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A tale of two soft-shell clams: an integrative taxonomic analysis confirms *Mya japonica* as a valid species distinct from *Mya arenaria* (Bivalvia: Myidae)

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The soft-shell clam *Mya arenaria* Linnaeus, 1758 is a commercially important fishery resource that occurs in boreal and temperate environments in the Northern Hemisphere. Whether the soft-shell clam is a single species with a circumboreal range or a species complex also comprising *Mya japonica* Jay, 1857 distributed in the north Pacific has long been debated by malacologists and palaeontologists based on slight differences in shell morphology. We used an integrative taxonomic approach incorporating available *Mya* spp. mitochondrial *COI* and 16S rRNA, and nuclear 28S rRNA gene sequences, as well as spermatozoan and shell morphological characters to test the validity of *M. japonica* and examine the range of soft-shell clam distribution. Although differences in shell morphology were minor, the results from tree topologies, pairwise uncorrected *p*-distances, Automatic Barcode Gap Discovery (ABGD) and spermatozoan ultramorphological data confirm the validity of *M. japonica* in both its endemic region in the northwest Pacific, and as here newly reported introduced populations in British Columbia in the northeast Pacific, and show that *M. arenaria* is distributed in the northeast Pacific, North Atlantic, Barents Sea (Arctic Ocean) and Mediterranean. We estimate these two closely related sister species diverged 4.1–12.5 Myr during early Pliocene to late Miocene, which is consistent with current evolutionary theory regarding *M. arenaria*. In addition, ABGD indicated the congener *Mya truncata* Linnaeus, 1758 may represent a species complex, but additional evidence is still needed to clarify its taxonomic status.

ADDITIONAL KEYWORDS: biogeography – cryptic species – DNA barcoding – molecular phylogeny – Mollusca – spermatozoan ultramorphology.

INTRODUCTION

The soft-shell clam *Mya arenaria* Linnaeus, 1758, described from Europe, is an edible bivalve currently found in boreal and northern temperate marine and estuarine environments with a complex, but widely accepted migration history (MacNeil, 1965; Laursen, 1966; Strauch, 1972; Strasser, 1999). *Mya arenaria* originated in the western Pacific probably around Japan during the Miocene, spreading to the eastern Pacific later in the Miocene and then to both coasts of the Atlantic Ocean during the Pliocene (MacNeil, 1965). It was extirpated in the eastern Pacific and eastern Atlantic during the Pleistocene glaciation, surviving only in the western Atlantic with uncertain status in the Bering Sea and western Pacific (MacNeil, 1965; Carlton, 1979; Strasser, 1999). It re-invaded

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the eastern Atlantic during the Middle Ages and the eastern Pacific during the late 1800s, both invasions mediated by anthropogenic activities (Fujie, 1957, 1962; MacNeil, 1965; Bernard, 1979; Strasser, 1999; Cross *et al.*, 2016). It has more recently appeared in the Mediterranean and Black Sea (Strasser, 1999; Gomoiu *et al.*, 2002). Historical Arctic records are now generally regarded as belonging to a congener, *Mya pseudoarenaria* Schlesch, 1931 (Laursen, 1966). *Mya arenaria* is a major fishery resource in the western Atlantic (Congleton *et al.*, 2006). However, in many parts of its introduced range *M. arenaria* can negatively impact native benthic communities (Streftaris & Zenetos, 2006; Crocetta & Turolla, 2011), disrupt native fisheries (Carlton, 1979) and represent a significant portion of benthic biomass (Strasser, 1999).

Within the genus *Mya* there is much disagreement regarding evolutionary history, species distributions and taxonomy (MacNeil, 1965; Bernard, 1979; Petersen, 1999; Huber, 2010; Bouchet & Gofas, 2013; ITIS, 2017), particularly regarding the number of extant species and the relationships between extant forms and species described from fossil materials (Petersen, 1999). The most extensively studied species (both ecologically and taxonomically) is *M. arenaria*, but questions regarding taxonomic status and geographical range remain. *Mya arenaria* is reported to have numerous synonyms: *M. communis* Megerle von Mühlfeld, 1811; *M. lata* Sowerby, 1815; *M. acuta* Say, 1822; *M. acuta mercenaria* Say, 1822; *M. subovata* Woodward, 1833; *M. subtruncata* Woodward, 1833; *M. alba* Agassiz, 1839; *M. corpulenta* Conrad, 1845; *M. japonica* Jay, 1856; *Sphenia ovoidea* Carpenter, 1864; *M. hemphilli* Newcomb, 1874; *M. declivis* Pennant, 1777; *M. elongata* Locard, 1886; *M. arenaria* var. *ovata* Jensen, 1900; *M. paternalis* Matsumoto, 1930; *M. arenaria oonogai* Makiyama, 1935; *M. japonica oonogai* Makiyama, 1935; *M. oonogai* Makiyama, 1935; and *M. arenaria corbuloides* Comfort, 1938 (Coan, Scott & Bernard, 2000; Zhang, Xu & Liu, 2012). One of the most contentious questions pertains to the validity of *M. japonica*, which has been reported in the western Pacific (Habe, 1955, 1977; Fujie, 1957, 1962; MacNeil, 1965; Okutani, 2000), with possible relict populations or adventitious individuals occurring in the Bering Sea (MacNeil, 1965; Carlton, 1979). Soft-shell clams in the western Pacific identified as *M. japonica* possess rough commarginal wrinkles, relatively shorter and more rounded posterior end and more impressed pallial line in comparison to *M. arenaria* (Jay, 1856; MacNeil, 1965). These differences have led some malacologists to accept *M. japonica* as a valid species (Habe, 1955, 1977; Fujie, 1957, 1962; MacNeil, 1965; Okutani, 2000), whereas others have reported that *M. arenaria* and *M. japonica* could not be separated by the statistical analysis of morphological characteristics, and therefore, *M. japonica* was a junior synonym of *M. arenaria* (Nagao & Inoue, 1941; Bernard, 1979, 1983; Coan *et al.*, 2000; Huber, 2010; Zhang *et al.*, 2012). Confounding these taxonomic uncertainties are conflicting descriptions of important morphological characters, such as the chondrophore in the left valve (MacNeil, 1965; Bernard, 1979). Although several studies have examined genetic relationships between North American and European populations of *M. arenaria* (Strasser & Barber, 2009; Layton, Martel & Hebert, 2014; Barco *et al.*, 2016; Cross *et al.*, 2016; Lasota *et al.*, 2016), none have conducted a rigorous genetic analysis of *M. arenaria s.l.* from across its entire range (i.e. including the western Pacific).

Recent molecular analyses have identified varying levels of cryptic diversity in several cosmopolitan and wide-ranging molluscs. For example, many temperate North American populations of the tellinid clam *Macoma balthica* Linnaeus, 1758 are now identified as *Macoma petalum* Valenciennes, 1821 (Väinölä, 2003; Nikula, Strelkov & Väinölä, 2007); the widespread African freshwater oyster *Etheria elliptica* Lamarck, 1807 consists of at least three distinct sibling species (Elderkin *et al.*, 2016); and the aeolidiid nudibranchs *Aeolidia papillosa* Linnaeus, 1761 and *Spurilla neapolitana* Delle Chiaje, 1841 now comprise complexes of four and five sibling species, respectively (Carmona *et al.*, 2014; Kienberger *et al.*, 2016). Molecular analyses can be particularly powerful when combined with morphological and other biological data (e.g. spermatozoan ultramorphology; Carstensen *et al.*, 2009) within a broader integrative taxonomic framework (Padial *et al.*, 2010; Schlick-Steiner *et al.*, 2010).

Spermatozoan ultramorphology appears to be highly conserved and has been used to resolve taxonomic and phylogenetic questions at the species level in many bivalve genera, including *Solen* (Hodgson, Devilliers & Bernard, 1987), *Donax* (Hodgson, Bernard & Vanderhorst, 1990; Introini, Passos & Recco-Pimentel, 2013), *Crassostrea* (Gwo, Liou & Cheng, 1996), *Bathymodiolus* (Eckelbarger & Young, 1999), *Nutricola* (Geraghty, Russell & Dollahon, 2008) and *Brachidontes* (Torroglosa & Gimenez, 2015). Fine organization of soft-shell clam spermatozoa from the western Pacific was studied in *M. japonica* (Drozdov, Sharina & Tyurin, 2009) and *M. arenaria oonogai* (Kim, Chung & Park, 2011). In both studies, spermatozoa were classified as ect-aquasperm with a conical curved head consisting of an acrosomal complex and nucleus, a midpiece with four mitochondria and a flagellum with a 9 × 2 + 2 organization of microtubules. However, no previous studies have described the morphology of *M. arenaria* spermatozoa from the greater Atlantic or preformed range-wide comparisons.

In this study, we report the first rigorous range-wide investigation of the soft-shell clam, using an integrative taxonomic approach based on mitochondrial cytochrome *c* oxidase subunit I (*COI*), mitochondrial 16S

rRNA (16S) and nuclear 28S rRNA (28S) gene data, and quantitative sperm ultramorphology and qualitative shell morphological comparisons. We also report the first detailed description of spermatozoan ultramorphology of *M. arenaria s.s.* and provide updated distributional data.

