion route originated from the Danube Delta, spreading along the Danube River down to the Rhine Delta, where intermixing between the various lineages has occurred (Audzijonyte et al., 2008). During the 1990s and 2000s the species spread throughout western Europe. In 1992 it was discovered in Finnish coastal waters of the Baltic Sea, Sweden in 1995, Poland in 2002, and the UK in 2004 (Salemaa and Hietalahti, 1993; Holdich et al., 2006; Audzijonyte et al., 2008). Additional populations were documented in inland waterways of Germany in 1998 and the Czech Republic during 2003 (Audzijonyte et al., 2008).

In North America, evidence to date indicates widespread invasion and ongoing colonization within the Great Lakes basin. The first North American records of *H. anomala* came from Lakes Michigan and Ontario in 2006 (Pothoven et al., 2007; Walsh et al., 2010). Subsequent

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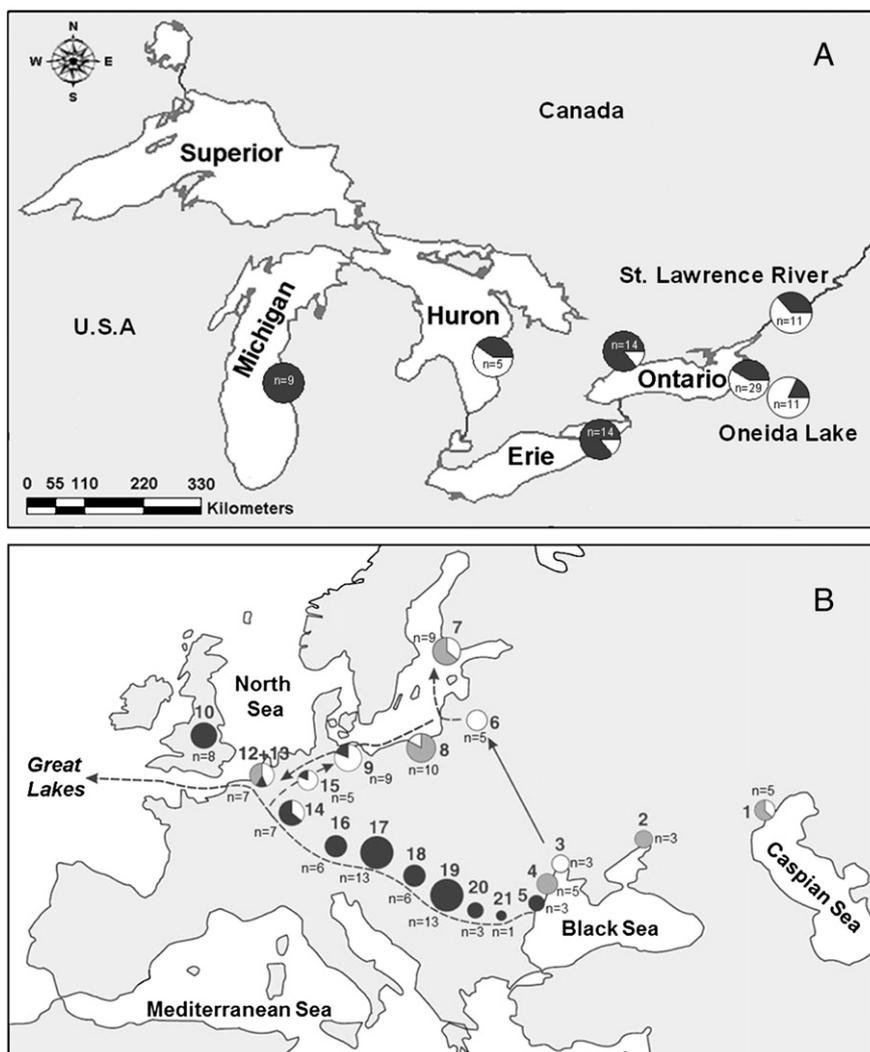


Fig. 1. A) Distribution of the two observed *H. anomala* haplotypes from the mitochondrial COI gene in the Laurentian Great Lakes. Data for Lake Michigan from Audzijonyte et al. (2008) and Oneida Lake from Brooking et al. (2010). B) Distribution of the three observed *H. anomala* haplotype clusters across Europe (modified from Audzijonyte et al., 2008). Dark gray pie slices represent the A1 haplotype, light gray represent the C haplotypes and white pie slices represent the B haplotypes. In all locations (except 1, 3, and 6 in Fig. 1B), the B cluster consisted solely of the B1 haplotype. Position of pie charts represents approximate sample locations.

sampling during 2007–2009 has confirmed *H. anomala* populations in Lakes Erie and Huron (Marty et al., 2010) and documented the spread of the species within the Lake Ontario watershed (Kestrup and Ricciardi, 2008; Brooking et al., 2010). A lack of positive findings of *H. anomala* in Lake Superior or other inland lakes in the Great Lakes basin may be attributed to a lack of sampling effort.

