In the Sea of Japan and the Adriatic Sea, a COX1 DNA test revealed genetic similarity of Manila clams, although shell parameters, spermatogenesis patterns, and sperm characteristics are area specific

In the Sea of Japan and the Adriatic Sea, a COX1 DNA test revealed genetic similarity of Manila clams, although shell parameters, spermatogenesis patterns, and sperm characteristics are area specific

Arkadiy Reunov[1*, 2], Evegenia Vekhova[2], Liliana Milani[3], Giovanni Piccinini[3], Mariangela Iannello[3], Yana Alexandrova[2], Yulia Reunova[2], Evgeny Zakharov[4], Anna Akhmadieva[2,5], Eugenia Pimenova[2]

[1] St. Francis Xavier University, Department of Biology, Antigonish, NS B2G 2W5, Canada;

[2] A.V. Zhirmunsky National Scientific Center of Marine Biology (NSCMB), Russian Academy of Sciences, Vladivostok, 690041, Russia;

[3]Department of Biological, Geological and Environmental Sciences (BiGeA), University of Bologna (Italy);

[4]Canadian Centre for DNA Barcoding, Biodiversity Institute of Ontario, Guelph, Canada;

[5]Far Eastern Federal University, School of Natural Sciences, 10 Ajax Bay, Russky Island, Vladivostok 690950, Russia

***Corresponding author:**

Arkadiy Reunov (e-mail: areunov@stfx.ca)

Present address: St. Francis Xavier University, Department of Biology, Antigonish, NS B2G 2W5, Canada.

Abstract

The two Manila clam forms living in the Sea of Japan and in the Adriatic Sea were compared genetically and morphologically to identify traits that could be used to authenticate these geographic forms. COX1 DNA barcoding confirmed that both forms of these venerid bivalve belong to the species *Ruditapes philippinarum* (Adams and Reeve, 1850). It has also been shown that, based on COX1 analysis, it is not possible to separate these forms into different clusters based on geographic origin. However, some morphological features make it possible to differentiate these forms. It is noted that the contour of the shells of the Adriatic and Pacific molluscs is rounded and oblong, respectively. Underwater analysis of Pacific clams showed that shell color may be specific to specimens found at certain collection sites and differ between specimens taken from different collection sites. In the spermatogenesis of Adriatic and Pacific clams, a difference was found in the cellular mechanism of interaction between germplasm granules and mitochondria, manifested by distant contact and direct contact. Spermiogenesis has three similar lines of acrosome development, but differs in the number of ways in which the nucleus is formed. Due to the variability of the ways of sperm formation, heteromorphic morphs of spermatozoa are formed, the total number of which is six for the species *R. philippinarum*. In the Adriatic Manila clam, five morphs from this set are expressed. The Pacific Manila clam has only three sperm morphs. Only two sperm morphs are similar in both forms. In addition, each form is distinguished by the presence of unique sperm morphs and each form has its own type of dominant spermatozoon. Thus, the contour and color of the shells, as well as cytologic markers that have been found in the mechanisms of differentiation of meiotic cells and the characteristics of spermatozoa, can be used to distinguish the Adriatic and Pacific forms of the bivalve mollusc *R. philippinarum*.

Keywords Manila clam, *Ruditapes philippinarum*, Adriatic Sea, Japan Sea, DNA barcoding, COX1, shells, spermatogenesis, sperm, geo-authentication.

Introduction

The Manila clam *Ruditapes philippinarum* (Adams and Reeve, 1850) is one of the most economically important marine bivalves in the world, and currently accounts for 20-25% of the world mollusc fisheries (Chiesa et al. 2016; Cordero et al. 2017). The history of this species is that of a biological invasion, and it is worth emphasizing that it is one of the most successful marine invaders ever known (Chiesa et al. 2016).

Historically, a Manila clam was at first collected on the island of Mindanao in the Philippines during the voyage of HMS Samarang, a 28-gun frigate of the British Royal Navy, which took place in 1843-1846 under the command of Captain Sir Edward Belcher. The biological collections were done by the ship surgeon Arthur Adams. Together with many other organisms, the new clam species was described in the scientific report of the expedition. At present, it is known that the Manila clam is distributed from the Zhuanghe River in Liaoning Province to the southern part of the Leizhou Peninsula in Guangdong Province in China, as well as along the coasts of the southern Sea of Okhotsk (Russia), Sakhalin (Russia), the Kuril Islands (Russia), Japan, Korean peninsula, Philippines, Pakistan, India, Sri Lanka, and Indonesia (Ponurovsky and Selin 1988; Low et al. 2020).

In the 1930s, this species was accidentally introduced to the Pacific coast of North America in shipments of the Pacific oysters *Crassostrea gigas* (Thunberg, 1793) imported from Japan. It subsequently spread rapidly along this coast, including north to British Columbia in Canada (Nosho and Chew 1972; Bourne 1982). Considering the positive economic impact of *R. philippinarum*, its next worldwide distribution was based mainly on deliberate introductions. It was introduced to Norway, Great Britain, Romania, France, Spain, and Italy (Mortensen 1993; Morgan 2002).

The genetic structure of *R. philippinarum* is of interest to geneticists. Population-genetic data have been obtained and individual populations of this species in North America and Europe have been described (Chiesa et al. 2016). By studying mitochondrial DNA and microsatellite markers in nine populations from Asia, North America and Europe, the current genetic structure has been shown to be in good agreement with the described history of cross-continental invasion of the clam, which did not lead to a deep genetic divergence between the invasive "North American/European" Manila clams and the original "Japanese" Manila clams (Cordero et al.

2017). Despite the fact that a number of factors, as well as local hybridization, can cause intraspecific genetic divergence in bivalves (Skurikhina et al. 2001; Ni et al. 2015; Chetoui et al. 2016), this is not the case for *R. philippinarum*. According to electrophoretic studies, natural hybridization of the Western Pacific *R. philippinarum* with the European native *R. decussatus* (Linnaeus, 1758) is impossible due to the degree of genetic dissimilarity (Fava and Meggiato 1995). Also, experimental work on obtaining hybrids by mixing mature gametes of *R. philippinarum* and *R. decussatus* showed the impossibility of successful hybridization (Markaide et al. 2021).

Considering the genetic similarity between the invasive ("North American/European") and the original ("Japanese") *R. philippinarum*, the question arises whether it is possible to distinguish the geographical forms of this species. This situation is becoming increasingly important at the front of today commercial challenges. For example, in the lagoons of the Adriatic Sea in Northern Italy, Manila clam farming provides important socio-economic benefits, and local clams must be registered in the Protected Designation of Origin system. Therefore, it is necessary to develop tests for tracking the commercial circulation of shellfish in order to ensure the identification of the origin of the product with the possible prevention of fraud (Bianchini 2021). In this regard, a comprehensive comparative study of the Adriatic Manila clam in relation to the original form of this species is necessary in order to find reliable criteria for identifying specimens.

When looking for criteria, it should be taken into account that the genetic analysis of *R. philippinarum* is incomplete, since not all original populations of this species have been genetically tested. For example, the wild Manila clam living in the Russian part of the Sea of Japan has never been studied. It seems appropriate to study this Pacific form in order to compare it with the Adriatic form.

It is not entirely clear whether shell color can be useful in identifying the geographic forms of the Manila clam. The shells of this species usually vary a lot in color: white, cream, yellow or light brown, sometimes with rays, streaks, blotches or zigzags of a darker brown/black, often with curved radiating darker bands or dark blotches (Yan et al. 2019; Ding et al. 2021). However, despite the diversity of coloration, there is probably a chance to use shell coloration to distinguish between geographic forms. Indeed, despite the wide variety of shell colors of the mactrid mollusc *Mactra chinensis* (Philippi, 1846), it has been shown that shell shade

corresponds to the shade of the seabed, and this phenomenon may help to establish the geographical origin of this mollusc (Reunov et al. 2021a). Thus, it is worth checking whether a similar phenomenon can be found in the Manila clam.

