

Research article

First Pacific record of the north Atlantic ascidian *Molgula citrina* – bioinvasion or circumpolar distribution?

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Abstract

The small brooding ascidian *Molgula citrina* Alder and Hancock, 1848 has long been known as a common inhabitant of shallow waters on both sides of the north Atlantic on subtidal natural hard substrates and also as a fouler of floating docks. There are published records from the White Sea (NW Russia), but none from the north Pacific. In May and August 2008, a number of adult brooding *Molgula* sp. specimens were collected from floating docks at the small fishing village of Seldovia on the Kenai Peninsula at Kachemak Bay, Alaska. Morphologically these individuals exactly match Atlantic specimens of *Molgula citrina* in all characters that were examined. The unique constellation of characters for this species differentiates it from all other *Molgula* species, as noted by Van Name (1945). In addition, the full-length 18S and 28S rDNA sequences are identical for both the Alaska specimens and New England *M. citrina*. Is this a new invasion, or is *M. citrina* a northern circumpolar species that was somehow overlooked? If this new record indicates a natural distribution, why has it not been collected before? If it is a recent introduction, it is unlikely that *M. citrina*, an inhabitant of cold waters, could survive in sea chests of ships from the N. Atlantic arriving in Alaska via the Panama Canal, but the intriguing idea exists of transport from Atlantic to Pacific through the Northwest or Northeast Passages. As global warming diminishes the ice cover in this region, more and more ships are traversing the Northwest Passage across northern Canada as well as the NE Passage across northern Russia, representing significant new routes for anthropogenic transport of marine species. The tadpoles of this small brooder are retained for some time after hatching, resulting in an extremely short free larval life, but could survive as metamorphosed juveniles attached in sea chests or free-floating in ballast water. They have a wide temperature tolerance and once they metamorphose can live free-floating in sea water for some time. They become very sticky and will ultimately stick to whatever they contact. Thus they could conceivably live for many generations in sea chests and sustain a viable population from which to invade new habitats.

Key words: fouling, marina, tunicate, ascidian, invasions, nonindigenous, Alaska, *Molgula citrina*, 28S rDNA, 18S rDNA, COI

Introduction

In May 2008, a number of *Molgula* sp. specimens were collected from the floating docks and associated ropes and lines at the Seldovia Marina, Kachemak Bay, Alaska (59.44°N, 151.71°W) (Figure 1). These were presumed to be a local species and were preserved in seawater formalin for later identification. The morphology proved to match exactly that of *Molgula citrina* Alder and Hancock, 1848, a widely distributed north Atlantic species long known from the Arctic Ocean, North Sea, Spitsbergen, Scandinavia, White Sea, British Isles and English Channel coast of France, as well as the NE coast of North America from Canada to Woods Hole,

Massachusetts (Alder and Hancock 1907; Huntsman 1911; Redikorzev 1916; Årnäck-Christie-Linde 1928; Thompson 1930; Van Name 1945; Berrill 1950; Millar 1966, 1970; Monniot 1969). The species has never been reported from the north Pacific (it was not included in any of the above references nor in Ritter 1899, 1900, 1901, 1913; Huntsman 1912; Tokioka 1953, 1963; Nishikawa 1991; Sanamyan 1993b; Sanamyan and Sanamyan 2007). Twenty-four species of ascidians are known to be circumpolar, and five other species may prove to be circumpolar after further examination (Table 1). The north Atlantic has been more thoroughly sampled during the past century than has the north Pacific, a vast region with many thousands of miles of unsampled or undersampled coastline