MATERIAL AND METHODS

Specimen collection

For DNA analyses, we collected soft-shell clams from Yellow Sea, Qingdao, China (n = 8); Barents Sea, Murmansk, Russia (n = 3); Vostok Bay, Sea of Japan, Russia (n = 1); and Chesapeake Bay, Maryland, USA (n = 10) (Fig. 1). We also collected a congener, *Mya uzenensis* Nomura et Zinbo, 1937 from Vostok Bay (n = 1). The authors follow Lutaenko & Noseworthy (2012) and accept *M. uzenensis* as a valid species and not a junior synonym of *M. pseudoarenaria*. Voucher specimens were deposited at the Marine Biological Museum, Chinese Academy of Sciences (MBM), Qingdao, China, and the Smithsonian Institution National Museum of Natural History (USNM), Washington, DC, USA (Table 1). For spermatozoan ultramorphological analyses, three males in each of the Yellow Sea, Barents Sea and Sea of Japan sampling sites were collected at pre-spawning and spawning stages of the reproductive cycle (for staging details, see Porter, 1974; Dzyuba & Maslennikova, 1987). Voucher specimens were deposited at the Zoological Museum, Far Eastern Federal University, Vladivostok, Russia (ZMFU). In addition to the samples mentioned above, we examined 135 lots of soft-shell clams (photographing a sub-set of samples), which included individuals previously identified as *M. arenaria* and *M. japonica*, from historical MBM and ZMFU collections to identify possible morphological differences between soft-shell clams collected in the western Pacific and northwestern Asia/northern Europe (e.g. Barents Sea, White Sea, North Sea, Baltic Sea; Fig. 2).

Prior to analyses, all specimens (field collected and museum vouchers) were identified based on shell morphological differences of these two putative species (*M. arenaria* or *M. japonica*) recognized by early malacologists (Habe, 1955, 1977; Fujie, 1957, 1962; MacNeil, 1965). Specifically, we examined shell shape, noting profile of the posterior end, shell texture (regularity and depth of commarginal growth lines), the pallial line, and the shape, size, and orientation of the chondrophore in the left valve. Soft-shell clams possessing an elongated shell shape with even commarginal growth lines and lightly impressed pallial line were classified as *M. arenaria*, whereas soft-shell clams with a more truncate shell shape with rough commarginal wrinkles and more deeply impressed pallial line were classified as *M. japonica*.

Molecular work

DNA extraction, amplification and sequencing

Thirteen specimens of *M. arenaria*, nine specimens of *M. japonica* and one specimen of *M. uzenensis* were collected for molecular analyses. We extracted total DNA from the samples using the Marine Animal

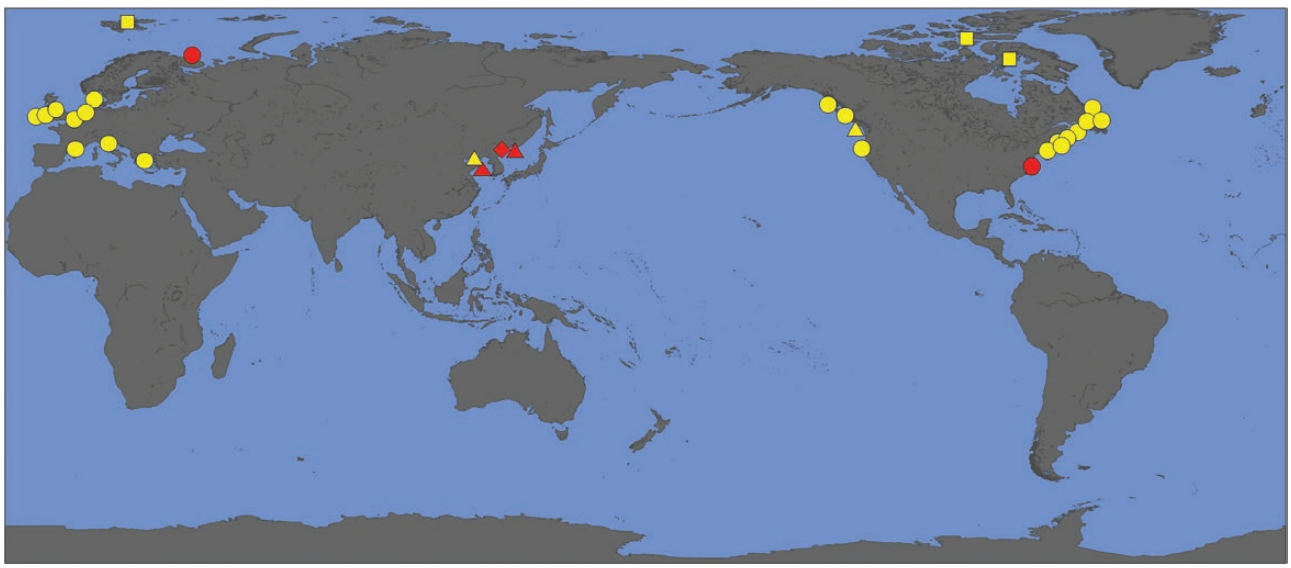


Figure 1. Locations of *Mya japonica* (red triangles), *Mya arenaria* (red dots) and *Mya uzenensis* (red diamond) collected in this study, and other known localities of *Mya japonica* (yellow triangles), *Mya arenaria* (yellow dots) and *Mya truncata* (yellow squares) of which sequences were used in genetic analyses.

Table 1. Sampling information of specimens collected for this study and GenBank accession numbers of their sequences

Species	Voucher	Location	GenBank numbers		
			COI	16S	28S
Mya arenaria	MBM229022	Murmansk, Barents Sea, Russia (Arctic)	KX534201	KX534176	KX534188
Mya arenaria	MBM229023	Murmansk, Barents Sea, Russia (Arctic)	KX534202	KX534177	KX534189
Mya arenaria	MBM229024	Murmansk, Barents Sea, Russia (Arctic)	KX534203	KX534178	KX534190
Mya arenaria	USNM:IZ:1286707	Chesapeake Bay, Maryland, USA (NW Atlantic)	KT959460	KT959520	–
Mya arenaria	USNM:IZ:1286708	Chesapeake Bay, Maryland, USA (NW Atlantic)	KT959399	KT959490	–
Mya arenaria	USNM:IZ:1286709	Chesapeake Bay, Maryland, USA (NW Atlantic)	KT959418	KT959498	–
Mya arenaria	USNM:IZ:1286710	Chesapeake Bay, Maryland, USA (NW Atlantic)	KT959394	KT959487	–
Mya arenaria	USNM:IZ:1286892	Chesapeake Bay, Maryland, USA (NW Atlantic)	KU906055	–	–
Mya arenaria	USNM:IZ:1286895	Chesapeake Bay, Maryland, USA (NW Atlantic)	KU906064	–	–
Mya arenaria	USNM:IZ:1286896	Chesapeake Bay, Maryland, USA (NW Atlantic)	KU905943	–	–
Mya arenaria	USNM:IZ:1286897	Chesapeake Bay, Maryland, USA (NW Atlantic)	KU905735	–	–
Mya arenaria	USNM:IZ:1287437	Chesapeake Bay, Maryland, USA (NW Atlantic)	KU906072	–	–
Mya arenaria	USNM:IZ:1287540	Chesapeake Bay, Maryland, USA (NW Atlantic)	KU906038	–	–
Mya japonica	MBM229013	Qingdao, Yellow Sea, China (NW Pacific)	KX534192	KX534167	KX534180
Mya japonica	MBM229014	Qingdao, Yellow Sea, China (NW Pacific)	KX534193	KX534168	–
Mya japonica	MBM229015	Qingdao, Yellow Sea, China (NW Pacific)	KX534194	KX534169	KX534181
Mya japonica	MBM229016	Qingdao, Yellow Sea, China (NW Pacific)	KX534195	KX534170	KX534182
Mya japonica	MBM229017	Qingdao, Yellow Sea, China (NW Pacific)	KX534196	KX534171	KX534183
Mya japonica	MBM229018	Qingdao, Yellow Sea, China (NW Pacific)	KX534197	KX534172	KX534184
Mya japonica	MBM229019	Qingdao, Yellow Sea, China (NW Pacific)	KX534198	KX534173	KX534185
Mya japonica	MBM229020	Qingdao, Yellow Sea, China (NW Pacific)	KX534199	KX534174	KX534186
Mya japonica	MBM229021	Vostok Bay, Peter the Great Bay, Sea of Japan, Russia (NW Pacific)	KX534200	KX534175	KX534187
Mya uzenensis	MBM229025	Vostok Bay, Peter the Great Bay, Sea of Japan, Russia (NW Pacific)	KX534204	KX534179	KX534191

Genomic DNA Extraction Kit (Tiangen Biotech, Beijing, China) according to manufacturer's instructions. North American samples were sequenced following Aguilar *et al.* (2016) using the Primer sets jgLCO1490/jgHCO2198 (*COI*) and 16Sar-L/16Sb (16S). For Eurasian samples, fragments of other specimens of the mitochondrial *COI* gene were amplified using primers of Lco1490: 5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′, Hco2198: 5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′ (Folmer *et al.*, 1994). Fragments of the mitochondrial 16S rRNA gene were amplified using 16Sa: 5′-CGC CTG TTT ATC AAA AAC AT-3′ (Xiong & Kocher, 1991), 16Sb: 5′-CTC CGG TTT GAA CTC AGA TCA-3′ (Edgecombe, Giribet & Wheeler, 2002). The nuclear 28S rRNA gene was amplified using 28Sa: 5′-GAC CCG TCT TGA AAC ACG GA-3′, 28Sb: 5′-TCG GAA GGA ACC AGC TAC-3′ (Whiting *et al.*, 1997). The 25-μL reaction for the amplification contained 12 μL 2×Es TaqMasterMix (CWBio Co., Ltd, Beijing, China), 2 μL of template DNA (50 ng/μL), 1 μL of each primer (10 M) and 9 μL dH_2O. Amplification reactions were conducted in a T100 Thermal Cycler (Bio-Rad Laboratories, Inc.) with the following thermal profile: 95 °C for 3 min; 35 cycles of 95 °C for 30 s, 50–55 °C for 45 s, 72 °C for 1 min, and a further 10-min elongation at 72 °C. The PCR products were purified and sequenced by BGI Tech Solutions Co., Ltd.