Recently, as a result of detailed re-examination of sperm, heteromorphism or plasticity of spermatozoa has been discovered as the normal physiological state of marine bivalves such as *C. gigas* and *M. chinensis*. Moreover, it was found that because the quantitative characteristics of heteromorphic sets of spermatozoa correlate with local environmental conditions, this characteristic can serve as a geographical marker (Reunov et al. 2018, 2021a). It is interesting to find out whether analysis of sperm morphs can help in establishing the geographical origin of the Manila clam. In addition, the ultrastructural features of the spermatogenesis of the Adriatic *R. philippinarum* have recently been studied (Reunov et al. 2019a, 2021b), and it would be relevant to study the spermatogenesis of the Pacific *R. philippinarum* to find out if there are any differences suitable for determining geographic forms.

The aim of this work was to: (1) compare the COX1 sequence between the Adriatic Manila clam and the wild Pacific Manila clam living in the Russian part of the Sea of Japan, and to compare the obtained data with already existing data for other populations of this species; (2) check by analysis directly on the seabed using scuba diving whether the shell color of Manila clams collected in different areas can be geographically specific; (3) find out if any differences in the development of spermatogenic cells and the structure of spermatozoa can be detected and used to distinguish between Atlantic and Pacific geographic forms. We hope that our study will help to find new criteria for geo-authentication of specimens of the bivalve mollusc *R. philippinarum*.

Materials and methods

Sample collection

Adriatic Manila clam

Specimens were obtained from the fish market in Bologna (Italy) in May-July 2019. According to the supplier, the specimens were collected from Sacca di Goro (Adriatic Sea, FE,

Italy). Geographic coordinate of the location cite is 44°49'N, 12°18'E. However, the origin of the specimens is not exactly known, as underwater research has not been carried out.

Pacific Manila clam

Specimens were collected in Peter the Great Bay (Sea of Japan, Russia) by scuba diving during May-July 2019 and May-July 2020 from three geographic locations (Fig. 1). At each point, shellfish were collected on an area of about 10 square meters. The first collection site (point # 1) was in Vostok Bay (coordinates: 42°54'13.1"N 132°43'35.7"E). The second and third locations (point # 2 and point # 3) were in the Amursky Bay (coordinates: 43°12'03.3"N 131°55'13.2"E and 43°11'53.6"N 131°55'09.7"E, respectively). Coordinates were identified using manual profiler Cast Away ctd. (SonTek, USA). To analyze the color of the shells, 30 individuals were collected at each collection point.

COX1 barcoding and phylogenetic analyses

Five specimens (Rph m1 cox1F, Rph m2 cox1F, Rph m3 cox1F, Rph m4 cox1F, Rph m5 cox1F) and eight specimens (CCDB-ST03131, CCDB-ST03132, CCDB-ST03133, CCDB-ST03136, CCDB-ST03137, CCDB-ST03138, CCDB-ST03140, CCDB-ST03141) were analysed for the Adriatic Manila clam and Pacific Manila clam, correspondingly. Extraction of DNA from the samples was performed with spin-column extraction kit (DongSheng Biotech) following producer instructions. For PCR amplification (with GoTaq Flexi2 kit, Promega), we used COX1 primer pairs designed on online available sequences to amplify the F-type mitochondrial gene (Left Primer: TTTATGGGGTTGGTGTTAAAAA; Tm: 58.3 °C; GC: 31.8 %; Right Primer: TAGTTAAACCCCCTGCCAAA, bp Tm: 59.4 °C; GC: 45.0 %; Product Size: 732 bp). The PCR cycle consisted in an initial denaturation step at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 73 °C for 90 s. At the end of the cycling steps, a final extension at 73 °C for 5 min was done. Both strands of amplicons were sequenced at Macrogen Europe (The Netherlands).

To the COX1 partial sequences of the present study, we added all available *R. philippinarum* COX1 sequences from the previous works of Cordero et al. (2017) and of Sekine et al. (2006).

Since we were interested in population structures only, we considered only F-type sequences and not M-type ones. Sequences were aligned with MAFFT (default parameters; Katoh and Standley 2013) and the alignment was manually trimmed to keep only positions shared by all samples, with a final length of 555 base pairs. A maximum likelihood tree was inferred from it with IQ-TREE (MG+F1X4+G4 codon model, inferred as best model of evolution by ModelFinder as implemented on IQ-TREE; 1000 bootstrap replicates were performed to retrieve node supports; Minh et al. 2020). A phylogenetic network was built on the same alignment with PopART (Leigh and Bryant 2015) using the median-joining method (default parameters).

Shell coloration comparison

For the Adriatic Manila clam, as well as for the Pacific Manila clam, the shells of genetically identified specimens were dried and photographed. The images were compared.

Transmission electron microscopy

Some of the genetically identified specimens were selected for study. Three specimens of the Adriatic Manila clam and three specimens of the Pacific Manila clam were analyzed. For the Pacific Manila clam, specimens collected only at point # 1 were used. The testes were removed, cut into small pieces and fixed overnight in primary fixative containing 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4) at 4 °C. Fixed tissues were washed in buffer, postfixed in 2% OsO_4 in 0.1 M cacodylate buffer for 2 h, rinsed in 0.1 M cacodylate buffer and distilled water, dehydrated in an ethanol series and acetone, infiltrated and embedded in Spurr resin. Ultra-thin sections were mounted on slot grids that were coated with formvar film. Sections were stained with 2% alcoholic uranyl acetate and aqueous lead citrate and were examined with a transmission electron microscope Zeiss Libra 120 (A Carl Zeiss SMT AG Company, Oberkochen, Germany) and Philips 410 Transmission Electron Microscope (Philips 123 Electronics, Eindhoven, The Netherlands).

Scanning electron microscopy

Some of the genetically identified specimens were selected for study. Three specimens of the Adriatic Manila clam and three specimens of the Pacific Manila clam were analyzed. For the Pacific Manila clam, specimens collected only at point # 1 were used. The testes were removed, cut into small pieces, and fixed for 2–3 h (in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4). Primary fixed materials were washed gradually in the same buffer. Washed samples were rinsed in buffer and distilled water, dehydrated in a graded series of ethanol solutions. Sperm suspension was prepared by crushing pieces of fixed materials. The suspension was pipetted onto a Thermanox coverslip (Cat. # 72280) and allowed to settle for 1 h. Coverslips with attached sperm cells were transferred to acetone and critical-point-dried in CO_2. Dried materials were mounted onto aluminum stubs, coated with gold, and examined with a scanning electron microscope LEO-430 (Horus Tech Inc., USA).

Quantitative analysis of sperm morphs studied by scanning electron microscopy

500 sperm cells of each clam specimen were analyzed. 1500 sperm cells were analysed for the Adriatic Manila clam and 1500 sperm cells were analysed for the Pacific Manila clams. Sperm phenotypes were identified and frequency of each phenotype was calculated. The results were analysed by the Microsoft Excel program using Student's t-test. All values are expressed as means with standard error of the mean (SEM). Differences between groups were calculated using Student's t-test. $p < 0.05$ was considered statistically significant.

Results

Partial COX1 population structure

Phylogenetic analysis carried out for five specimens of the Adriatic Manila clam and eight specimens of the Pacific Manila clam confirmed that all specimens belonged to the species *R. philippinarum*. Both the maximum-likelihood phylogenetic tree (Fig. 2) and the median-joining phylogenetic network of the partial F-type COX1 sequences (Fig. 3) agreed in clustering our sample sequences among specimens belonging to the mitochondrial haplogroup A (referring to the nomenclature adopted by Cordero et al. 2017). Within this haplogroup, our samples from the

Sea of Japan and from the Adriatic Sea could not be clustered separately. Indeed, we did not observe any haplotype of the network nor any clade of the tree that comprehended samples belonging exclusively to a specific geographical origin.

Adriatic Manila clam shells

These were (1) a shell, the color of which is represented by brownish squares on a yellow background (Fig. 4a), (2) a shell with a gray background, on which there are several radial purple stripes, and there is also a slight yellowish tint on the side (Fig. 4b), (3) a shell with many purple zigzags on a gray background (Fig. 4c), (4) a shell with a slightly yellowish background without stripes (Fig. 4d) and (5) a shell with a yellowish background, on which there are radial brownish stripes (Fig. 4e). The shell contour is relatively round, and the top of the shell (umbo) is more centered (Fig. 4a-e) than in the shells of Pacific Manila clams (Fig. 4f-m).