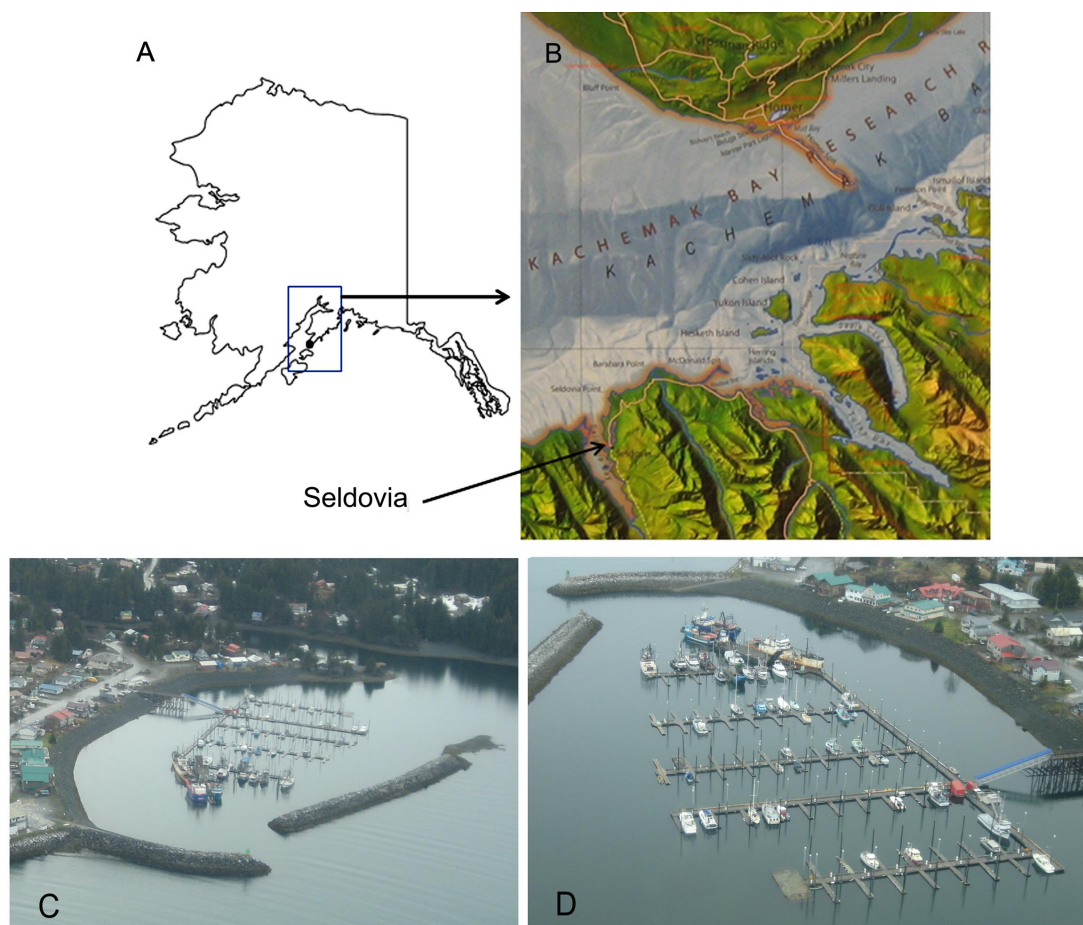


Figure 1. A) Outline map of Alaska (<http://www.worldatlas.com/webimage/countrys/namerica/usstates/outline/ak.gif>); B) Relief map of Seldovia region (Tibor G. Toth, Toth Graphix); C, D) aerial views of Seldovia Marina (photographs by GL).

especially in northern British Columbia, Alaska, and NE Russia. Thus the N. Pacific invertebrate fauna and distributions are still poorly known. A number of new species and new distribution records have been published in the past 20 years (Lambert 1989, 2003; Lambert and Sanamyan 2001; numerous publications by Sanamyan listed in References).

A second larger collection from the Seldovia, AK *Molgula* population was made on August 15, 2008 and the animals were preserved in 95% ethanol for DNA sequencing to augment the morphological analysis and help determine if the Alaskan *Molgula* are a genetically distinct cryptic species, an undiscovered natural Pacific population of a circumpolar species distribution or a recent invasion from the Atlantic.

Materials and methods

Animals were collected during May and August of 2008 from floating docks and associated ropes at the Seldovia Marina, Kachemak Bay, Alaska, latitude 59.44° N, longitude 151.71° W (Figure 1). They were preserved in 10% seawater formalin for morphological analysis and in 95% ethanol for DNA sequencing. The morphology was compared with previously collected formalin-fixed *M. citrina* from Journey's End Marina (on mussels), Rockland, Maine, latitude 44.103° N, longitude 69.109° W (collected July 30, 2007). The DNA sequences of 5 individuals from Alaska (GenBank accession # HM807350 for the 28S rDNA, HM807351 for the 18S rDNA) were compared with sequences from Woods Hole, MA, *M. citrina* already listed in

Table 1. Circumpolar ascidian species naturally distributed in the northern hemisphere (not considered to have been transported anthropogenically).