Advances in molecular techniques have created tools useful in phylogeography and population genetics for interpreting the mechanisms and biology behind pre- and post-settlement of NIS. Within the Laurentian Great Lakes, genetic methods have been applied in a number of studies to evaluate the origin and invasion history of NIS including sea lamprey (*Petromyzon marinus*, Bryan et al., 2005), round gobies (Dillon and Stepien, 2001) and ruffe (*Gymnocephalus*, Stepien et al., 1998). Mitochondrial DNA (mtDNA) is a widely accepted tool for assessing genetic patterns and structure. Comparison of sequences from mtDNA to native and nonindigenous populations has aided in identifying invasion pathways, source populations, and the number of separate introduction events, as well as recognizing key variables that help to enhance the success of establishment and spread of NIS in a novel environment (Kelly et al., 2006; Stepien and Tumeo, 2006; Facon et al., 2008).

Invasion history and relationships among *H. anomala* populations across Europe, the UK, and into Lake Michigan were evaluated in a previous study by Audzijonyte et al. (2008) using information from

the mitochondrial cytochrome oxidase I (COI) gene. Nine haplotypes among three groups (A, B, C) were identified (GenBank accession numbers EU02162–EU029170) from the species' native range and the Kaunas Reservoir (site of first intentional introduction; Audzijonyte et al., 2008). Presence and proportions of these nine haplotypes among populations in the invaded areas were used to track the spread of *H. anomala* in western Europe. Only haplotype A1 was found throughout the Danube River drainage, and this was also the only haplotype found from the Lake Michigan population, leading Audzijonyte et al. (2008) to report the Danube River lineage as the likely source of introduction into the Great Lakes. Our work expands upon the study of North American populations to i) identify any additional haplotypes for *H. anomala* in Lakes Ontario, Erie, Huron and the St. Lawrence River and ii) compare haplotype frequencies observed in these areas with those previously observed in Lake Michigan, Oneida Lake (New York), and European populations of *H. anomala*.

Methods

Sample collection

We analyzed a total of 73 *H. anomala* collected during 2007–2009 at 5 localities encompassing Lake Ontario (northwestern: n = 14,

Table 1
Observed haplotype frequencies of mtDNA COI gene in *Hemimysis anomala* across the Laurentian Great Lakes. Haplotypes B2–C4 were not observed in the North American populations. *n*, number of individuals sequenced per population for the COI gene; year, year sampled. Data for Lake Michigan from Audzijonyte et al. (2008) and Oneida Lake from Brooking et al. (2010).

Location	Latitude (°N)	Longitude (°W)	Year	Haplotype frequencies		
				A1	B1	<i>n</i>
Southeastern Lake Ontario						
Sunset Bay area (USA)	43.53	–76.38	2007	0.41	0.59	29
Northwestern Lake Ontario						
Cobourg, Ontario (Canada)	43.95	–78.15	2007	0.71	0.29	14
Lake Erie						
Port Maitland (Canada)	42.85	–79.58	2008	0.86	0.14	14
Lake Huron						
Kincardine (Canada)	44.18	–81.64	2008	0.40	0.60	5
St. Lawrence River						
Montreal Port (Canada)	45.50	–73.55	2009	0.36	0.64	11
Lake Michigan						
Muskegon Channel (USA)	43.23	–83.34	2006–07	1.00	0.00	9
Oneida Lake (USA)	43.20	–75.90	2009	0.18	0.82	11
Total						93

southeastern: *n* = 29), the St. Lawrence River (*n* = 11), Lake Erie (*n* = 14), and Lake Huron (*n* = 5; Fig. 1, Table 1). Samples from southeastern Lake Ontario (east of Oswego, New York) were collected by horizontal tows at night using a 505-µm mesh zooplankton net and a benthic sled with a 1-m² frame, 1000-µm mesh zooplankton net (as in Walsh et al., 2010). Samples from the other sites were collected using vertical tows with a 400-µm mesh zooplankton net (as in Marty et al., 2010). Samples from Oneida Lake, New York (*n* = 11) were analyzed concurrently with this study but reported previously as the first record of that population (Brooking et al., 2010). All *H. anomala* collected were preserved in 95% ethanol. All samples were collected during ongoing studies of the species and provided by collaborators;

at the time, we were not able to obtain any additional collections from Lake Michigan.