Pacific Manila clam shells

Three shell colors have been found among the genetically identified specimens. These were: (1) gray shells (Fig. 4f-h), (2) ocher-colored shells (Fig. 4i-k) and cream-colored shells (Fig. 4l, m). Each of the three collection sites (Fig. 1) corresponded to one of the three revealed colors. Apart from the genetically determined specimens, the same shell color was found in each of the thirty specimens collected at each collection point. Among the 90 specimens, shell colors did not overlap between collection sites and were geographically specific.

The shells of the Pacific Manila clams are more oblong and have a more laterally positioned umbo (Fig. 4f-m) compared to the relatively rounded shells with the umbo in a more central position, which is characteristic of the Adriatic Manila clams (Fig. 4a-e).

Early spermatogenic cells

In the testes of the Manila clam collected both in the Sea of Japan and the Adriatic Sea, early spermatogenic cells (spermatogonia and spermatocytes), identified according to the previously described features (Reunov et al. 2019a), are tightly packed in groups (Fig. 5a). In the testes of

both clam forms, spermatogonia and spermatocytes are similar in that they have the so-called germplasm granules (GG), which are located in the cytoplasm. In the spermatogonia of the Manila clam of the Sea of Japan, GG are irregularly shaped groups of granules (Fig. 5b). These granules tend to be in contact with mitochondria (Fig. 5c, d). Due to the interaction of GG with mitochondria, mitochondrial clusters arise, which are characteristic of the close contact of GG with mitochondrial membranes (Fig. 5e). In the spermatogonia of the Adriatic Manila clams, GG are less common, have a larger size, and never come into direct contact with mitochondria. (Fig. 5f, g).

Early spermiogenesis

During early spermiogenesis of both forms of Manila clam, an acrosome vesicle produced by the Golgi complex (not shown) was found in the basal part of the cell near the flagellum and mitochondria (Fig. 6a). It is surrounded by a membrane and filled with an electron-dense material that features a more electron-light central part (Fig. 6b). Initially located in the middle part of the spermatid (Fig. 6a), the acrosomal vesicle then moves in the lateral part of the spermatid (Fig. 6c). Finally, it is located in the apical cellular region (Fig. 6d). In spermatids of both forms of Manila clam, during the next differentiation, the apically located acrosomal vesicle changes its shape from round to flat (Fig. 6e, f). Flat acrosomal vesicles show an invagination filled with loose periacrosomal material (Fig. 6g). In more advanced spermatids, the acrosome vesicle acquires a conical shape, and its invagination contains periacrosomal material that contacts the surface of the nucleus (Fig. 6h). The next stage of differentiation is characterised by elongation of the periacrosomal material body and upward ascent of the acrosomal vesicle (Fig. 6i).

Late spermiogenesis

During late spermiogenesis, both the Adriatic Manila clam and the Pacific Manila clam were found to have three patterns of acrosome formation. The first was distinguished by the formation of a straight acrosome. This is found with a gradual elongation of the apical part of the spermatid. This elongation occurs due to the expansion of the column of periacrosomal material

with the gradual ascent of the cone-shaped acrosomal vesicle (Fig. 7a-c). This pattern of spermiogenesis culminates in the formation of sperm with straight or slightly curved acrosomes, which are located along the anteroposterior axis of the spermatozoa (Fig. 7d). The second pattern is usually found with the acrosome having a strong bend during its elongation (Fig. 7e). The third is a pattern recorded as acrosome folding, that occurs at the onset of acrosome elongation (Fig. 7f, g). During the next formation, the acrosome gradually rises upward (Fig. 7h-j). As a result of this pattern, undulating acrosomes were recorded, which have a bend in the middle part (Fig. 7k).

In the spermiogenesis of the Manila clam, the presence of two nuclear shapes has been recorded. Both late spermatids and spermatozoa with a straight nucleus were found (Fig. 7a, b). Also, both late spermatids and spermatozoa with a curved nucleus were recorded (Fig. 7l, m). Typically, both nuclear shapes coexist in late spermiogenesis of the Adriatic Manila clam. In the spermatids of the Pacific Manila clam, only a curved shape of nucleus was found.

Structure of spermatozoa

Previous examination of ultrathin sections using a transmission electron microscope has shown that in samples of the Adriatic Manila clam, sperm cells have a bullet-shaped nucleus, the apical end of which is covered with an acrosome. The mitochondria are located under the basal (wide) end of the nucleus (midpiece). These mitochondria surround the distal centriole, which is the basal body of the flagellum that grows out of the sperm body. Thus, mitochondria are assembled into a ring of four organelles, and this number is fairly constant in mature sperm (Reunov et al. 2019a, 2021b). The ultrastructure of the spermatozoa of the Pacific Manila clam on ultrathin sections looks the same, no differences were found (not showed).

Using scanning electron microscopy, three sperm morphs unique to the Adriatic Manila clam were found. These were (1) spermatozoa with a straight nucleus and a straight acrosome (Fig. 8a); (2) spermatozoa with a straight nucleus and curved acrosome (Fig. 8b); (3) spermatozoa with a straight nucleus and wavy acrosome (Fig. 8c). Also, the Adriatic Manila clam have (4) spermatozoa with a curved nucleus and a slightly curved acrosome (Fig. 8d), and (5) spermatozoa with a curved nucleus and a strongly curved acrosome (Fig. 8e).

The Pacific Manila clam specimens also have spermatozoa with a curved nucleus and a slightly curved acrosome, as well as spermatozoa with a curved nucleus and a highly curved

acrosome (Fig. 8f, g), which morphologically correspond to the sperm models 4 and 5 found in the Adriatic Manila clam (Fig. 8d, e). Moreover, the Pacific Manila clam have (6) a sperm morph with a curved nucleus and undulating acrosome (Fig. 8h). Thus, the intraspecific set of spermatozoa of *R. philippinarum*, found on the basis of the analysis of the sperm of two geographic forms of this species, includes six morphs, which we designated here as SPERM1, SPERM2, SPERM3, SPERM4, SPERM5, and SPERM6 (Fig. 8i).

Quantitative analysis showed that the proportions of sperm samples differ. Adriatic Manila clams have different percentages of sperm morphs: 12%, 22%, 17%, 40% and 9% are accounted for by SPERM1, SPERM2, SPERM3, SPERM4 and SPERM5, respectively (Fig. 8j). In the Pacific Manila clams: 20%, 14%, 66% correspond to SPERM4, SPERM5 and SPERM6 (Fig. 8k).

Discussion

Manila clam from the Sea of Japan and the Adriatic Sea belong to the same haplogroup of COX1 genes and cannot be separated based on geographic origin

By analysing the COX1 partial DNA sequences of our specimens, we could confidently assess that they belonged to the species *R. philippinarum.* Moreover, we were also able to confirm the genetic similarity of such species in the Russian part of the Sea of Japan and in the Adriatic Sea. Indeed, all our samples belonged to haplogroup A (referring to the nomenclature adopted by Cordero et al. 2017). This haplogroup was characteristic of *R. philippinarum* inhabiting the Japanese part of the Sea of Japan, and from our data it now also includes samples from the Russian part of the Sea of Japan. The clustering of these samples together with those from the Adriatic Sea is coherent with the fact that the colonization of *R. philippinarum* in America, and subsequently in the Adriatic Sea, involved individuals belonging to haplogroup A (Cordero et al. 2017).

However, the haplogroup A is separated by haplogroups B and C, that are more spread in the Chinese coast (Cordero et al. 2017). Indeed, the indigenous population of *R. philippinarum* studied in East Asia is divided into three geographic populations. These wild populations have large genetic differences, which may be due to geographic isolation (Tan et al. 2020). Thus,

although COX1 testing is not useful to differentiate samples from the Adriatic Sea from those of the Sea of Japan, geo-authentication of samples from south areas of China is possible.

It should be noted that other molecular approaches work well for Manila clam geo-authentication. Using a dual-labelled PNA-probe-based melting curve analysis the foreign (Chinese) and domestic (Korean) Manila clams having similar morphology were discriminated (Kim et al. 2015). In addition, elemental analysis coupled with isotope ratio mass spectrometry has been used to identify isotopic fingerprints of *R. philippinarum* collected from three Adriatic lagoons, and this method appears promising for tracking the geographic origin of the Manila clam at a regional level (Bianchini et al. 2021).

The color of the shell may be promising for determining the origin of specimens

During this study, five color patterns were found in specimens of the Adriatic Manila clam. Three variants of shell coloration were found in the Pacific Manila clam. Thus, both forms of *R. philippinarum* are characterized by the phenomenon of intraspecific shell plasticity.