Taxon	Brooder	Reference
Aplousobranchia 7		
<i>Aplidium glabrum</i> (Verrill, 1871) (?)	x	Van Name 1945, Sanamyan 1998a
<i>Aplidium spitzbergense</i> Hartmeyer, 1903	x	Van Name 1945, Sanamyan 1998a
<i>Eudistoma vitreum</i> (Sars, 1851)	x	Sanamyan 1993a
<i>Synoicum pulmonaria</i> (Ellis & Solander, 1786) (?)	x	Van Name 1945
<i>Synoicum turgens</i> Phipps, 1774	x	Sanamyan 1998a
<i>Didemnum albidum</i> (Verrill, 1871) (?)	x	Van Name 1945, Millar 1966, Nishikawa 1990
<i>Trididemnum tenerum</i> (Verrill, 1871)	x	Van Name 1945, Sanamyan 1999
Phlebobranchia 4		
<i>Ascidia callosa</i> Stimpson, 1852	x	Van Name 1945, G. Lambert unpubl.
<i>Ascidia prunum</i> Müller, 1776		Van Name 1945, Sanamyan 1998b
<i>Chelyosoma macleayanum</i> Broderip & Sowerby, 1830		Huntsman 1922b, Van Name 1945, Sanamyan 1998b
<i>Ciona gelatinosa</i> Bonnevie, 1896		Van Name 1945, Sanamyan & Sanamyan 2007
Stolidobranchia 18		
<i>Cnemidocarpa finmarkiensis</i> (Kiaer, 1893)		Van Name 1945, Millar 1966
<i>Cnemidocarpa rhizopus</i> (Redikorzev, 1907)		Huntsman 1922b, Van Name 1945, Sanamyan 2000
<i>Dendrodoa aggregata</i> (Rathke, 1806)		Van Name 1945
<i>Dendrodoa grossularia</i> (Van Beneden, 1846)	x	Millar 1966
<i>Dendrodoa lineata</i> (Traustedt, 1880)		Van Name 1945, Sanamyan 2000
<i>Dendrodoa pulchella</i> (Verrill, 1871)		Van Name 1945
<i>Halocynthia aurantium</i> (Pallas 1787)/ <i>H. pyriformis</i> (Rathke, 1806) (?) (possibly synonymous)		Van Name 1945
<i>Kükenthalia borealis</i> (Gottschaldt, 1894)	x	Van Name 1945, Sanamyan 2000
<i>Peloniaia corrugata</i> Forbes & Goodsir, 1841		Van Name 1945, Millar 1966, Sanamyan 2000
<i>Styela coriacea</i> (Alder & Hancock, 1848)		Van Name 1945, Millar 1966, Sanamyan 2000
<i>Styela rustica</i> (Linnaeus, 1767)		Van Name 1945, Millar 1966
<i>Styela sigma</i> Hartmeyer, 1906 (?)		Van Name 1945 & Millar 1966 (as <i>S. atlantica</i>), Nishikawa 1991, Sanamyan 2000
<i>Boltenia echinata</i> (Linnaeus, 1767)	x	Huntsman 1922a, b, Van Name 1945, Millar 1966
<i>Boltenia ovifera</i> (Linnaeus, 1767)		Huntsman 1922b, Van Name 1945, Millar 1966, Sanamyan 1996
<i>Eugyra glutinans</i> (Müller, 1842)		Van Name 1945, Millar 1966, Sanamyan 1993b
<i>Molgula griffithsii</i> (Macleay, 1825)		Huntsman 1922b, Van Name 1945, Millar 1966, Sanamyan 1993b
<i>Molgula retortiformis</i> Verrill, 1871		Van Name 1945, Millar 1966, Sanamyan 1993b, G. Lambert unpubl.
<i>Rhizomolgula globularis</i> (Pallas, 1776)		Huntsman 1922b, Van Name 1945, Millar 1966

GenBank (accession # L12420, L12421) and 3 individuals from the Rockland, Maine collection. Additional specimens from Rockland, Maine (SBMNH # 149492) and Seldovia, Alaska (SBMNH # 149490 collected May 2008, 149491 collected August 2008) have been deposited at the Santa Barbara Museum of Natural History.