Species delimitation analyses

To discriminate species, we used an integrative taxonomic approach including pairwise uncorrected *p*-distances, phylogenetic trees, Automatic Barcode Gap Discovery (ABGD) and spermatozoan ultramorphological and qualitative shell morphological data. In addition to sequences generated from our field collections, we included all *Mya COI* (> 500 bp), 16S and 28S gene sequences available in GenBank and BOLD (Ratnasingham & Hebert, 2007) in the generation of *p*-distances and in the ABGD analyses, most of which were associated with published articles (Supporting Information, Table S1). In total, 175 *COI* sequences (including four *Potamocorbula* sequences, which were originally submitted under the name *Corbula amurensis* in GenBank as an outgroup) were used for ABGD and *p*-distance analyses, and 26 16S sequences (including four outgroup *Potamocorbula* sequences) were also used for *p*-distance analyses. To improve clarity,

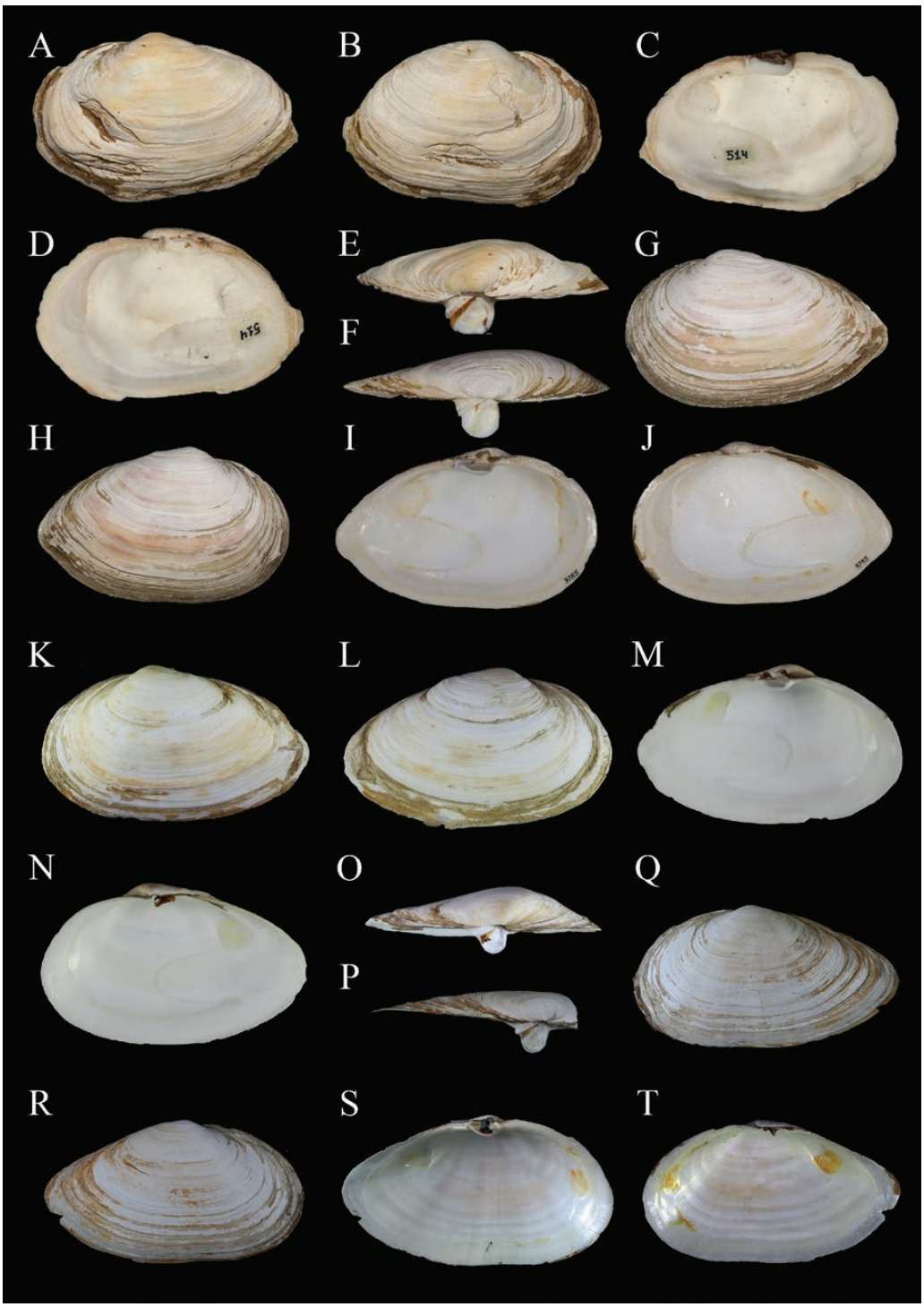


Figure 2. *Mya* shell morphology. A–E, *Mya japonica* Jay, 1857, ZMFU no. 10069/Bv-514, sampled from the open part of Peter the Great Bay, Bolshoy Pelis Island, Russia. F–J, *Mya japonica* Jay, 1857, ZMFU no. 23301/Bv-3785, sampled from semi-enclosed part of Peter the Great Bay, Vityaz Bay, Russia. K–O, *Mya japonica* Jay, 1857, MBM229023, sampled from Qingdao, Yellow Sea, China. P–T, *Mya arenaria* Linnaeus, 1758, MBM229013, sampled from Murmansk, Barents Sea, Russia.

sequences without associated publications or location information were omitted (n = 49) from phylogenetic trees. The ABGD (Puillandre *et al.*, 2012) settings were as follows: steps = 10, X = 1, P_{min} = 0.001, P_{max} = 0.1, Nb bins = 20, and Jukes-Cantor (JC69) and Kimura (K80) TS/TV. The p-distances within and among each species

grouping were estimated with MEGA v.6 (Tamura *et al.*, 2013).

Phylogenetic analyses

Sequences of each region were aligned independently using MAFFT v.7 (Katoh & Standley, 2013) with the G-INS-i and Q-INS-i algorithms for the protein-coding and ribosomal regions. The alignments were further checked by eye. A combined analysis was conducted with concatenated sequences from the three genes. Three genes from the same individual were concatenated together as a sequence in SequenceMatrix v 1.8 (Vaidya, Lohman & Meier, 2011). Sequences from *Potamocorbula amurensis* (Schrenck, 1861) in the same superfamily, Myoidea, were selected as an outgroup. The best-fitted evolutionary models were selected by Akaike information criterion as implemented in jModeltest2 (Darriba *et al.*, 2012). Maximum likelihood (ML) analysis was carried out using PhyML-3.1 (Guindon *et al.*, 2010). Node support came from a majority-rule consensus tree of 1000 bootstrap replicates. For the ML bootstraps, we consider values < 70 as low, 70–95 as moderate and ≥ 95 as high, following Hillis & Bull (1993). Bayesian inference (BI) analysis was carried out using MrBayes v3.2.6 (Ronquist *et al.*, 2012) in CIPRES Science Gateway. We estimated posterior probability (PP) using four chains running 10 000 000 generations sampling every 100 generations. The first 25% of sampled trees were considered burn-in trees, and Tracer v1.6 was used to confirm that post-burn-in trees yielded an effective sample size of > 200 for all parameters. All remaining trees were used to calculate PPs using a 50% majority-rule consensus. For the Bayesian PPs, we considered values < 94% as low and ≥ 95% as high following Alfaro, Zoller & Lutzoni (2003).

Divergence time estimates

We used a Bayesian Markov chain Monte Carlo method implemented in Beast version 2.4.0 (Bouckaert *et al.*, 2014) to estimate the evolutionary ages of internal nodes in the tree topology derived from the combined phylogenetic analyses. The time to most recent common ancestor (T), that is the time of divergence, was estimated as $T = K/2r$, where K is the K2P genetic distance, r is the nucleotide substitution rate. Substitution rates (r) were estimated as percentage per lineage per million years (Myr), so they equal one half the pairwise sequence divergence per Myr, that is divergence rate. Our estimates of T are based on the mitochondrial *COI* divergence between cognate species of Arcidae isolated across the Isthmus of Panama at a rate of 0.7–1.2%/Myr (Marko, 2002); thus, the substitution rate (r) equals 0.35–0.6%/Myr. A relaxed, uncorrelated lognormal clock with a general time reversible (GTR) substitution model was inferred for each partition, and a Yule speciation process was assumed for the tree prior. The analysis was run for 100 million generations, sampled every 1000 generations and a burn-in of 25%. Convergence and mixing were assessed using Tracer v. 1.6 by examining log-likelihood values across generations and ensuring that all remaining trees yielded an effective sample size for all parameters. Results were visualized using Tracer v. 1.6 and FigTree v. 1.4.2.