This conclusion is consistent with previous data on the Manila clam. It is known that the shells of this mollusc can have a variety of colors, which can be complemented by rays, stripes, spots and zigzags (Yan et al. 2019; Ding et al. 2021). This color diversity is provided by the differential expression of genes such as Tyr and Mitf (Yan et al. 2019), and miRNAs, which have been shown to play a role in the production and regulation of shell pigments (Ding et al. 2021). With regard to the role of environmental factors, it has been suggested that various biotic and abiotic factors can influence shell color in molluscs (Sokolova and Berger 2000; Williams 2017; Yuan et al. 2020). Certainly, the color of the substrate is very important in determining the color of the shells (Morton 1976; Moss 1990; Sigurdson and Sundari 1990; Reunov et al. 2021a).

For the Pacific Manila clam, we found that shell color is specific to each collection site. Divers who collected clams for this project reported that the type of substrate was different at the three collection sites, and the overall color tone of the seafloor was specific to each site. We hypothesize that shell colors correlate with seafloor hue and find it reasonable to suggest that shell color can be used as a geographically specific marker. A more detailed study aimed at identifying the correlation between the color of the shell and the seabed is planned by us for the Manila clam living in the Sea of Japan.

Also, we have not been able to verify whether the color of the shells of the Adriatic Manila clams is related to the specifics of the seabed, since the samples were taken at the fish market. In this regard, it will be useful to plan scuba diving explorations in various geographic habitats of the Manila clam in the Adriatic lagoons.

The shell shape can be useful for distinguishing the geographic forms of the Manila clam

Although our study did not include an analysis of shell morphology, we observed that the shell outline and position of the umbo were different in the Manila clams of the Adriatic and the Japan Seas. The oblong shape and lateral position of the umbo were more characteristic of the shells of the Pacific Manila clam, while the rounded shape with a more central location of the umbo was more characteristic of the Adriatic Manila clam. It is possible that the morphology of the shell can also help distinguish the geographical forms of this mollusc. Indeed, along the coast of Jeju Island in the Korean part of the Pacific Ocean, the shell shape of *R. philippinarum* varied significantly between sites (Silina 2010). Identification of phenotypic variations of shell contour has been proposed in order to distinguish *R. philippinarum* collected from several points on the Atlantic coast of France (Caill-Milly et al. 2014). In the Matsukawaura Lagoon (Japan), the shell shapes of wild Manila clams also varied significantly depending on the habitat (Tomiyama 2021).

Using morphological differences observed in differentiating meiotic cells, it is possible to distinguish the geographical forms of the Manila clam

We recently analyzed the oogenesis and spermatogenesis of the Adriatic Manila clam, focusing on the study of the so-called germplasm granules (GG), cytoplasmic structures that are involved in the transition from mitosis to meiosis (Reunov et al. 2019a). In the present work, devoted to the analysis of two forms of this mollusc, we compared the morphological character of the meiotic differentiation of the Adriatic Manila clam with this process occurring in the Manila clam living in the Sea of Japan. Interestingly, a clear difference was found. It was figured out that instead of "remote interaction" with mitochondria, which is characteristic of the GG of the Adriatic Manila clam, the pre-meiotic cells of the Manila clam living in the Sea of Japan are

characterized by direct contact of the GG with mitochondria (Fig. 9a, a'). In our opinion, this difference can serve as a cytological marker for distinguishing the forms of *R. philippinarum*.

It is difficult to explain the difference in the spatial localization of GG and mitochondria interacting in meiotic cells. Probably, these organelles can carry out the same molecular event both at a distance and in contact with each other. On samples of the Adriatic Manila clam, it was shown that the GG/mitochondrial interaction is accompanied by the penetration of the VASA protein from the VASA-positive GG to the mitochondria, which, in turn, release their matrix into the cytoplasm, as evidenced by the cytoplasmic localization of CYTB (Reunov et al. 2019a, 2021b). It remains to be clarified whether the same phenomenon takes place during the meiotic differentiation of the Pacific Manila clam. Probably, such interaction of GG with mitochondria is also possible in the Pacific form, since the cytoplasmic localization of mitochondrial content is considered to be universal for meiotic differentiation of various animal species. Its role appears to be to provide mitochondrial ribosomes for the translation of cytoplasmic proteins during the transition from mitosis to meiosis, as has been suggested for *Drosophila*, mouse, and zebrafish (for review Reunov et al. 2019b).

The number of sperm morphs, the presence of unique sperm morphs, and the type of dominant sperm variant allow to distinguish between the geographical forms of the Manila clam

Interestingly, both forms of Manila clam lack the monomorphism of the last phase of spermatogenesis – i.e. spermiogenesis. Indeed, during spermiogenesis of both forms, three types of acrosomes are formed: straight, curved, and folded. In addition, the Adriatic Manila clam has two patterns of nucleus development (straight and curved). Thus, in *R. philippinarum*, spermiogenesis is heteromorphic.

Heteromorphism in the mechanisms of sperm formation is not unique to Manila clams. Reunov et al. (1999) found two patterns of acrosome development in spermiogenesis in the mussel *Perna viridis* (Linneaus, 1758). Au et al. (1998) described four lines of spermiogenesis in the sea urchin *Anthocidaris crassispina* (A. Agassiz, 1864). Considering that the number of patterns of nucleus formation is different in the two forms of the Manila clam, we assume that

this feature is applicable as a cytological marker for the geographical forms of this differentiating mollusc (Fig. 9b, b').

We also investigated whether the structure of the sperm could be used to recognize the geographic forms of the Manila clam. In the Adriatic Manila clam, a bullet-shaped nucleus cupped with a thin acrosome was found in spermatozoa (Reunov et al. 2019a, 2021b). This general model is consistent with sperm found in samples of Manila clams collected in the Pacific region (this article), as well as sperm of Manila clams found along the Korean coast (Kim et al. 2013; Gwo et al. 2021). However, our study provided more detailed data on the morphology of spermatozoa and revealed their heteromorphism.

Interestingly, sperm heteromorphism was recently found in other bivalve molluscs, such as the Pacific oyster *C. gigas*, the heteromorphic set of spermatozoa of which consists of six morphologically stable morphs (Reunov et al. 2018), and in the surf clam *M. chinensis*, in which the set of spermatozoa consists of four morphologically stable morphs (Reunov et al. 2021a). We believe that *R. philippinarum* can also be attributed to the group of bivalves with heteromorphic spermatozoa.

The phenomenon of intraspecific heteromorphism of spermatozoa is known in animals (Morrow and Gage 2001). The functional parameters of heteromorphic spermatozoa can vary, for example, the cells having higher speed may have shorter life span and the cells having lower speed may have higher life span (Levitan 2000). Also, spermatozoa of different phenotypes can be genetically different, ensuring different success in sperm competition (Borowsky et al. 2018). In *C. gigas* oysters, which reproduce by external fertilization in seawater, the causes of sperm plasticity are associated with reproductive adaptation to the aquatic environment, which can be influenced by intense water current or turbulence, unstable temperature and salinity, anthropogenic pollution. The dominant expression of one or another variant of spermatozoa may be associated with a greater degree of adaptability of this variant to a certain type of environment. Parallel production of additional sperm variants can increase the chances of reproductive success in *C. gigas* oysters living in unstable conditions (Reunov et al. 2018). Because *R. philippinarum* also has external fertilization, a link between environmental conditions and sperm shape in this species seems likely. Given that the total number of sperm morphs is higher in the Adriatic Manila clam (Fig. 9c, c'), it can be suggested that the fertilization conditions may be more varied in the case of the Adriatic Manila clam than those

experienced by the Pacific Manila clam. Considering that both forms of molluscs have unique sperm morphs (Fig. 9d, d'), it seems possible that unique conditions for fertilization can be found in both cases. Given that the dominant sperm variant of the two forms of Manila clam is different (Fig. 9e, e'), it would be tempting to speculate that the dominant ecological factor influencing fertilization is different for these two forms. In any case, we believe that parameters such as different numbers of sperm morphs (Fig. 9c, c'), the presence of unique sperm morphs (Fig. 9 d, d'), and a difference in the dominant sperm pattern (Fig. 9e, e') can be used as cytological markers to distinguish geographic forms of Manila clam.