DNA was isolated from dissected gonads using a DNeasy Tissue extraction kit (Qiagen, Valencia, CA). Approximately 1,800 bp of 18S rDNA and a region of about 400 bp in the D2 loop region of the 28S rDNA were amplified by

polymerase chain reaction (PCR) using the primers described in Hadfield et al. 1995 (for 28S rDNA) and Swalla et al. 2000 (for 18S rDNA). PCR was performed in a 25- μ L total-reaction volume with 0.25 μ L of each primer (1 μ g/ μ L), 0.5 μ L dNTPs (10mM), 5 μ L PCR buffer, 1.5 μ L MgCl₂ (25mM), 0.25 μ L Taq polymerase (Promega) and 5 μ L template DNA. A single incubation at 94°C for 4 min was followed by 35 cycles (denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min) and a final extension at

Figure 2. A) Five individuals from Seldovia AK collected May 2008; B) left side of one of the specimens in A removed from tunic; C) right side of specimen from Rockland ME removed from tunic; D) left side of different specimen from Rockland ME removed from tunic. **em:** brooded embryos; **es:** excurrent siphon; **i:** intestine; **is:** incurrent siphon; **k:** kidney; **o:** ovary; **t:** testis. Scale bars 5 mm. Photos by GL.

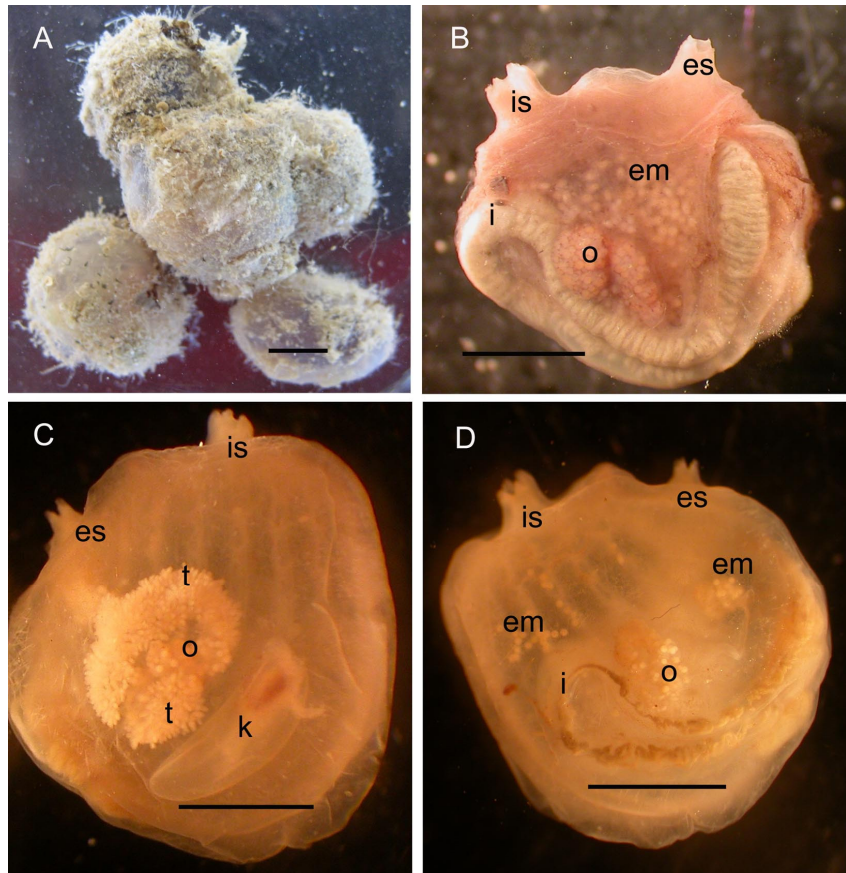
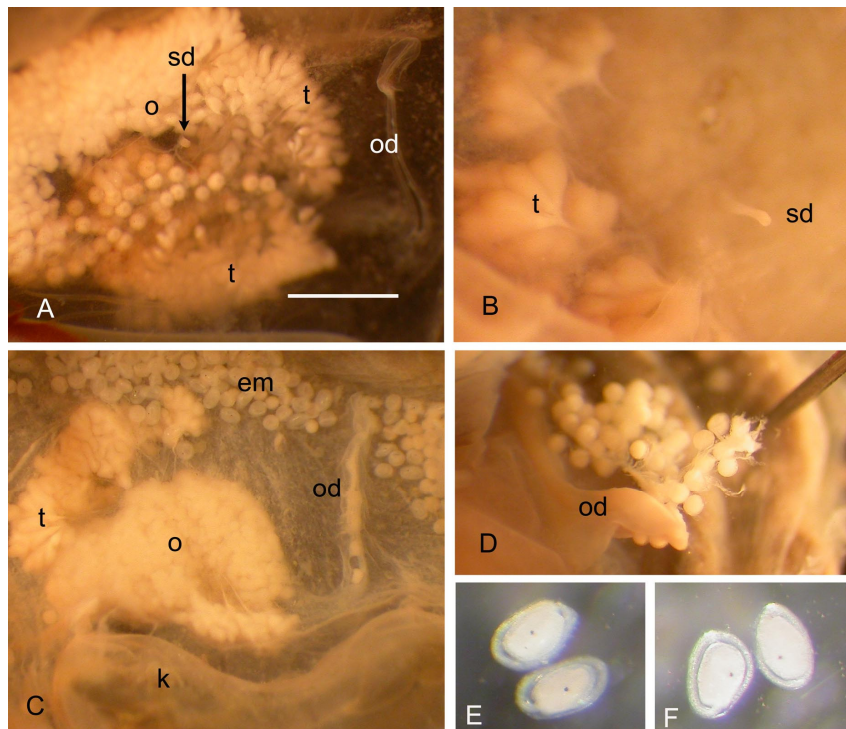