SPERMATOZOAN ULTRAMORPHOLOGY

Transmission electron microscopes preparation

Small pieces of gonadal tissues were pre-fixed in 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.5), with the addition of NaCl to provide appropriate tonicity of sea water, for 2 h at 4 °C. The specimens were then rinsed several times in the same buffer and held at 4 °C until analysis at the National Scientific Center of Marine Biology (Vladivostok, Russia). In the laboratory, gonadal tissue samples were post-fixed in 2% (w/v) osmium tetroxide in 0.1 M cacodylate buffer (pH 7.5) for 2 h in the dark at room temperature. The specimens were washed in distilled water and then dehydrated using a graded ethanol series and acetone. The dehydrated specimens were embedded in Araldite-EMbed-812 resin. Ultrathin sections (*c*. 50 nm) were obtained using a Leica UC6 ultramicrotome equipped with a diamond knife. The sections were stained with 2% aqueous uranyl acetate and Reynolds' lead citrate (Reynolds, 1963) and were observed under Zeiss Libra 120 and Zeiss Libra 200FE transmission electron microscopes (Far East Center of Electron Microscopy, National Scientific Center of Marine Biology FEB RAS).

Measurements of spermatozoa

To investigate possible differences in spermatozoan ultramorphology among soft-shell clams, we examined three males (five spermatozoa per male) from each species/geographical groupings: *M. arenaria* from the Barents Sea, *M. japonica* from the Sea of Japan and *M. japonica* from the Yellow Sea. Only those spermatozoa in which the following compartments could be simultaneously observed in a longitudinal projection were measured: (1) acrosomal complex with cylindrical subacrosomal space, from the base of anterior nuclear fossa to the apex of acrosomal vesicle; (2) cone-shaped nucleus with the gradual dilatation from the apical to the basal part without any narrowing; and (3) midpiece with two centrioles. Because of the curved shape of the nucleus, we measured the maximal Feret's diameter to estimate the nuclear length. We measured the length of the acrosomal complex from the invagination of the anterior nuclear fossa to the terminal point at the apical part of the acrosomal vesicle, and the width of the acrosomal

complex at its base. All measurements were calculated with ImageJ version 1.49b (Schneider, Rasband & Eliceiri, 2012). To compare the size and shape of the acrosomal complex, we calculated volumes and length to width ratios. Based on the close-to-conical profile of the acrosomal complexes in the sections, we used the following formula to estimate volume: $V = 1/3\pi R^2 H$, where R is ½ of the width of the acrosomal complex and H is the length of the acrosomal complex.

Statistical analyses

We used one-way analysis of variance (ANOVA) to test for significant differences in mean nucleus length, mean acrosomal volume and mean acrosomal length to width ratio among the three species/geographical groupings: *M. arenaria* from the Barents Sea, *M. japonica* from the Sea of Japan and *M. japonica* from the Yellow Sea. Effect size (η^2) for ANOVA was calculated using the corresponding function of R add-on package 'lsr' version 0.5 (Navarro, 2014). Multiple comparisons among geographical areas were made using Tukey's honestly significant difference (Tukey's HSD) tests. Prior to analysis, all data sets were tested for normality with Shapiro-Wilk tests and for homogeneity of variance using Levene's tests and were determined to meet these assumptions of parametric analyses. Differences were considered statistically significant at $P < 0.05$ for all tests. We performed all statistical analyses using R version 3.2.4 (R Core Team, 2015) running on a Debian GNU/Linux workstation.

RESULTS

Morphology

Shell characteristics and morphological identifications

Based on our visual examinations, the samples from the western Pacific morphologically corresponded well with *M. japonica* and the northeast Pacific/Atlantic/Arctic soft-shell calms were morphologically identical with *M. arenaria* (Fig. 2). Detailed statistical analyses of morphological characteristics of soft-shell clams classified as *M. arenaria* and *M. japonica* have been reported previously (see Bernard, 1979), but here we describe minor differences in shell characteristics between them. In general, *M. arenaria* possessed a more elongated shell, with a longer and thinner posterior end and regular thin commarginal growth lines (Fig. 2P–T). *Mya japonica* was higher, with rough commarginal wrinkles and a relatively shorter and more rounded posterior end (Fig. 2A–O). In addition, the pallial line of *M. arenaria* was less impressed than in *M. japonica*. However, there was considerable variation in the shape, relative size and orientation of the left valve chondrophore among all the clams examined and not useful for discriminating between species.

Two distinct morphological forms of *M. japonica* were observed in Peter the Great Bay. A thin, 'typical' form of *M. japonica*, which was elongated with regular growth lines, occurring mostly in semi-enclosed bays and other protected seashore areas, often with decreased salinity and a low energy environment (Fig. 2F–J). Conversely, a 'thick-shelled' form with irregular growth lines occurred in open, wave-influenced bays (Fig. 2A–E). Intermediate specimens were also found.

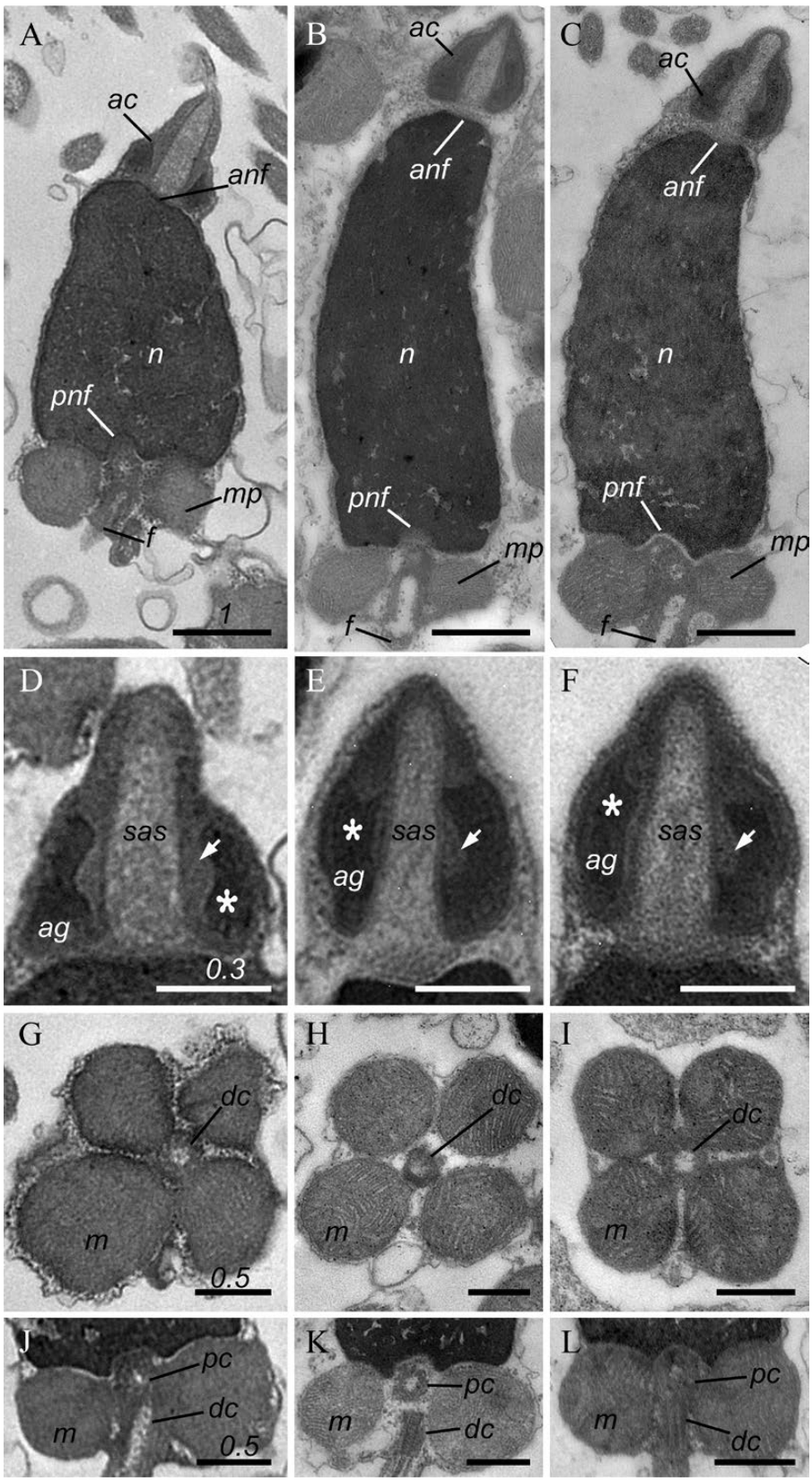
Comparison of spermatozoan ultramorphology

All spermatozoa examined in the present study were ect-aquasperm, a presumed primitive sperm type often associated with aquatic invertebrates with external fertilizing, free-swimming gametes (Eckelbarger & Young, 1999). Spermatozoa of all the specimens examined consisted of an acrosomal complex, nucleus, midpiece and flagellum. The data on dimensions of nuclei and acrosomal complexes are summarized in Table 2. The sperm were all rotationally asymmetrical due to the curvature of the nucleus or the position of the acrosomal complex (Fig. 3A–C). The midpieces of all

Table 2. Descriptive statistics of spermatozoan compartment dimensions in *Mya arenaria* sampled from the Barents Sea and in *Mya japonica* sampled from the Sea of Japan and the Yellow Sea (mean ± SD)

	Mya arenaria from the Barents Sea	*Mya japonica* from the Sea of Japan	*Mya japonica* from the Yellow Sea
Nucleus length (μm)	1.98 ± 0.16	2.97 ± 0.13	3.08 ± 0.18
Acrosomal complex length (μm)	0.74 ± 0.06	0.80 ± 0.05	0.82 ± 0.04
Acrosomal complex width (μm)	0.61 ± 0.05	0.55 ± 0.04	0.57 ± 0.03
Acrosomal complex length to width ratio	1.22 ± 0.11	1.46 ± 0.08	1.46 ± 0.09
Acrosomal complex volume (μm^3)	0.073 ± 0.015	0.064 ± 0.012	0.069 ± 0.010

Number of samples: 15 spermatozoa in each sampling site.



spermatozoa examined were similar, containing four spherical mitochondria (Fig. 3G–I), and orthogonally arranged proximal and distal centrioles (Fig. 3J–L). In addition, the flagellum of all spermatozoa possessed a typically organized axoneme (9 × 2 + 2), posterior to the distal centriole.