Conclusion

Commercial certification of the Manila clam *R. philippinarum* requires reliable methods to trace the geographic origin of this mollusc specimens. The study of the mitochondrial COX1 gene showed that the Adriatic Manila clam and Manila clam collected in the Russian part of the Sea of Japan are genetically indistinguishable. However, shell color and morphology can be used to determine specimen origin. Besides, spermatogenesis of Adriatic and Pacific Manila clams differs in the spatial distribution of germ plasm granules and mitochondria. Moreover, spermatid development differs in the number of nuclear formation patterns. Manila clam forms also differ in the number of sperm morphs, the presence of unique sperm morphs, and the type of dominant sperm morphs. Although an electron microscopy laboratory is required to compare reproductive cells, their differences can be used as cytological markers to determine the geographical origin of Manila clam.

Acknowledgements

Many thanks to Diving Department Head A. Goloseev and to divers N. Prohorov, K. Dudka, A. Oskolkov, M. Eremenko, I. Krasilnikov, S. Izrailskiy (National Scientific Centre of Marine Biology, Vladivostok, Russia) for very professional collection of *R. philippinarum*. Many thanks to Elizabeth Alexandrov for photographing mollusc shells. We are deeply indebted to Denis Fomin for the helpful assistance during observations with the scanning electron microscope and transmission electron microscope in the Microscopy Core Facility of the National Scientific

Centre of Marine Biology (Vladivostok, Russia). A part of the electron microscopy study was done in the Microscopy Facility at St. Francis Xavier University (Antigonish, NS, Canada).

Author contributions

All authors contributed to the study. AR conceived, design the study, analyzed the data, wrote the paper. LM analyzed the data, revised the paper. GP, MI collected the samples, performed genetic analyses, revised the paper. YR participated in the work using scanning electron microscopy and performed statistical analyses. EZ participated in genetic analyses. EV, YA, AA, EP collected samples, performed routine work using scanning electron microscopy and transmission electron microscopy. All authors read and approved the final version of the manuscript.

Funding

Part of the genetic testing was funded by the Italian Ministry of Education, University and Research MIUR-SIR Programme (Grant Number RBSI14G0P5) funded to LM.

Availability of data and material

The datasets generated and analysed during the current study are available from the corresponding author on request, and will be available in ResearchGate after publication of the article.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethics approval

Not applicable.

References

Au D, Reunov A, Wu S (1998) Four lines of spermatid development and dimorphic spermatozoa in the sea urchin *Anthocidaris crassispina* (Echinodermata, Echinoida). Zoomorphology 118:159–168. https://doi.org/10.1007/s004350050065

Bianchini G, Brombin V, Carlino P, Mistri E, Natali C, Salani GM (2021) Traceability and authentication of Manila clams from North-Western Adriatic Lagoons Using C and N Stable Isotope Analysis. Molecules 26:1859. https://doi.org/10.3390/ molecules26071859

Borowsky R, Luk A, He X, Kim RS (2018) Unique sperm haplotypes are associated with phenotypically different sperm subpopulations in Astyanax fish. BMC Biology 16:72. https://doi.org/10.1186/s12915-018-0538-z

Bourne N (1982) Distribution, reproduction and growth of Manila clam, *Tapes philippinarum* (Adams and Reeve, 1850), in British Columbia. J Shellfish Res 2:47-54

Caill-Milly N, Bru N, Barranger M, Gallon L, D'amico F (2014) Morphological trends of four Manila clam populations (*Venerupis philippinarum*) on the French Atlantic coast: identified spatial patterns and their relationship to environmental variability. J Shellfish Res 33(2):355-372

Chetoui I, Denis F, Boussaid M, Telahigue K, El Cafsi M (2016) Genetic diversity and phylogenetic analysis of two Tunisian bivalves (Mactridae) *Mactra corallina* (Linnaeus, 1758) and *Eastonia rugosa* (Helbling, 1799) based on COI gene sequences. C R Biol 339:115–122. https://doi.org/10.1016/j.crvi.2016.02.001

Chiesa S, Lucentini L, Freitas R, Nonnis Marzano F, Breda S, Figueira E, Caill-Milly N, Herbert RJH, Soares AMVM, Argese E (2016) Genetic diversity of introduced Manila clam. Bull Jap Fish Res Edu Agen 42:55－65

Cordero D, Delgado, M, Liu B, Ruesink J, Saavedra C (2017) Population genetics of the Manila clam (*Ruditapes philippinarum*) introduced in North America and Europe. Sci Rep 7:39745. https://doi.org/10.1038/srep39745

Ding J, Wen Q, Huo Z, Hongtao H, Qin Y, Yan X (2021) Identification of shell-color-related microRNAs in the Manila clam *Ruditapes philippinarum* using high-throughput sequencing of small RNA transcriptomes. Sci Rep 11:8044. https://doi.org/10.1038/s41598-021-86727-9

Fava G, Meggiato L (1995) Genetica biochimica e caratteri morfologici in popolazioni alto adriatiche di *Ruditapes philippinarum* e *R. decussatus*. Technical Report Ricerche e Sperimentazioni, Programma Integrato Mediterraneo per le zone lagunari dell'Adriatico settentrionale, pp 203-209

Gwo J-C, Huang Y-S, Kuo T-Y (2021) Sperm ultrastructure of *Ruditapes variegata* and *Tapes literatus* (Mollusca, Bivalvia, Veneridae, Tapetinae) from Pescadores, Taiwan. Tissue and Cell 71:101575. https://doi: 10.1016/j.tice.2021.101575

Katoh K, Standley DM (2013). MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. Mol Biol Evol 30(4):772–780. https://doi.org/10.1093/molbev/mst010

Kim JH, Chung JS, Lee K-Y (2013) Ultrastructural characteristics of the testis, spermatogenesis and taxonomic values of sperm morphology in male *Ruditapes philippinarum* in Western Korea. Dev Reprod 17 (2):121-132. https://doi: 10.12717/DR.2013.17.2.121

Kim EM, Song MS, Hur DH, An CM, Kang JH, Park JY (2015) Easy method for discriminating the origins of Manila clam *Ruditapes philippinarum* with a dual-labelled PNA-probe-based melting curve analysis. Biochip J 9:247-258. https://doi.org/10.1007/s13206-015-9402-1

Leigh JW, Bryant D (2015) PopART: Full-feature software for haplotype network construction. Methods Ecol Evol 6(9):1110–1116. https://doi.org/10.1111/2041-210X.12410

Levitan D (2000) Sperm velocity and longevity trade off each other and influence fertilization in the sea urchin *Lytechinus variegates*. Proc R Soc Lond B 267:531–534

Low M, Ng Pkl, Clark PF (2020) Additional notes on the publication of the narrative, zoology and notes from a journal of research into the natural history of the voyage of H.M.S. Samarang and its consequences for the nomenclature of decapod crustaceans and other taxa. Zootaxa 4809(2):zootaxa.4809.2.3. https://doi.org/10.11646/zootaxa.4809.2.3

Markaide P, Gairín I, Cordero D, Ibarrola I, Saavedra C (2021) No hybridization and marked interspecific differences in individual growth rate in mixed cultures of Manila clam (*Ruditapes philippinarum*) and grooved carpet-shell clam (*R. decussatus*). Aquaculture 541:736824. https://doi.org/10.1016/j.aquaculture.2021.736824

Minh BQ, Schmidt HA, Chernomor O, Schrempf D, Woodhams MD, von Haeseler A, Lanfear R (2020) IQ-TREE 2: New models and efficient methods for phylogenetic inference in the genomic era. Mol Biol Evol 37:1530-1534. https://doi: 10.1093/molbev/msaa015

Morgan C (2002) Naturalisation of an introduced species, the Manila clam, *Tapes philippinarum*, in Poole Harbour, UK. Special Publication - Aquaculture Association of Canada 6:53-55

Morrow EH, Gage MJG (2001) Consistent significant variation between individual males in spermatozoal morphometry. J Zool 254(2):147−153

Mortensen SH (1993) A health survey of selected stocks of commercially exploited Norwegian bivalve molluscs. Diseases of Aquatic Organisms 16(2):149-156

Morton B (1976) The biology, ecology and functional aspects of the organs of feeding and digestion of the S.E. Asian mangrove bivalve, *Enigmonia aenigmatica* (Mollusca: Anomiacea). J Zool Lond 179:437–466