Figure 3. A) Mesial view of right side of Rockland ME specimen showing ovotestis, sperm duct and oviduct, embryo brood removed. Scale bar 2 mm; B) mesial view of testes and sperm duct of Seldovia AK specimen collected August 2008; C) mesial view of right side of Seldovia AK specimen showing ovotestis, oviduct and brooded embryos; D) end of oviduct and group of ovulated eggs in Seldovia AK specimen (different from the individual in C); E) two brooded embryos from a Rockland ME specimen; F) two brooded embryos from a Seldovia AK specimen. **em:** brooded embryos; **k:** kidney; **o:** ovary; **od:** oviduct; **sd:** sperm duct; **t:** testis. E, F: 394 μ m. Photographs by GL.



72°C for 10 min, on an MJ Research Dyad Thermocyclers (Bio-Rad, CA). PCR products were run on a 1% TAE agarose gel and the bands were excised and purified using a QIAquick Gel Extraction Kit (Qiagen). Purified PCR products were ethanol precipitated and bidirectionally sequenced on an ABI 3130 sequencer (Foster City, CA) in the Univ. of Washington's Biology Department Comparative Genomic Center. The complete 18S rDNA region was compiled and compared to sequences of *M. citrina* from Woods Hole (GenBank number L12420) using Macvector 10.5. The 28S rDNA from the Alaska samples was compared with the *M. citrina* from Woods Hole (GenBank # L12421), and to the *M. citrina* from Rockland, Maine using ClustalX version 2 (Larkin et al. 2007). The mitochondrial cytochrome oxidase I gene (COI) was amplified from the Alaska and Maine samples using the protocol described in Stefaniak et al. (2009).

A phylogenetic tree was inferred based on the 28S rDNA sequences deposited in GenBank (Appendix 1) and the new sequences obtained from the current study, using the maximum parsimony (MP) algorithms within the PAUP package (version 3.0; Swofford 1990). The sequences were analyzed by heuristic search with 100 replicates under the random addition option to increase the likelihood of finding the most parsimonious tree. The degree of support for internal branches of the tree was assessed in 1,000 bootstrap pseudoreplicates (Felsenstein 1985) using as an out group the sequence of the D2 loop region of *Herdmania momus* and *Styela plicata* (Appendix 1).

Results

The Alaska *Molgula citrina* are identical in all morphological characters to specimens from the NW and NE Atlantic (Figures 2, 3), confirmed by dissections of the material collected as described in the Materials and Methods, and also described by Redikorzev (1916), Ärnback-Christie-Linde (1928), Van Name (1945), Berrill (1950) and Monniot (1969). Characters compared (see Figures 2 and 3, though not all characters are shown) included size, body shape, tunic, mantle musculature, oral tentacles (number and branching pattern), branchial sac folds and infundibula, number of longitudinal vessels, wide flattened curvature pattern of gut, shape of ovaries, shape and arrangement of

testes, single opening of the short common sperm duct in most cases (a very small second sperm duct in one animal) and location of the opening approximately over the center of the ovary on the mesial side, the long distinctive oviduct with its right angle bend and long distal portion ending in an expanded flared portion opening subterminally (compare Figure 3A, C), and the brooded tailed embryos with a size/shape combination different from other molgulids (Figure 3E, F; mean size 394 µm). None of the above authors mention that the distal end of the oviduct is expanded, or illustrate this feature. This distal expansion is more developed in the largest individuals (Figure 3D). The oviduct opening is not terminal but faces the dorsal lamina, as stated by Redikorzev (1916). The eggs appear to be held together in a sticky mucus as they are ovulated (Figure 3D).