DNA extraction and COI sequencing

Total genomic DNA was extracted from chopped whole specimens using Genra Puregene tissue kits, according to the manufacturer's protocol. Isolated DNA was quantified using a fluorometer. A 549-bp fragment of the 3' part of the mitochondrial cytochrome oxidase I (COI) subunit gene was amplified. PCR amplification was performed using primers HemiHatF and HemiHatR (Audzijonyte et al., 2008). In samples of lower quality, internal primers were used as described in Audzijonyte et al. (2008). Amplifications were performed in 20 µL volumes containing 0.5 U of Hot Start Taq DNA polymerase (Qiagen), 1 × buffer, 3 mM MgCl₂, 0.2 mM dNTP, 10 pmol of each primer, and 10 ng of genomic DNA. Thermal cycler conditions included a 95 °C activation for 15 min followed by 35 cycles for 1 min at 95 °C, 1 min at 50 °C, and 1 min at 72 °C, with a final extension for 5 min at 72 °C.

PCR products were purified using either Agencourt AMPure (Beckman Coulter Genomics) or QIAquick PCR cleanup kit (Qiagen) and directly sequenced in either the forward direction or both directions (depending on sequence quality), using the DTCS Quick Start Kit (Beckman Coulter) in 10 µL reaction volumes, according to manufacturer's protocol. Sequencing products were purified with either ethanol precipitation or CleanSEQ (Beckman Coulter Genomics) and capillary electrophoresis was conducted on a Beckman Coulter CEQ8000 genetic analysis system.

Data analysis

Mitochondrial COI sequences were aligned using BIOEDIT Ver. 7.0.9.0 (Hall, 1999) and compared to the 9 haplotypes observed by Audzijonyte et al. (2008). Frequencies of haplotypes were then compared to published data from Lake Michigan (Audzijonyte et al., 2008), Oneida Lake (Brooking et al., 2010) and European populations (Audzijonyte et al., 2008), with the Danube River and Rhine Delta locations in the European populations pooled to increase sample sizes. Population differentiation between North American and European populations was measured using an exact test of population differentiation (Raymond and Rousset, 1995) using 30,000 Markov steps and a chain length of 100,000 steps. A sequential Bonferroni correction was used to account for multiple comparisons (Rice, 1989). The analysis

Table 2
Probability values for exact tests of population differentiation between North American and Ponto-Caspian locations. Site locations correspond to numbers on Fig. 1B; sites 1–5 represent the native range of *H. anomala*, 6–21 represent range expansion. Bold values are significant (*p* < 0.0006) after a sequential Bonferroni correction.

Site location	SE Lake Ontario	NW Lake Ontario	St. Lawrence	Oneida Lake	Lake Erie	Lake Huron	Lake Michigan
SE Lake Ontario							
NW Lake Ontario	0.125						
St. Lawrence	1.000	0.094					
Oneida Lake	0.275	0.020	0.636				
Lake Erie	0.007	1.000	0.016	0.001			
Lake Huron	1.000	0.266	1.000	0.542	0.086		
Lake Michigan	0.002	0.469	0.004	0.000	0.503	0.028	
1	0.000	0.001	0.000	0.000	0.000	0.015	0.001
2	0.000	0.009	0.003	0.006	0.003	0.054	0.004
3	0.048	0.045	0.225	0.245	0.017	0.642	0.004
4	0.000	0.000	0.000	0.000	0.000	0.008	0.001
5	0.092	1.000	0.192	0.026	1.000	0.193	1.000
6	0.042	0.011	0.214	0.374	0.002	0.449	0.001
7	0.000	0.001	0.001	0.004	0.000	0.027	0.000
8	0.000	0.000	0.000	0.000	0.000	0.007	0.000
9	0.439	0.059	0.642	1.000	0.006	0.580	0.002
10	0.004	0.469	0.012	0.001	0.515	0.034	1.000
12 & 13	0.007	0.020	0.074	0.084	0.002	0.434	0.001
14	0.217	1.000	0.335	0.048	0.575	0.558	0.177
15	0.628	0.091	1.000	1.000	0.018	1.000	0.005
16–21	0.000	0.028	0.000	0.000	0.058	0.001	1.000

was conducted using the software ARLEQUIN (Excoffier and Lischer, 2010).

Results

Two different haplotypes, A1 and B1, were observed for *H. anomala* from Lakes Ontario, Erie, Huron, Oneida, and the St. Lawrence River (Table 1; Fig. 1). *H. anomala* in Lake Michigan (n=9) were reported by Audzijonyte et al. (2008) to exhibit only the A1 haplotype. Our study found northwestern Lake Ontario (n=14) and Lake Erie (n=14) to have a higher frequency of the A1 haplotype than the B1 haplotype. In contrast, the St. Lawrence River (n=11), southeastern Lake Ontario (n=29) and Lake Huron (n=5) had a higher frequency of the B1 haplotype than the A1 haplotype, as did Oneida Lake (n=11, 0.82; Brooking et al., 2010). However, exact tests of population differentiation revealed Lake Michigan and Oneida Lake as the only North American populations to be statistically different ($p < 0.0006$) (Table 2). Although other comparisons had low p-values, they were not significant after correcting for multiple comparisons.