Moss SM (1990) *Enigmonia aenigmatica*: an enigmatic molluscan chameleon. The marine biology of the South China Sea. In: Morton B (ed) Proceedings of the first international conference of the marine biology of Hong Kong and the South China Sea. Hong Kong, 28 October – 3 November 1990, Hong Kong University Press, Hong Kong

Ni G, Li Q, Lehai Ni, Kong L, Yu H (2015) Population subdivision of the surf clam *Mactra chinensis* in the East China Sea: Changjiang River outflow is not the sole driver. PeerJ 3:e1240. https://doi.org/10.7717/peerj.1240

Nosho TY, Chew KK (1972) The setting and growth of the Manila clam, *Venerupis japonica* (Deshayes), in Hood Canal, Washington. Proc Natl Shellfish Assoc 62:50-58

Ponurovsky SK, Selin NI (1988) Distribution, population structure, and growth of the bivalve molusk *Ruditapes philippinarum* in Vostok Bay, Sea of Japan. Sov J Mar Biol 1:11-15

Reunov A, Alexandrova Y, Komkova A, Reunova Y, Pimenova E, Vekhova E, Milani L (2021b) VASA-induced cytoplasmic localization of CYTB-positive mitochondrial substance occurs by destructive and nondestructive mitochondrial effusion, respectively, in early and late spermatogenic cells of the Manila clam. Protoplasma 258(4):817-825. https://doi.org/10.1007/s00709-020-01601-1

Reunov A, Alexandrova Y, Reunova Y, Komkova A, Milani L (2019a) Germ plasm provides clues on meiosis: the concerted action of germ plasm granules and mitochondria in

gametogenesis of the clam *Ruditapes philippinarum*. Zygote 27(1):25-35. https://doi.org/10.1017/S0967199418000588

Reunov A, Au D, Wu R (1999) Spermatogenesis of the green-lipped mussel *Perna viridis* with dual patterns of acrosome and tail development in spermatids. Helgol Mar Res 53:62–69

Reunov A, Lutaenko K, Vekhova E, Zhang J, Zakharov E, Sharina S, Alexandrova Y, Reunova Y, Akhmadieva A, Adrianov A (2021a) In the Asia-Pacific region, the COI DNA test revealed the divergence of the bivalve mollusc *Mactra chinensis* into three species; can these species be distinguished using shell coloration and sperm structure? Helgol Mar Res 75:7. https://doi.org/10.1186/s10152-021-00553-0

Reunov A, Vekhova E, Zakharov E, Reunova Y, Alexandrova Y, Sharina S, Adrianov A (2018) Variation of sperm morphology in Pacific oyster precludes its use as a species marker but enables intraspecific geo-authentification and aquatic monitoring. Helgol Mar Res 72:8. https://doi.org/10.1186/s10152-018-0510-x

Reunov A, Yakovlev K, Hu J, Reunova Y, Komkova A, Alexandrova Y, Pimenova E, Tiefenbach J, Krause H (2019b) Close association between vasa-positive germ plasm granules and mitochondria correlates with cytoplasmic localization of 12S and 16S mtrRNAs during zebrafish spermatogenesis. Differentiation 109:34-41. https://doi.org/10.1016/j.diff.2019.08.002

Sekine Y, Yamakawa Y, Takazawa S, Yingping L, Toba M (2006). Geographic variation in the COXI gene of the Short-neck clam *Ruditapes philippinarum* in coastal regions of Japan and China. Venus 65:229–224

Sigurdson JB, Sundari G (1990) Shellular changes in the colour in the tree-climbing bivalve *Enigmonia aenigmatica* (Holten 1802) (Annomiidae). Raffles Bull Zool 38:213–218

Silina AV (2010) Population characteristics of the bivalve *Ruditapes philippinarum* from Cheju Island coasts, Korea. Korean J Malacol 26(3):227-234

Skurikhina LA, Kartavtsev YF, Chichvarkhin AY, Pankova MV (2001) Study of two species of mussels, *Mytilus trossulus* and *Mytilus galloprovinciallis* (Bivalvia: Mytilidae), and their hybrids in Peter the Great Bay of the Sea of Japan with the use of PCR markers. Genetika 37:1717–1720

Sokolova IM, Berger VJ (2000) Physiological variation related to shell colour polymorphism in White Sea *Littorina saxatilis*. J Exp Mar Biol Ecol 245(1):1–23. https://doi.org/10.1016/S0022-0981(99)00132-X

Tan Y, Fang L, Qiu M, Huo Z, Yan X (2020) Population genetics of the Manila clam (*Ruditapes philippinarum*) in East Asia. Sci Rep 10:21890. https://doi.org/10.1038/s41598-020-78923-w

Tomiyama T (2021) Ecophenotypic plasticity in shell growth direction of asari clam *Ruditapes philippinarum*. J Mar Biol Assoc United Kingdom 101(3):555-560. https://doi.org/10.1017/S0025315421000412

Williams ST (2017) Molluscan shell colour. Biol Rev 92:1039-1058. https://doi: 10.1111/brv.12268

Yan X, Nie H, Huo Z, et al (2019) Clam genome sequence clarifies the molecular basis of its benthic adaptation and extraordinary shell color diversity. iScience 19:1225-1237. https://doi.org/10.1016/j.isci.2019.08.049

Yuan H, Xu X, Yang F, Zhao L, Yan X (2020) Impact of seawater acidification on shell property of the Manila clam *Ruditapes philippinarum* grown within and without sediment. J Oceanol Limnol 38(1):236-248. https://doi.org/10.1007/s00343-019-8281-z

Legends

Fig. 1 Map of Peter the Great Bay (Sea of Japan, Russia) showing diving sites where the Manila clam *Ruditapes philippinarum* was collected. 1 – collection point #1 (Vostok Bay); 2, 3 – collection points #2 and #3 (Amursky Bay).

Fig. 2 Manila clam *Ruditapes philippinarum*; maximum-likelihood phylogenetic tree of COX1 partial sequences. Tree was calculated on 555 base pairs of COX1 (IQ-TREE software; MG+F1X4+G4 model; 1000 bootstrap replicates). Branch lengths with supports lower than 70 were collapsed (other values not shown). Samples included those sequenced in the present analysis, and those sequenced by Cordero et al. (2017) and Sekine et al. (2006). Red clade: sequences belonging to haplogroup A (as defined in Cordero et al. 2017). Haplogroup A included all samples belonging to the present study (bold orange tips - Rph m1 cox1F, Rph m2 cox1F, Rph m3 cox1F, Rph m4 cox1F, Rph m5 cox1F): Adriatic Sea samples; bold red tips - CCDB-ST03131, CCDB-ST03132, CCDB-ST03133, CCDB-ST03136, CCDB-ST03137, CCDB-ST03138, CCDB-ST03140, CCDB-ST03141: Sea of Japan samples), that however could not be separated in monophyletic clusters based on geographical origin. Branches are not scaled for branch lengths. Bold black tip: outgroup (belonging to *Mactra chinensis*).

Fig. 3 Manila clam *Ruditapes philippinarum*; phylogenetic network of COX1 partial sequences. Median-joining network calculated on 555 base pairs of COX1 (PopART software). Samples included those sequenced in the present analysis, and those sequenced by Cordero et al. (2017) and Sekine et al. (2006). Each colored dot represents a haplotype with size scaling based on the number of samples belonging. Each bar in the connecting lines represents a single nucleotide substitution. Each haplogroup (A, B, C) is highlighted with a colored square that refers to haplogroups defined in Cordero et al. (2017). Samples from the Sea of Japan (yellow dots) and samples from the Adriatic Sea (orange dots) are included in haplogroup A and cannot be divided into subgroups based on geographic origin. The haplogroup A is separated by haplogroups B and C, that are more spread in the Chinese coast (Cordero et al. 2017).

Fig. 4 Intraspecific variability of the shells of the Manila clam *Ruditapes philippinarum* that was revealed in genetically identified individuals. **a-e** Shell variants of the Adriatic Manila clam; **f-m** shell variants of the Pacific Manila clam. The arrowheads show shell umbos. Scale bar 1 cm.