Monniot (1969) reported the size of European *M. citrina* as 1-2 cm, a range that covers all the New England and all but a very few of the Alaska samples. The Alaskan *Molgulas* collected in May 2008 are all fairly small, the largest being about 15 mm in diameter. However, in the August 2008 collection, which was in excess of 50 adult specimens, the majority are up to 18 mm, with a few at 2 cm, and three are 22-23 cm, most of the "extra" being extra-thick tunic. There are also many very small individuals attached to the tight clumps of adults, most likely settled from brooded larvae released by the adults. The largest of the 2007 Rockland, Maine specimens is 18 mm, though Van Name (1945) reported that the largest ones he collected were 16 mm. Several other authors give a maximum size of 18 mm (Ärnback-Christie-Linde 1928; Thompson 1930).

The 28S rDNA sequences generated from Alaska specimens were 100% identical to sequences generated from specimens from Woods Hole, MA and Rockland, Maine (Figure 4). The 18S rDNA of the individuals from Alaska specimens are 99.9% identical to the Woods Hole samples; they differ in only one bp (out of an 1809 bp sequence) in position 1464, with a 'T' in the Alaska individuals vs. 'G' in the Woods Hole individuals. The new *Molgula citrina* sequences from Alaska have been deposited in GenBank (accession # HM807350 for the 28S rDNA, HM807351 for the 18S rDNA).

Seven COI sequences (2 from Maine and 5 from Alaska) were obtained (A. Gittenberger

unpublished data). The two Maine sequences are consistently different from the 5 Alaska sequences at 19 different base-positions (out of about 600), a difference of about 3%.

Discussion

There are no North Pacific records of *Molgula citrina* by the early ascidian taxonomists such as Redikorzev, Ritter, Huntsman, Tokioka, Van Name and others, or in the numerous recent excellent studies of NE Russia ascidians by Sanamyan and monographs on Japanese species (Tokioka 1953; Nishikawa 1991). Sanamyan (pers. comm.) stated after examining a few specimens that “it differs from all species we have on western side of N. Pacific.” There is still a possibility, though, that it could be a naturally occurring circumpolar species that was never encountered, as suggested by Huber et al. (2000). The existence of the Pacific tailless molgulids *M. pacifica* (Huber et al. 2000) and *M. pugetiensis* (Zeng et al. 2006) that are most closely related to the Roscovita clade of molgulids found in France, suggests that there are likely to be (or were at one time) tailed molgulid species from the same clade in the Pacific. The 3% COI difference between the Alaska and Maine specimens could be an intra-specific variation large enough to suggest a polar migration to the Pacific long ago rather than a recent anthropogenic invasion, but to prove that, the COI from many more *M. citrina* specimens will have to be sequenced and compared with specimens from other *Molgula* species. This marker has frequently been used in studies on introductions of ascidians (Rius et al. 2008; Stefaniak et al. 2009).

The north Pacific is a vast region, with large regions of undersampled coastline especially in northern British Columbia, Alaska, and NE Russia, and thus its invertebrate fauna and distributions are still incompletely known. New species and distributions continue to be described (Lambert 1989, 2003; Lambert and Sanamyan 2001; numerous publications by Sanamyan listed in References). A perusal of numerous taxonomic monographs yielded 24 northern circumpolar species, including 4 other molgulids, whose identifications appear to be unequivocally valid, plus 5 additional species with very similar Atlantic and Pacific representatives that may prove to be circumpolar (Table 1) (the table does not include any species

known to have achieved circumpolar distribution via anthropogenic transport). Interestingly, the majority are stolidobranch ascidians, with representatives from all three families; in general the members of this order are known to have a wider tolerance to environmental changes in temperature and salinity than do most of the members of the other two orders. All but the aplousobranchs are solitary species except *Kükenthalia borealis*. *Molgula citrina* is a brooder; only three stolidobranchs in Table 1 are brooders although all the aplousobranchs are brooders.