Nuclei were curved in all three geographical groups, but in *M. arenaria* from the Barents Sea, the curvature of the nuclei was less obvious than that in *M. japonica* from the Sea of Japan and the Yellow Sea (Fig. 3A–C). All nuclei possessed electron-dense chromatin with irregularly shaped electron-lucent lacunae. Anterior portions of the nuclei were slightly invaginated and formed an anterior nuclear fossa that was located subterminally (in *M. arenaria*) or apically (in *M. japonica*; Fig. 3A–C). Posterior portions had a well-defined invagination (posterior nuclear fossa) where a proximal centriole was located, and indentations were in the regions of contact of the nucleus and mitochondria (Fig. 3A–C, J–L). There were statistically significant differences in mean nuclei length (ANOVA: $F(2,42) = 219.51$, $P < 0.05$, $\eta^2 = 0.91$). The Tukey's HSD test indicated that *M. arenaria* (Barents Sea) had significantly shorter nuclei compared to *M. japonica* from the Sea of Japan and Yellow Sea ($P < 0.05$; Fig. 4A). No statistically significant difference in nuclei length between *M. japonica* collected from the Sea of Japan and Yellow Sea was found ($P = 0.165$).

In all spermatozoa examined, the acrosomal complexes were cone-shaped and consisted of a membrane-bounded acrosomal vesicle and subacrosomal space (Fig. 3D–F). Within the acrosomal vesicle, two regions with different electron density were distinguished: the inner with moderate electron density and the outer with high electron density (Fig. 3D–F). The acrosomal vesicle had a deep posterior invagination that extended to its apical portion and formed a subacrosomal space filled with flake-like subacrosomal material of low electron density. The distance between the acrosomal complex and the nucleus was narrow in *M. arenaria* (Barents Sea), but wider *M. japonica* (Sea of Japan and Yellow Sea; Fig. 3D–F). This is most likely the reason why the acrosomal complex lengths were generally larger relative to width in *M. japonica* (Sea of Japan and Yellow Sea) than *M. arenaria* (Barents Sea; Table 2). There was a statistically significant difference in mean acrosomal length to width ratios (ANOVA: $F(2,42) = 30.83$, $P < 0.05$, $\eta^2 = 0.60$). A Tukey's HSD test indicated that acrosomal complexes of the spermatozoa in *M. arenaria* (Barents Sea) were shorter relative to width than those in *M. japonica* (Sea of Japan and Yellow Sea; Table 2; $P < 0.05$), while no statistically significant difference in length to width ratios between *Mya* from the Sea of Japan and Yellow Sea was found ($P = 1$; Fig. 4B). There were no significant differences in mean acrosomal complex volumes among the different geographical regions (ANOVA: $F(2,42) = 1.94$, $P = 0.156$, $\eta^2 = 0.08$).

Molecular analyses

Barcoding analyses

In total, 52 new sequences were obtained and deposited in GenBank (Table 1). The alignment data set comprised 637 nucleotide positions for *COI*, 496 positions for 16S and 297 positions for 28S. The concatenated data set yielded a sequence alignment of 1430 positions. The ABGD analysis based on the *COI* alignment produced five distinct groupings, with *P* values ranging from 0.022 to 0.100 based on both the Jukes-Cantor (JC69) and Kimura (K80) TS/TV models (Table 3). These groupings corresponded with the five clades/subclades in the *COI* trees (Table 3; Fig. 5B–D). The *COI* *p*-distances within the former four groups were low (0–2.39%), while the *p*-distances among the five groups ranged from 7.17 to 19.12% (Table 3; Supporting Information, Table S2). The minimum interspecific *p*-distance among these groups was much higher than the maximum one within ABGD Group 1 (7.17 vs. 2.39%; Table 3), which indicated all the western Pacific soft-shell clams (those morphologically identified *M. japonica* by the authors and those classified as *M. arenaria* – the valid species name at the time of identification), plus two individuals from British Columbia (DFO097-11 + RBCMI219-14) represent a single species (i.e. *M. japonica*). After reassigning all western Pacific *M. arenaria* and DFO097-11 + RBCMI219-14 as *M. japonica*, the *COI* intraspecific *p*-distances within *Mya* were 0–7.57%, while the interspecific *p*-distances among *Mya* species ranged from 11.16 to 19.12% (Fig. 5A; Table 3; Supporting Information, Table S2), indicating a barcoding gap among *Mya* species (Fig. 5A). However, if the

Figure 3. Sperm fine morphology in the studied *Mya* species/populations. A, D, G, J, *Mya arenaria* sampled from the Barents Sea. B, E, H, K, *Mya japonica* sampled from the Sea of Japan. C, F, I, L, *Mya japonica* sampled from the Yellow Sea. A–C, longitudinal section of spermatozoon consisted of acrosomal complex (ac), nucleus (n) with anterior (anf) and posterior nuclear fossae (pnf), midpiece (mp) and flagellum (f), scale bar = 1 μm. D–F, acrosomal complex included acrosomal vesicle (av) with an inner moderate electron-dense layer (asterisk) and outer electron-dense layer (arrow) and subacrosomal space (sas) filled with subacrosomal material, scale bar = 0.3 μm. G–I, transversal section of midpiece showing four mitochondria (m) around distal centriole (dc), scale bar = 0.5 μm. J–L, longitudinal section of midpiece. Proximal (pc) and distal centrioles (dc) located orthogonally to each other, scale bar = 0.5 μm.

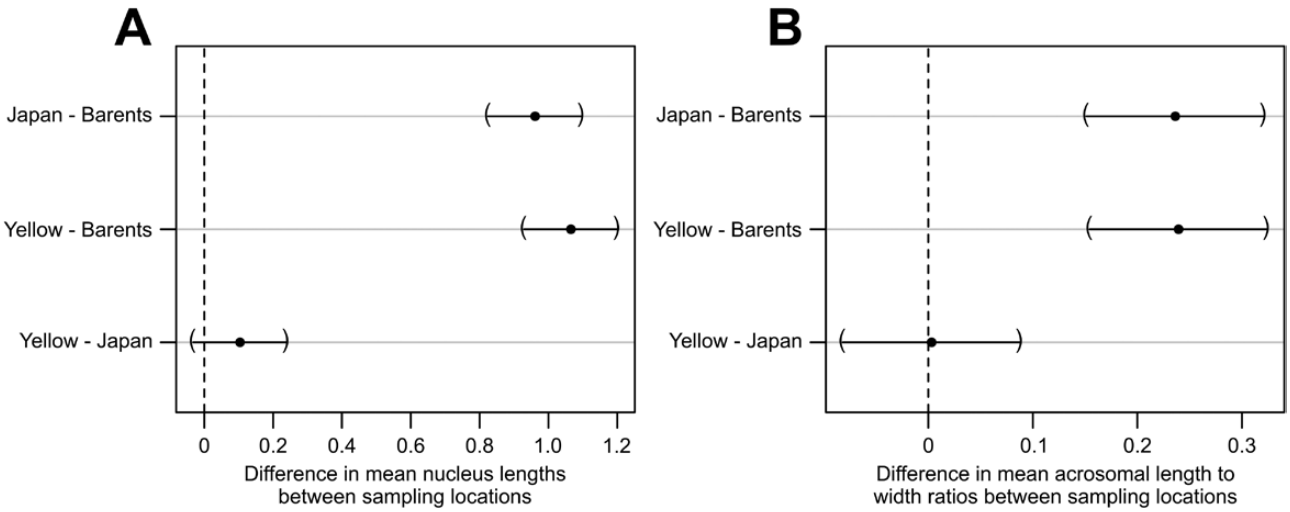


Figure 4. Whisker plots showing results of Tukey's honestly significant difference test for difference in mean nucleus lengths (A) and acrosomal L/W ratios (B) among groups of *Mya*. Black dots are the difference in pairwise comparisons between sampling locations of the means and the whiskers are 95% confidence intervals of these differences.

Table 3. Uncorrected pairwise distances at *COI* within and between the ABGD groups

		Group 1	Group 2	Group 3	Group 4	Group 5
Group 1	All *Mya japonica* + *Mya arenaria* QWEAS092-15, QWEAS093-15, DFO097-11, RBCMI219-14, KP976290, KP976289, KP976288, KP976287, KP976286, KJ125420, KJ125421, JQ267795	0–2.39%				
Group 2	Remaining *Mya arenaria* (142 sequences)	11.55–13.94%	0–1.99%			
Group 3	*Mya truncata* KF644116, KF644129	17.53–18.33%	15.54–17.53%	0.40%		
Group 4	*Mya truncata* KF644154, KF643769, KF643675, KF643403	17.53–18.73%	17.53–19.12%	7.17–11.55%	0	
Group 5	*Mya uzenensis* KX534204	17.53–18.33%	16.33–17.93%	11.16–11.55%	13.15%	–

two *Mya truncata* clades (ABGD Groups 3 and 4) are considered distinct species, intraspecific *p*-distances decrease to 0–2.39%, while interspecific distances range from 7.17 to 19.12%. For 16S, the intraspecific *p*-distances ranged from 0 to 0.97% within *M. arenaria* and *M. japonica*, while the interspecific *p*-distances ranged from 5.3 to 13.80% (Supporting Information, Table S3), which also indicates a barcoding gap between *M. arenaria* and *M. japonica*.