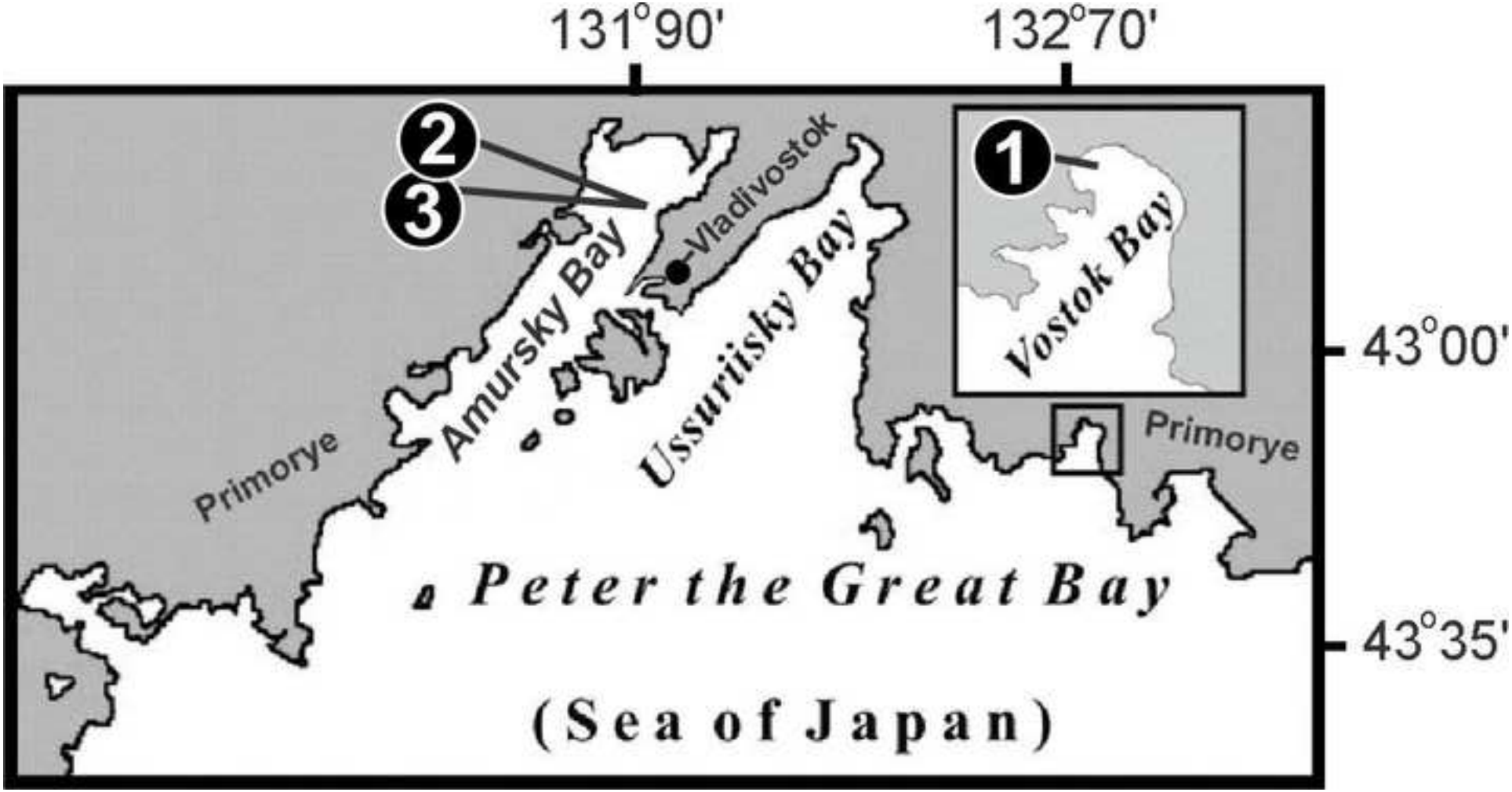
Fig. 5 Early spermatogenic cells of the Adriatic and Pacific forms of the Manila clam *Ruditapes philippinarum* by transmission electron microscopy. **a** Early spermatogenic cells in the testis of the Pacific Manila clam; **b** germ plasm granules (GG) in the spermatogonia of the Pacific Manila clam; **c** mitochondria that bind to GG in the cytoplasm of the spermatogonium of the Pacific Manila clam; **d** an enlarged version of a part of a cell, shown as a square in the previous image; **e** mitochondrial cluster in the spermatogonium of the Pacific Manila clam; notice the mitochondria, which are tightly attached to the GG by their membrane; **f** mitochondrial cluster in spermatogonium of the Adriatic Manila clam; **g** an enlarged version, shown by a square in the previous image; pay attention to mitochondria that are located next to the GG, but do not form direct contact with this structure. Sg – spermatogenic cells, m – mitochondrion, gg – germ plasm granule, nu – nucleus. Scale bars 3 µm (**a**), 0.5 µm (**b-g**).

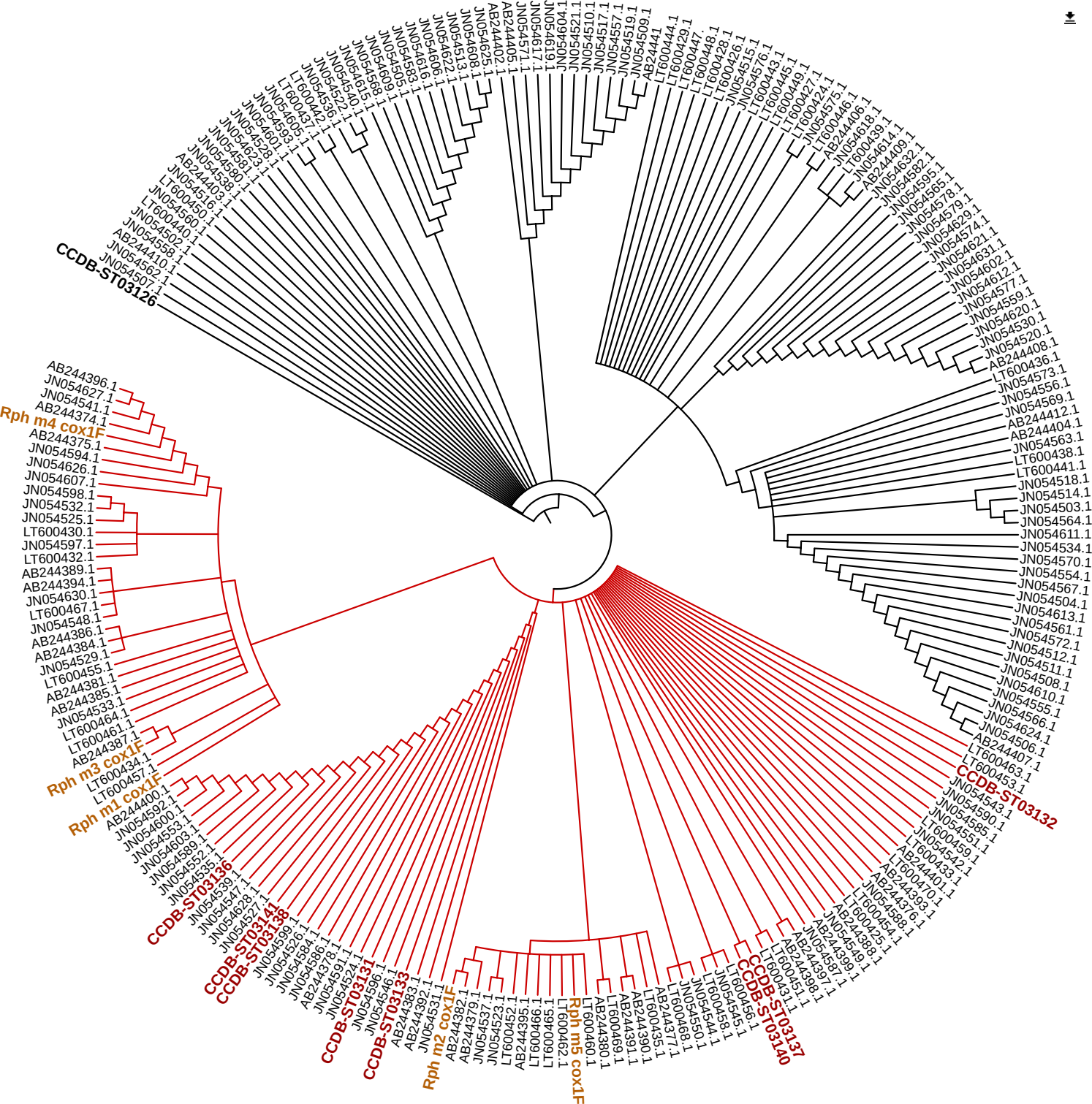
Fig. 6 Early spermiogenesis of the Manila clam *Ruditapes philippinarum* by transmission electron microscopy. The cell ultrastructure is the same in both forms of the Manila clam and is shown here mainly on the example of the Adriatic Manila clam. **a**, **b** The acrosomal vesicle in the basal part of the early spermatid (**a**); enlarged image of the acrosomal vesicle, shown by the arrowhead in the previous image (**b**); **c** acrosomal vesicle in the lateral part of early spermatid; **d** acrosomal vesicle in the apical part of early spermatid; **e**, **f** acrosomal vesicles, which are equally flat in spermatids from the Pacific Manila clam and the Adriatic Manila clam, respectively; **g-i** the initial stage of elongation of the acrosome in the spermatid; note invagination of the acrosomal vesicle, which is filled with loose periacrosomal material (**g**), the cupped acrosomal vesicle, which is filled with periacrosomal material that contacts the nucleus (**h**), and elongation of the periacrosomal material with an elevated acrosomal vesicle (**i**). Nu – nucleus, m – mitochondrion, f – flagellum, star shows acrosomal vesicle, arrowheads show acrosomal vesicles, arrows show periacrosomal material. Scale bar 0.5 µm.