If the Alaska *M. citrina* population is part of a natural distribution, some variations in the DNA would be expected. According to Vermeij and Roopnarine (2008), with reference primarily though not exclusively to molluscs, “Most trans-Arctic lineages with temperate Atlantic members show genetic and geographic gaps between Pacific and Atlantic populations, indicating that post-Pliocene sea-ice expansion in the coastal Arctic Ocean ended trans-Arctic dispersals in these lineages.” However, the prevailing oceanic circulation in the Arctic is from Pacific to Atlantic, thus arguing against a natural expansion of *M. citrina* from the Atlantic to the Pacific. Vermeij and Roopnarine (2008) indicate that most of the circumpolar species in the north Atlantic are derived from the north Pacific, reflecting the prevailing oceanic circulation in the far north. In predicting future inter-oceanic invasions in the far north due to climate warming, the authors state: “As in the past, few Atlantic to Pacific invasions are expected.” The same conclusion was made by Reid et al. (2007) in reporting the recent expansion of a N. Pacific diatom into the N. Atlantic. Negotiations are underway to obtain specimens of *M. citrina* from the White Sea in Russia, the furthest east known location of this species and a region now open during the summer months to shipping across the NE Passage to the Pacific, as well as from various northern European locations.

In the present study of *Molgula citrina*, not only the morphology but also both the 18S and 28S rDNA sequences are identical between the NW Atlantic and the Alaska specimens, confirming that they belong to the same species. The same genetic markers confirmed recently that *Molgula echinosiphonica* Lacaze-Duthiers, 1877 from Roscoff, France and *Molgula citrina* from the northeast coast of the U.S. and northern France Atlantic coasts are two populations of the

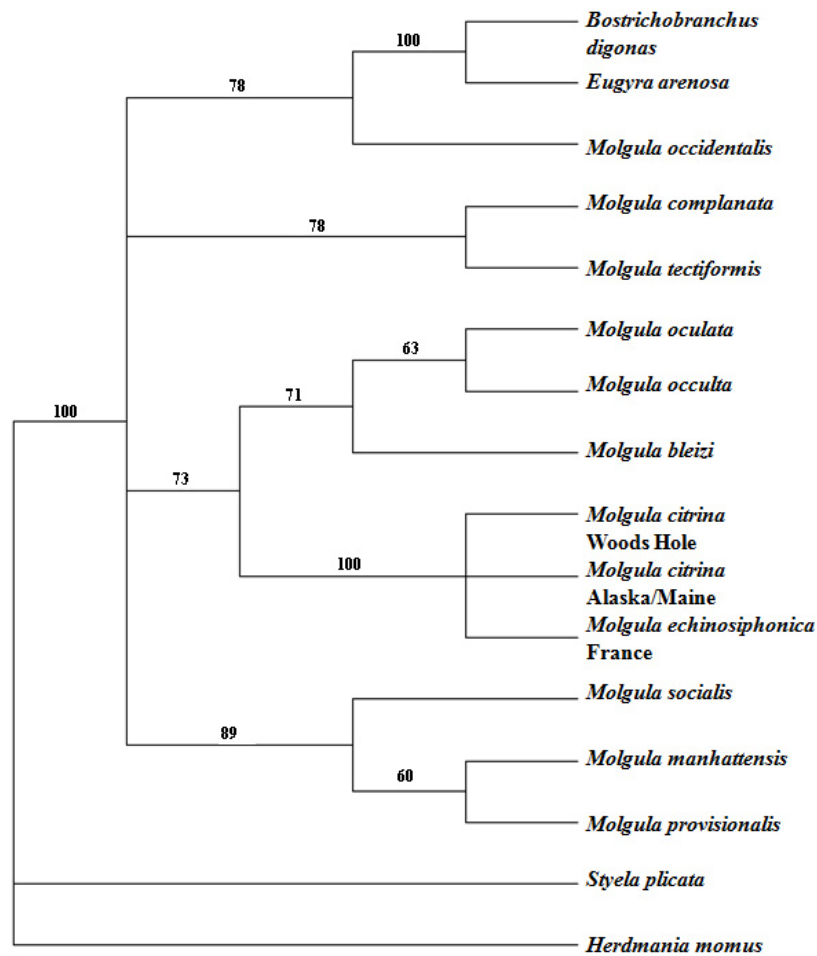


Figure 3. Phylogenetic relationship between species of the Molgulidae inferred from the 28S (D2 loop) rDNA sequences with the MP method (modified from Hadfield et al. 1995). Phylogenetic GenBank accession numbers are in Appendix 1.

same species, *Molgula citrina* (Shenkar and Swalla 2010). The 18S rDNA and 28S rDNA are good markers to distinguish species within the Molgulidae family (Figure 4; Hadfield et al. 1995; Zeng et al. 2006).