Phylogenetic analyses

For *COI*, 16S rDNA, 28S rDNA and the concatenated alignments, the TIM3 + I + G, TPM1uf + I, HKY and TVM + G evolutionary models were the best-fitted models, respectively. We report only the results of trees generated from *COI* sequences (Fig. 5B, D), which better resolved relationships at species level, and trees from the concatenated regions (Fig. 6). Trees constructed from 28S were uninformative with low support values at all levels and were omitted. Trees constructed from 16S were nearly identical in topology with the concatenated regions (except for differences in support values) and were also omitted. The resulting two consensus trees generated using BI and ML analyses were generally consistent; thus, a single topology was presented for the concatenated regions with support values indicated on branches (Fig. 6). This topology was in agreement with the *COI* trees and showed species-level groupings with high support (Fig. 6).

In all *COI* trees, the ABGD Group 1 (all western Pacific soft-shell clams and two individuals of *M. arenaria* from the northeast Pacific) formed a clade with high node support (BI 1.00, ML 100%) and the ABGD Group 2 (remaining *M. arenaria*) grouped together with low to high node support before clustering to other species (BI 1.00, ML 42%, Fig. 5B, D). In the *COI* BI tree and the concatenated trees, *M. arenaria* clustered with *M. japonica* with high support, while *M. truncata* formed a sister clade with *M. uzenensis* with full node support

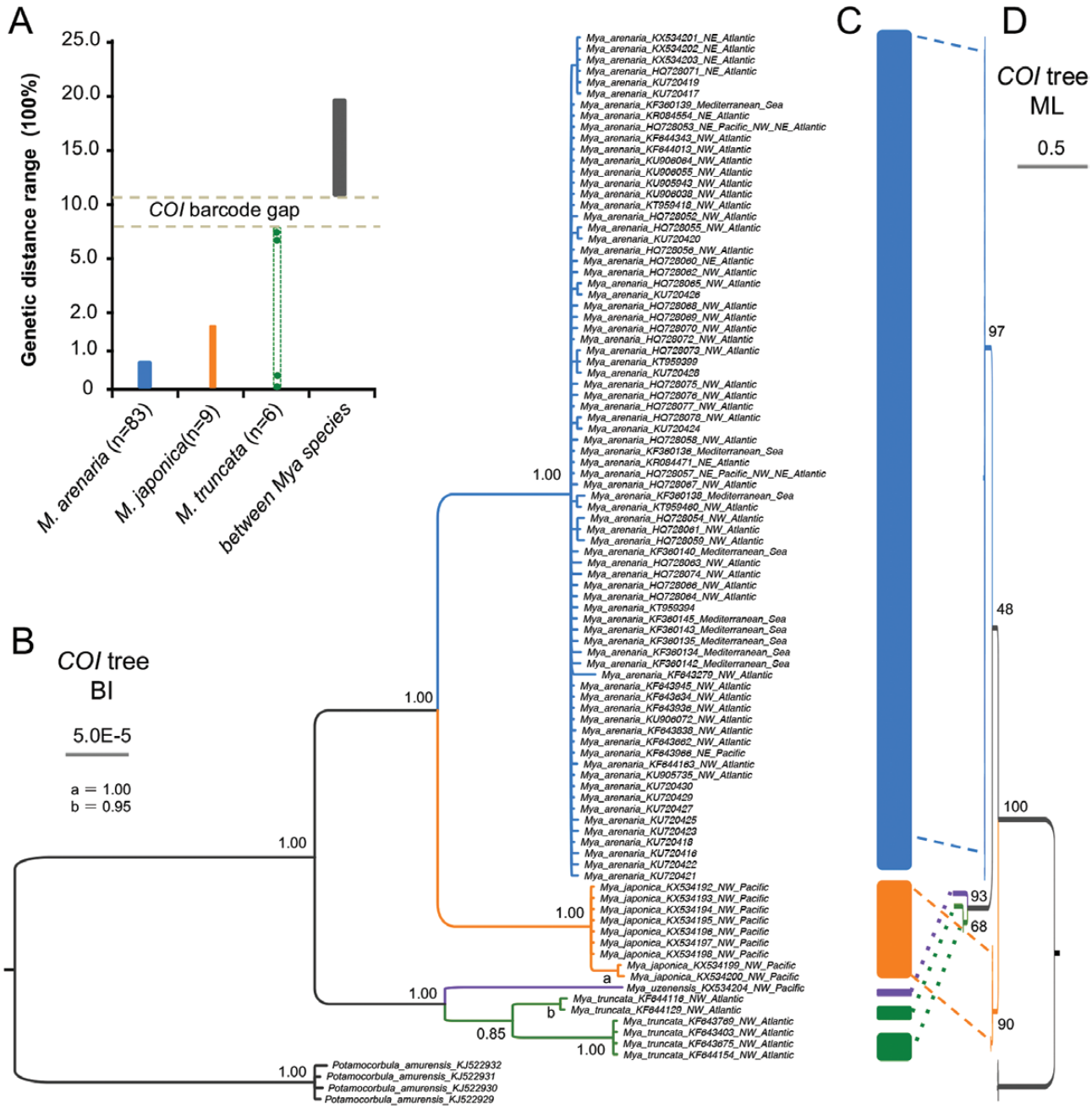


Figure 5. Genetic distance range (A), phylogenetic trees (B, D) and candidate species inferred from Automatic Barcode Gap Discovery (ABGD) (C). A, the *COI* intraspecific distance values with *M. truncata* are 0, 0.33, 6.93 and 7.26% marked with green spots. B, phylogenetic tree inferred by Bayesian analysis (BI). C, ABGD, based on the *COI* alignment, with both models of Jukes-Cantor (JC69) and Kimura (K80) TS/TV. D, phylogenetic tree inferred by maximum likelihood (ML).

(Figs 5B, 6). In the *COI* BI tree, *M. japonica* and *M. arenaria* QWEAS092-15 + QWEAS093-15 + DFO097-11 + RBCMI219-14 branched early within *Mya* with high node support (ML 95%), while the remaining *M. arenaria* clustered with the clade *M. truncata* + *M. uzenensis* with low support (ML 25%, Fig. 5D). In all *COI* trees, *M. truncata* were separated into two subclades, KF644116 + KF644129 and KF644154 + KF643769 + KF643675 + KF643403 (BI 0.86, ML 88).

Divergence time estimates

Assuming r_1 = 0.35%, *M. japonica* would have split from *M. arenaria* ~9.4 Myr [95% highest posterior

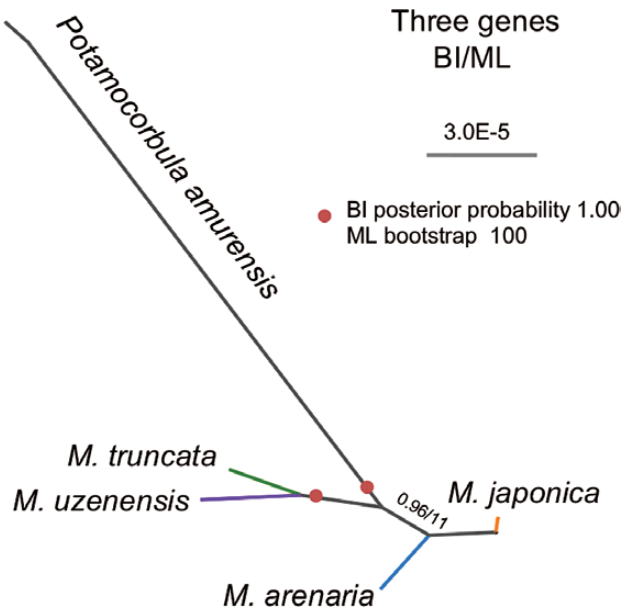


Figure 6. Bayesian (BI) and maximum likelihood (ML) consensus tree inferred from concatenated sequences of *COI*, 16S and 28S genes.

density (HPD) bounds: 6.7–12.5 Myr]. *Mya uzenensis* and *M. truncata* separated 8.1 Myr (95% HPD bounds: 4.0–11.9 Myr; Fig. 7). Time (*T*) to the most recent common ancestor for the *Mya* would be 9.9–20.0 Myr. Alternatively, assuming a faster substitution rate (r_2 = 0.6% per lineage per Myr), *M. japonica* would have split from its congener, *M. arenaria*, ~5.7 Myr (95% HPD bounds: 4.1–7.5 Myr). *Mya uzenensis* and *M. truncata* separated 4.7 Mya (95% HPD bounds: 2.4–6.8 Myr). *T* for *Mya* would be 5.8–11.4 Myr (Fig. 7).

DISCUSSION

MYA JAPONICA IS A VALID SPECIES

Although the morphological differences between the clams identified as *M. arenaria* and *M. japonica* in the present study were minor, the ABGD delimitation, reciprocal monophyly, genetic divergence and spermatozoan ultramorphology of soft-shell clams from the western Pacific strongly support the recognition of *M. japonica* as a valid species. Within *Mya*, we found two clades, one comprising *M. arenaria* and *M. japonica* and the other containing both other *Mya* species examined. While *M. arenaria* and *M. japonica* were most related to each other, there was deep divergence between *M. arenaria* from the Northeast Pacific, Atlantic, Mediterranean and Barents Sea (Arctic) and *M. japonica* from the Pacific, which was further supported by ABGD. Furthermore, we found significant differences in spermatozoan ultramorphology between *M. arenaria* (Barents Sea) and *M. japonica* (Sea of Japan and Yellow Sea). The spermatozoan differences and genetic divergence reported in this study suggest long-term reproductive isolation between these two morphologically similar species.