Based on exact tests of population differentiation, several Ponto-Caspian populations appeared to be unlikely sources for introduction into the Great Lakes (Table 2). The Volga Delta, Don Delta, Dniester Delta, Gulf of Finland, and Gulf of Gdansk locations each had significant genetic differences from the sampled North American locations (Table 2). The Danube, the purported source lineage for introductions into the Great Lakes (Audzijonyte et al., 2008), had significant genetic differences from the St. Lawrence River, southeastern Lake Ontario, Oneida Lake and Lake Huron (Table 2). High probability values indicate which of the sampled locations were most similar and highlight potential source populations. Within the Great Lakes, general patterns based on high probability values were observed, grouping southeastern Lake Ontario, the St. Lawrence River, Oneida Lake, and Lake Huron together; and grouping northwestern Lake Ontario, Lake Erie, and Lake Michigan together. When compared to Ponto-Caspian sources, relatively high probability values were observed between southeastern Lake Ontario/St. Lawrence River/Oneida Lake/Lake Huron and Lake Schwerin/the Mittellandkanal; and between northwestern Lake Ontario/Lake Erie and the Danube Delta/Nottingham/Lower Rhine. Low probability values were observed between the groups described above; however, most are not significant after correcting for multiple comparisons.

Discussion

Our results document the occurrence of two haplotypes (A1 and B1) in the Great Lakes and associated watersheds, in contrast to the single haplotype (A1) that was detected in Lake Michigan (Audzijonyte et al., 2008). The presence of the B1 haplotype demonstrates that the Danube River lineage, also characterized by only the A1 haplotype, was unlikely the sole source of introduction into the Great Lakes (Audzijonyte et al., 2008). Instead, based on existing data, locations where the B1 haplotype occurs (e.g., the Rhine and Baltic drainages in Germany) may be potential source populations. However, definitive source population(s) have yet to be confirmed with available data. *H. anomala* has expanded its range throughout Europe and all potential source populations were not represented in this analysis. More variable genetic markers, such as microsatellites, and larger sample sizes (to ensure sampling of all haplotypes present in the population) are necessary to identify source populations and understand migration pathways.

A possible invasion scenario to explain our results is that multiple, independent introductions of *H. anomala* into the Laurentian Great Lakes occurred from separate Ponto-Caspian populations or other regions where they had become established. Differences in haplotype frequencies would then be a result of the genetic differences between the various source populations. An alternative invasion scenario would be a single introduction from a source other than the Danube River lineage, followed by subsequent spread throughout the Great

Lakes. Given the large populations observed to date in the Lake Ontario watershed (Walsh et al., 2010) and their tendency to swarm, it seems most likely that populations within the Great Lakes basin are being established from a large number of founding individuals as populations expand and colonize new areas through either active movement or transport by currents. It might be possible for small numbers of individuals to be transported to new areas accidentally via recreational boating, but little is known about the likelihood of this vector for dispersing *H. anomala*, and the large geographic distance between some populations evaluated in this study reduces the plausibility of that scenario. The southeastern Lake Ontario site is the area where the species was first reported in that Lake (Walsh et al., 2010), and movement of animals from this site to Oneida Lake and the St. Lawrence River via active or passive movement of animals, or transport by recreational or commercial boat traffic, is plausible because of the geographic proximity and location of all sites in the same watershed.

Understanding invasion pathways and migration patterns of *H. anomala* may help predict whether other nonnative species will become invasive. If introductions from multiple sources have occurred, the invasive species may be more likely to become established. Multiple introductions from different source populations can potentially enhance the establishment and spread of an invasive species by reducing the effect of a population bottleneck and increasing gene flow (Kelly et al., 2006; Suarez and Tsutsui, 2007). Genetic data can then help determine the risk of *H. anomala* to the Great Lakes ecosystem, thereby enhancing the risk analysis processes for NIS (as in Stepien and Tumeo, 2006).

Acknowledgments

Funding for this study was provided by the New York Great Lakes Protection Fund through the Great Lakes Research Consortium as well as the Scholarly and Creative Activities Grant through the State University of New York at Oswego. Support for sample collection in Sunset Bay, Lake Ontario was provided by the Doug Halliday research fellowship through the Department of Biological Sciences at the State University of New York at Oswego. Samples were provided by Kelly Bowen of the Department of Fisheries and Oceans Canada, Ted Schaner of the Ontario Ministry of Natural Resources, and Jerome Marty of the St. Lawrence River Institute. We would also like to thank Richard Back for guidance with sampling protocols and taxonomic identification and Patrick Wallace for volunteer fieldwork in Sunset Bay, Lake Ontario. This article is Contribution 1667 of the U.S. Geological Survey Great Lakes Science Center. Use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by any parties to this work.

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