Possible modes of introduction of non-indigenous ascidians are: 1) in ballast water; 2) on the hulls of barges, pleasure craft and other boats; 3) as epifauna on shells and shellfish transported between mariculture operations (Lambert 2002); and 4) in sea-chests (Coutts and Dodgshun 2007). Transport of *M. citrina* in ship ballast water is not a likely vector. This species broods its embryos past the hatching stage, so the tadpole has a very short free-swimming

phase usually ranging from minutes to perhaps an hour or two. Sometimes the tadpoles metamorphose prior to hatching (Berrill 1950; B.J. Swalla, unpublished observations), or prior to settlement, and thus could live as free floating juveniles for a time in ballast water. They ultimately become very sticky, and will stick to any surface encountered. Pre-settlement metamorphosis, reviewed by Feng et al. (2010), is known to occur in a number of ascidians and may be a fairly important mode of dispersal.

A more likely vector than ballast water is sea chests, which are “recesses built into a ship’s hull located beneath the waterline on the side and/or on the bottom near the engine room. They

are designed to reduce water cavitation, and thus increase pumping efficiency when seawater is pumped aboard the vessel for engine cooling, ballast, and fire fighting purposes” (Coutts et al. 2003). There are several sea chests in very large ships and they may be several meters in length, width and height. They are always open to the sea, covered only by a grating, and accessible only during dry docking. Hundreds of species of live marine organisms have been recorded during recent careful examination of these compartments (Coutts et al. 2003; Coutts and Dodgshun 2007). Most if not all of these organisms would not survive transoceanic transport if they were fouling the hull, but in the sea chests they are well protected. Once the ship docks, spawned gametes or released brooded embryos can leave the ship via the openings in the grating. While most of the fouling is by sessile organisms, many small fish, crabs and other motile animals appear to survive well. *Molgula citrina*, being a brooder, could conceivably survive for many generations in sea chests. It is doubtful whether they could survive a ship’s passage to and through the warm waters of the Caribbean and Panama Canal. But as global warming causes thinning of the Arctic sea ice, ice-free conditions across the NW Passage have occurred for several weeks during the past four summers enabling the beginning of commercial shipping (National Geographic online News Sept. 17, 2007 <http://news.nationalgeographic.com/news/2007/09/070917-northwest-passage.html>; Scientific American online Aug 27, 2008 <http://www.scientificamerican.com/>), and the first commercial ships traversed the NE Passage during 2009 (New York Times http://www.nytimes.com/2009/09/11/science/earth/11passage.html?_r=4&hp). Military ice breakers have travelled these waters for several decades, but now there is much interest in, and demand for increasing usage of, these routes by various shipping companies, as it will shorten the shipping distance between the N. Atlantic and N. Pacific by many thousands of miles. This also increases the threat of anthropogenic transport of non-native species between the North Atlantic and North Pacific (see Ruiz and Hewitt 2009 for review). It is a requirement of the U.S. Coast Guard that the sea chests of all ships in ice-clogged U.S. waters must be equipped with heat exchangers in order not to damage or shut down the engines. Most likely all ships traversing the NE or NW Passage are equipped similarly. This would greatly enhance the survivability of the organisms fouling the sea chests. Thus while the

question posed in this paper, “Is the Alaska population of *Molgula citrina* a result of natural circumpolar distribution or anthropogenic transport?”, cannot yet be answered until we learn more about populational differences, the threat of ship transport of nonindigenous species across the NW and NE Passages is a real and ever-increasing problem that should be addressed immediately, as global warming continues to quickly open up these waterways.

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Appendix 1. GenBank accession numbers.

Species	GenBank numbers
<i>Bostrichobranchus digonas</i> Abbott, 1951	L12415
<i>Eugyra arenosa</i> Alder & Hancock, 1848	L12417
<i>Molgula bleizi</i> (Lacaze-Duthiers, 1877)	L12419
<i>Molgula citrina</i> Alder & Hancock, 1848	L12421(Woods Hole)
<i>Molgula complanata</i> Alder & Hancock, 1870	L12423
<i>Molgula echinosiphonica</i> Lacaze-Duthiers, 1877	L12425
<i>Molgula manhattensis</i> (De Kay, 1843)	L12427
<i>Molgula occidentalis</i> Traustedt, 1883	L12429
<i>Molgula occulta</i> Kupffer, 1875	L12431
<i>Molgula oculata</i> Forbes, 1848	L12433
<i>Molgula provisionalis</i> Van Name, 1945	L12435
<i>Molgula socialis</i> Alder, 1863	L12437
<i>Molgula tectiformis</i> Nishikawa, 1990	L12439
<i>Herdmania momus</i> (Savigny, 1816)	X53538
<i>Styela plicata</i> (Lesueur, 1823)	AF158724