The fine morphology of spermatozoa from *M. japonica* examined in this study was very similar to *M. japonica* from Peter the Great Bay, Sea of Japan (Drozdov *et al.*, 2009) and '*M. arenaria oonogai*' from the coastal Korea Strait of Samcheonpo, Korea (Kim *et al.*, 2011), which should all represent *M. japonica s.s.* Similarly, previous junior synonyms of *M. arenaria* described from the western Pacific, such as *M. oonogai* and *M. arenaria oonogai*, probably represent *M. japonica*.

SHELL MORPHOLOGICAL EXAMINATIONS AND INTRASPECIFIC VARIATION OF *M. JAPONICA*

Although individuals identified as *M. arenaria* and *M. japonica* were similar in appearance, there were recognizable differences in general shell shape, shell texture and appearance of the pallial line. Although the shape, size and orientation of the left valve chondrophore are often important diagnostic characters among *Mya* and related bivalves (Bernard, 1979), we found considerable variation in the characteristics of the chondrophore among all the material examined and it was not useful in discriminating between species. Given the comparative nature of the characteristics reported here (e.g. less/more impressed, elongated/shorter etc.) and overall degree of intraspecific variation, it may be difficult to identify individual clams using the descriptions contained herein, particularly for those who have not had the benefit of studying many specimens. Thus, we strongly recommend molecular or other analyses to confidently separate *M. arenaria* and *M. japonica*.

Makiyama (1934, 1935) reported two forms of soft-shell clam in Japan, a northern form described as *M. japonica* and the southern form described as *M. oonogai*. The latter was referred to as *M. japonica oonogai* by Habe (1955) and Fujie (1957, 1962) and *M. arenaria oonogai* by Kwon *et al.* (2001), Kwon, Park & Lee (1993) and Yoo (1967). Fujie (1957) not only agreed that northern forms were *M. japonica* but also described a forma 'α', which was shorter and more ovate. Nagao & Inoue (1941) described differences between northern and southern Japanese forms and noted many intermediate specimens. The northern group, which possessed a thick, relatively short and nearly equilateral shell that was somewhat well rounded or slightly truncated posteriorly, was considered *M. arenaria* (due to synonymy with *M. japonica*). The southern group, which was characterized by its generally thin, long and somewhat inequilateral shell with a narrowly rounded

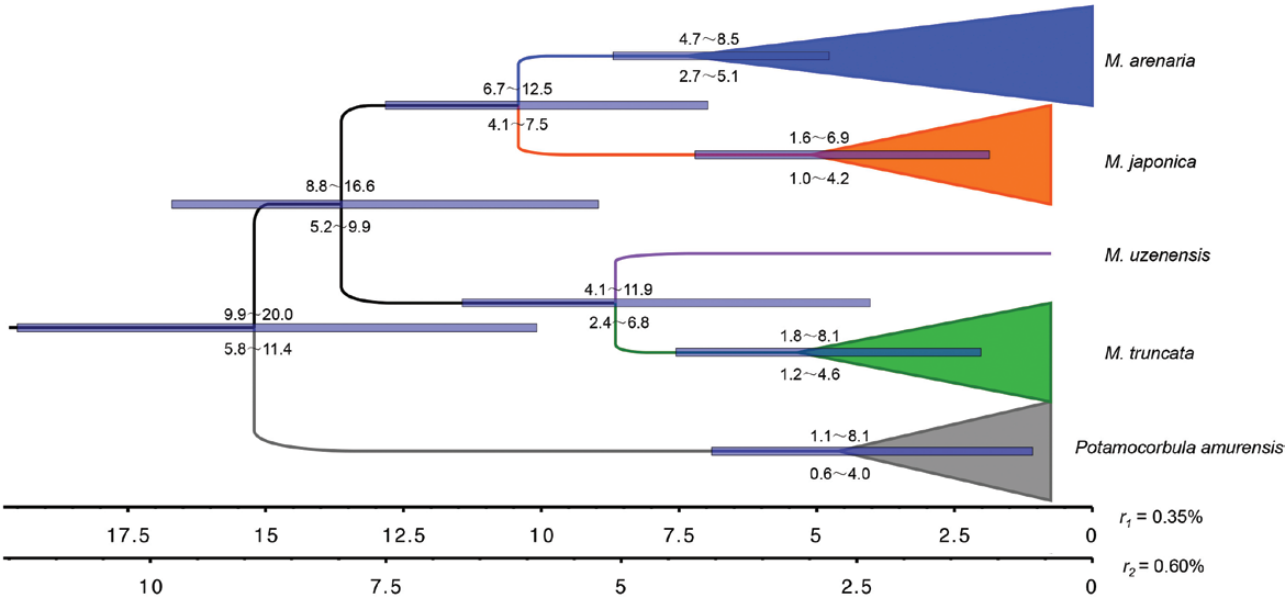


Figure 7. Phylogenetic relationships among bivalve molluscs in *Mya* with estimates of divergence times, based on *COI* sequences. Bars in the tree correspond to 95% credibility intervals. Values above the bars were estimated by substitution rate r_1 = 0.35% and below by substitution rate r_2 = 0.6%. Scar bars on the bottom indicate time before present (Myr) derived from the two substitution rates (r_1 and r_2).

or frequently acuminated posterior extremity, was considered an unnamed species. Nagao & Inoue (1941) also reported that the chondrophore in the northern group was nearly perpendicular to the antero-posterior diameter of the shell, and was narrower and more deeply excavated, with its inner border less convex than in the southern group. The posterior ridge was also not well differentiated from the chondrophore.

Similarly, Scarlato (1981) noted two morphological forms of *M. japonica* in Russian Far Eastern seas. One form is distributed in low-boreal (temperate) waters (Aniva Bay in southern Sakhalin, southern Kurile Islands and Peter the Great Bay), where this species reaches its maximum size and possesses the typical shape with a smooth surface and fine, regular growth lines. The other form was found in the northern part of its distributional range (Bering and Chukotsk seas, eastern Kamchatka and northern Sea of Okhotsk), where *M. japonica* never reaches its maximum length and often has an atypical shape, sometimes appearing misshapen or deformed. However, our examination of *M. japonica* from Peter the Great Bay indicates that these variations also occur in the same geographical locality and are related to different environments (Fig. 2) along with intermediate specimens. The co-occurrence of different morphological forms and intermediate specimens in a single geographical region strongly suggests that these forms are ecophenotypes. It should be noted that the 'typical' thin and regular form reported here fully complies with '*M. japonica oonogai*' collected from Hokkaido and Nagasaki, Japan, and our 'thick-shelled' form is very similar to *M. japonica* forma 'α', as illustrated by Fujie (1957). In addition, the *M. japonica* of Fujie (1957) is intermediate between the two forms we reported here. The results of our phylogenetic and morphological analyses indicate that *M. japonica* is a single species, which displays a high degree of phenotypic plasticity in shell morphology, and many previously reported forms/species combinations probably reflect ecophenotypic variation or represent natural intraspecific variation.

Distribution of *M. arenaria* and *M. japonica*

The soft-shell clam *M. arenaria* is widely accepted to have a circumboreal distribution (Bernard, 1979, 1983; Strasser, 1999; Coan *et al.*, 2000; Huber, 2010; Zhang *et al.*, 2012). Previous molecular studies indicated a complex colonization history between Europe and North America, but these analyses lacked samples from the northwest Pacific – the putative range of *M. japonica* (Strasser & Barber, 2009; Layton *et al.*, 2014; Barco *et al.*, 2016; Cross *et al.*, 2016; Lasota *et al.*, 2016). Our genetic and spermatozoan ultramorphological analyses strongly suggest that soft-shell clams in the northwest Pacific represent *M. japonica*. Two publicly available sequences identified as '*M. arenaria*' (the valid species name at the time of identification) from China (QWEAS092-15 + QWEAS093-15) were highly similar (< 2% difference *COI*; Supporting

Information, Table S2) to western Pacific *M. japonica*. These individuals are probably *M. japonica* and do not reflect the presence of *M. arenaria* in the western Pacific. MacNeil (1965) reported that *M. arenaria* occurred in Japan only in the middle Miocene and that later and recent specimens from the Okhotsk Sea, west coast of Japan and China are *M. japonica*, which was supported by Lutaenko & Noseworthy (2012) and Scarlato (1981). Numerous other authors have also reported *M. japonica* from China, Japan, Korea and the Russian Far Eastern seas (Jay, 1856; Makiyama, 1935; Habe, 1955, 1977; Tchang, Qi & Li, 1955; Fujie, 1957; Scarlato, 1981; Okutani, 2000). While both *M. arenaria* and *M. japonica* were present along western North America at one time, there is no palaeontological or archaeological evidence that either species survived the Pleistocene glaciation, with the possible exception of relict populations in the Bering Sea (Carlton, 1979). *Mya arenaria* was first introduced into the northeastern Pacific via Eastern Oyster *Crassostrea virginica* Gmelin, 1791 seed plantings in San Francisco Bay, California, during the 1860–1870s (Carlton, 1979). Since that time, it has spread northward, by either intentional plantings or natural dispersal, to southern Alaska (Carlton, 1979; Strasser, 1999; Powers *et al.*, 2006). In contrast, until the present study, there were no recent eastern Pacific records of *M. japonica* or any evidence of intentional or accidental introductions of western Pacific *Mya* into the eastern Pacific. Two publicly available sequences identified as '*M. arenaria*' from the British Columbia, one collected from Quascilla Bay (DFO097-11) and the other from Graham Island (RBCMI219-14), were highly similar (< 2% difference *COI*; Supporting Information, Table S2) to all western Pacific *M. japonica*. These individuals, both collected in June 2011, are probably *M. japonica* and went unnoticed because of morphological similarities to *M. arenaria*, the expected species in British Columbia. Thus, these records represent the first modern documented occurrence of *M. japonica* in the northeast Pacific. In addition, after examining photographs of these two clams, the authors are confident in classifying them as *M. japonica* based on the morphological criteria used in this study.

We are uncertain of the source of introduction or status of possible eastern Pacific *M. japonica* populations. However, similar to *M. arenaria*, *M. japonica* was probably introduced via oyster plantings, but in this case, the Pacific Oyster *Crassostrea gigas* Thunberg, 1793. These plantings, which occurred from the early 1910s until after the Second World War (Carlton, 1979; Lavoie, 2005), are also thought to have led to other bivalve introductions, such as the Japanese Littleneck *Ruditapes philippinarum* Adams & Reeve, 1850 and Quadrate Trapezium *Neotrapezium liratum* Reeve, 1843. Alternatively, *M. japonica* may have been introduced by ballast water, as is speculated with the Asian Semele *Theora lubrica* Gould, 1861 and Asian brackish-water clam *P. amurensis* in California (Fofonoff *et al.*, 2003). In either case, *M. japonica* was probably introduced decades after *M. arenaria* became widespread and well established in the eastern Pacific (Carlton, 1979), which reduced the likelihood of detection of *M. japonica* in areas where *M. arenaria* was already present or in nearby localities. Furthermore, both individuals were larger-sized adults collected nearly 400 km apart, which suggests there may be viable populations of *M. japonica* in at least British Columbia or possibly other Pacific Northeast locations. Albeit unlikely, the presence of individuals with high *COI* similarly to *M. japonica* could represent F1 hybrids with maternal *M. japonica* contribution or possibly reflect historical or cryptic hybridization (Pfenninger, Reinhardt & Streit, 2002; Rees, Dioli & Kirkendall, 2003) between *M. arenaria* and *M. japonica*. While there were significant differences in sperm morphology between *M. arenaria* and *M. japonica*, we are uncertain if these differences (or ecological/behavioural differences) would prohibit hybridization.

According to Laursen (1966), historical reports of *M. arenaria* from the Arctic Ocean are erroneous and should represent *M. truncata ovata* Jensen, 1900, which is currently recognized as a junior synonym of *M. pseudoarenaria* (see Huber, 2010). However, we identified *M. arenaria* from the Russian waters of the Barents Sea, a marginal sea of the Arctic Ocean, and it has also been reported further west near Forsøl, Norway (Crocetta & Turolla, 2011). A rigorous genetic analysis (incorporating both nuclear and mitochondrial markers) of *M. arenaria* and *M. japonica* collected from across the eastern and western Pacific, Bering Sea, Arctic Ocean and greater Atlantic is required to fully resolve the zoogeography of both species, determine the extent of *M. japonica* introductions in the eastern Pacific, identify any possible cryptic introductions of *M. arenaria* in the western Pacific, determine the identity of soft-shell clams in the Bering Sea and examine possible hybridization of *M. arenaria* and *M. japonica*.

Divergence time and evolutionary/migration history

We estimate that *M. japonica* diverged from *M. arenaria* 4.1–12.5 Myr; this agrees with the evolution of *M. arenaria*, which evolved during early Pliocene to late Miocene from its ancestor *Mya fujiei* MacNeil, 1965, which arose during middle Miocene (MacNeil, 1965; Strauch, 1972). *Mya arenaria* first appeared in the fossil record from Japan in late Miocene formations and almost at the same time from the eastern Pacific in California (Strauch, 1972). *Mya japonica* was also probably present in the eastern Pacific at least during the

Pleistocene (MacNeil, 1965; Carlton, 1979). Although the formation of the Bering Strait during the Pliocene overlapped with the divergence of *M. arenaria* and *M. japonica*, it is likely that only *M. arenaria* crossed the Arctic to reach the western and eastern Atlantic (MacNeil, 1965; Bernard, 1979; Vermeij, 1989). Strauch (1972) suggested *M. arenaria* may also have migrated through the Central American Passage from the Pacific to the Atlantic, where it then spread to Europe in the late Pliocene (Strasser, 1999), but fossil evidence for this migratory route is lacking (Bernard, 1979). During the Pleistocene glaciation, *M. arenaria* was extirpated from all areas except the western Atlantic and *M. japonica* was extirpated from all areas except the western Pacific (Strasser, 1999). However, the status of fossil, archaeological and recent soft-shell clams in the Bering Sea requires further investigation, which could represent relict populations of *M. arenaria* or *M. japonica*, adventitious individuals of *M. japonica* from the western Pacific or an undescribed cryptic species (MacNeil, 1965; Bernard, 1979; Carlton, 1979). The evolutionary history of *Mya* is complex and there is much uncertainty regarding the identification and evolutionary relationships of fossil material (MacNeil, 1965) and their connection with extant forms, particularly as it relates to the synonymy of extant species and species identified solely from fossil material (Petersen, 1999). Due to the morphological similarities between *M. arenaria* and *M. japonica* and levels of phenotypic plasticity, it may be difficult to fully resolve their evolutionary and migration history.

MacNeil (1965) and Strauch (1972) reported that the oldest records of *M. truncata* and *Mya cuneiformis* Böhm 1915 were from the middle Miocene, both of which were direct descendants of the late Oligocene or early Miocene species, *Mya salmonensis* Clark, 1932, while *M. pseudoarenaria* and *Mya priapus* Tilesius, 1822 derived from *M. cuneiformis* in the late Miocene. Nakashima (1999) determined that the oldest records of *M. pseudoarenaria*, *M. cuneiformis* and *M. truncata* were from the early Miocene and *M. uzenensis* may have directly evolved from *M. salmonensis*. Our results indicate that the separation time of *M. uzenensis* and *M. truncata* (3.9–21.0 Myr) is nearly the same as *M. japonica* and *M. arenaria* and that *M. uzenensis* and *M. truncata* share a common ancestor, but it is unclear if *M. uzenensis* directly evolved from *M. salmonensis* or *M. cuneiformis*.

MYA TRUNCATA MAY REFLECT A SPECIES COMPLEX

The ABGD analysis indicated that *M. truncata* was a candidate species complex (Fig. 5C), which included two distinct groups (ABGD Groups 3 and 4; Table 3). The *COI* genetic distances between individuals of these groups ranged from 6.93 to 7.26% (Table 3). While these are intermediate values between the maximum intraspecific *p*-distance (1.70%) within *M. japonica* + *M. arenaria* and the minimum interspecific *p*-distance (10.68%) among the other *Mya* species, this may reflect a true species-level difference, which is consistent with Petersen (1999) and Layton *et al.* (2014). At present, we are unable to fully resolve the taxonomic status of *M. truncata*. All the *M. truncata COI* sequences examined in this study were obtained from individuals collected in Nunavut, Canada (Layton *et al.*, 2014). However, individuals in ABGD Group 3 were collected near Cornwallis Island, whereas individuals in ABGD Group 4 were collected around Igloolik Island, indicating possible spatial structure to the putative species complex. Specimens collected from the entire range of *M. truncata* (eastern Pacific, Arctic and northern Atlantic) and additional data (e.g. spermatozoan ultramorphology) are needed to clarify the taxonomic status of *M. truncata*.

CONCLUSIONS

Our results indicate that (1) *M. japonica* is a valid species, distributed in the northern Pacific, with introduced populations of unknown size and distribution in the northeast Pacific; (2) *M. arenaria* is distributed in the northeast Pacific, northern Atlantic, Mediterranean and Barents Sea (Arctic); (3) there were significant species-level differences in the spermatozoan ultramorphology of *M. arenaria* and *M. japonica*; (4) differences in shell morphology were minor, and given the levels of intraspecific variation noted among varying habitats, shell morphology alone may not be an effective method to differentiate *M. arenaria* and *M. japonica*; (5) the congeners, *M. arenaria*, *M. japonica*, *M. uzenensis* and *M. truncata* were all differentiated by DNA barcoding, with evidence of cryptic diversity in *M. truncata* from the Canadian Arctic; and (6) the timing of divergence between *M. arenaria* and *M. japonica* supports the evolutionary hypothesis that *M. arenaria* evolved during early Pliocene to late Miocene. Further collections of *Mya* species from across their entire range (especially Asia and Arctic Ocean/Bering Sea where collections have been scarce) and a rigorous analysis incorporating an integrative taxonomic approach using ecological, molecular, morphological and/or other biological data are required to fully resolve taxonomic relationships and distributions of these highly morpho-variable species.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article at the publisher's web-site:

Table S1. List of all specimens used for molecular analyses.
Table S2. Interspecific and intraspecific uncorrected pairwise distances at *COI* among species of *Mya* and *Potamocorbula*.
Table S3. Interspecific and intraspecific uncorrected pairwise distances at 16S among species of *Mya* and *Potamocorbula*.