Fig. 7 Late spermiogenesis, of the Manila clam *Ruditapes philippinarum*. The data were obtained by transmission electron microscopy (**a-c**, **e**, **f**, **j**, **l**, **m**) and scanning electron microscopy (**d**, **g-i**, **k**). **a-d** Development of a straight acrosome; **e** curved acrosome formed during spermiogenesis; **f-k** acrosome, which is formed during spermiogenesis by unfolding; **l, m** spermiogenesis accompanied by nuclear bending. Nu - nucleus, a – acrosome, arrows point to periacrosomal material, arrowhead shows acrosomal vesicle. Scale bar 1 µm.

Fig. 8 Intraspecific diversity of spermatozoa of the Manila clam *Ruditapes philippinarum* revealed by the analysis of external cell morphology by scanning electron microscopy. **a-e** Sperm morphs characteristic of the Adriatic Manila clam; **f-h** sperm morphs characteristic of the Pacific Manila clam. **i** Schematic representation of a set of heterogeneous spermatozoa characteristic of the Manila clam, consisting of (1) SPERM1, (2) SPERM2, (3) SPERM3, (4) SPERM4, (5) SPERM5 and (6) SPERM6. **j, k** The diagrams show the proportions of sperm morphs in the Adriatic Manila clam, which has sperm morphs 1-5 (**j**), and in the Pacific Manila clam, which has sperm morphs 4-6 (**k**). a – acrosome; n – nucleus; m – mitochondrial area; f – flagellum. Scale bar – 1 µm.

Fig. 9 Schematic representation of the structural differences found in the spermatogenesis of the Adriatic and Pacific forms of the Manila clam *Ruditapes philippinarum*.
These forms differ in: (1) remote interaction of germ plasm granules (GG) with mitochondria, characteristic of premeiotic cells of the Adriatic Manila clam (**a**) and contact interaction of GG and mitochondria, typical of the Pacific Manila clam (**a'**); (2) by the number of ways of nucleus formation, of which two (straight nucleus and curved nucleus) are found in the Adriatic Manila clam (**b**) and only one way (curved nucleus) is found in the Pacific Manila clam (**b'**); (3) by the number of heteromorphic sperm samples, of which five morphs (**c**) were found in the Adriatic Manila clam and three morphs were found in the Pacific Manila clam (**c'**); (4) by the number of unique sperm samples, which is three in the Adriatic Manila clam (**d**) and only one in the Pacific Manila clam (**d'**); (5) by the type of the dominant sperm sample, which is SPERM5 in the Adriatic Manila clam (**e**) and SPERM6 in the Pacific Manila clam (**e'**).

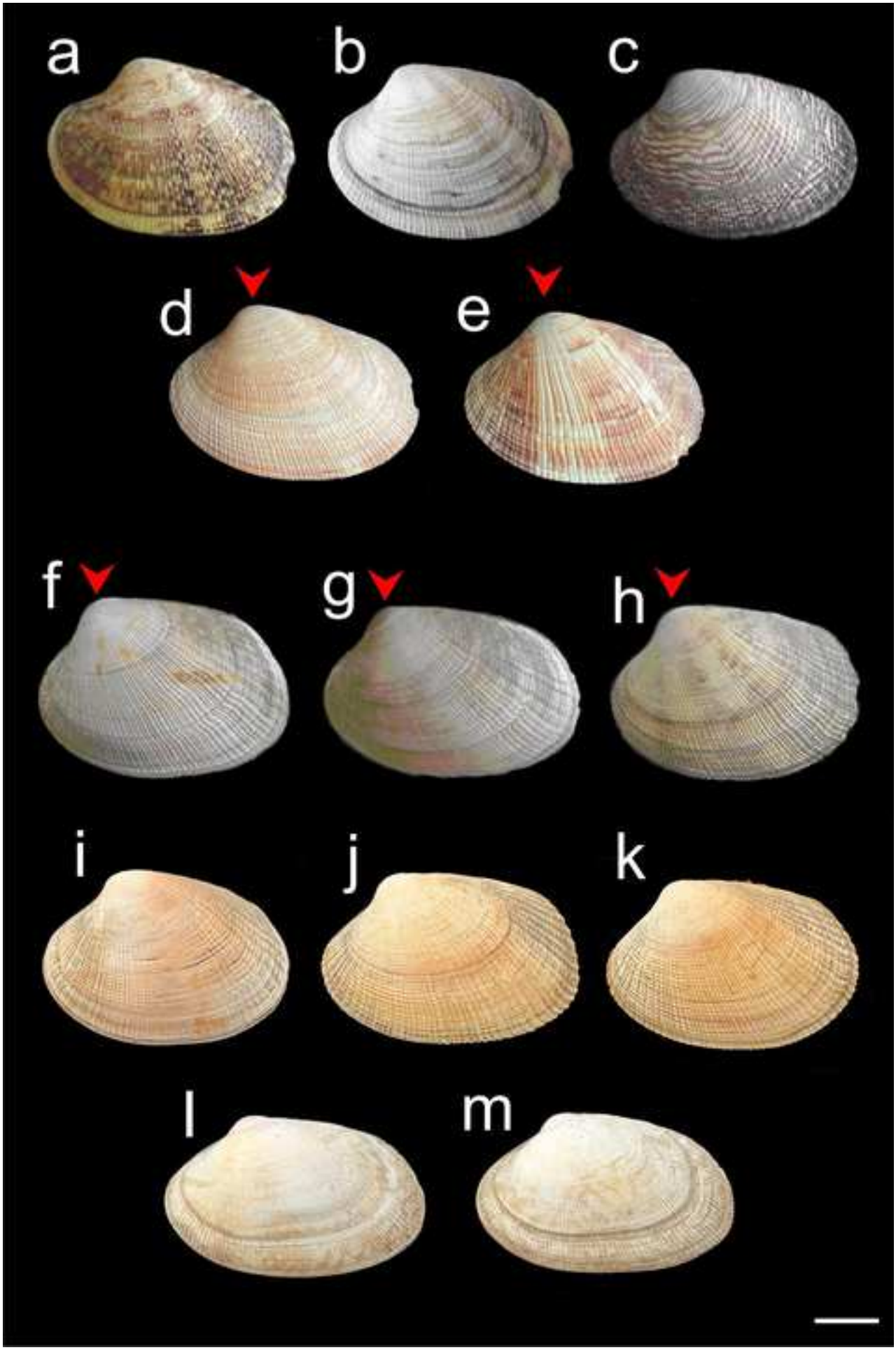

131°90'
132°70'
43°00'
43°35'
1
2
3
Vladivostok
Amursky Bay
Ussuritisky Bay
Vostok Bay
Primorye
Primorye
Peter the Great Bay
(Sea of Japan)

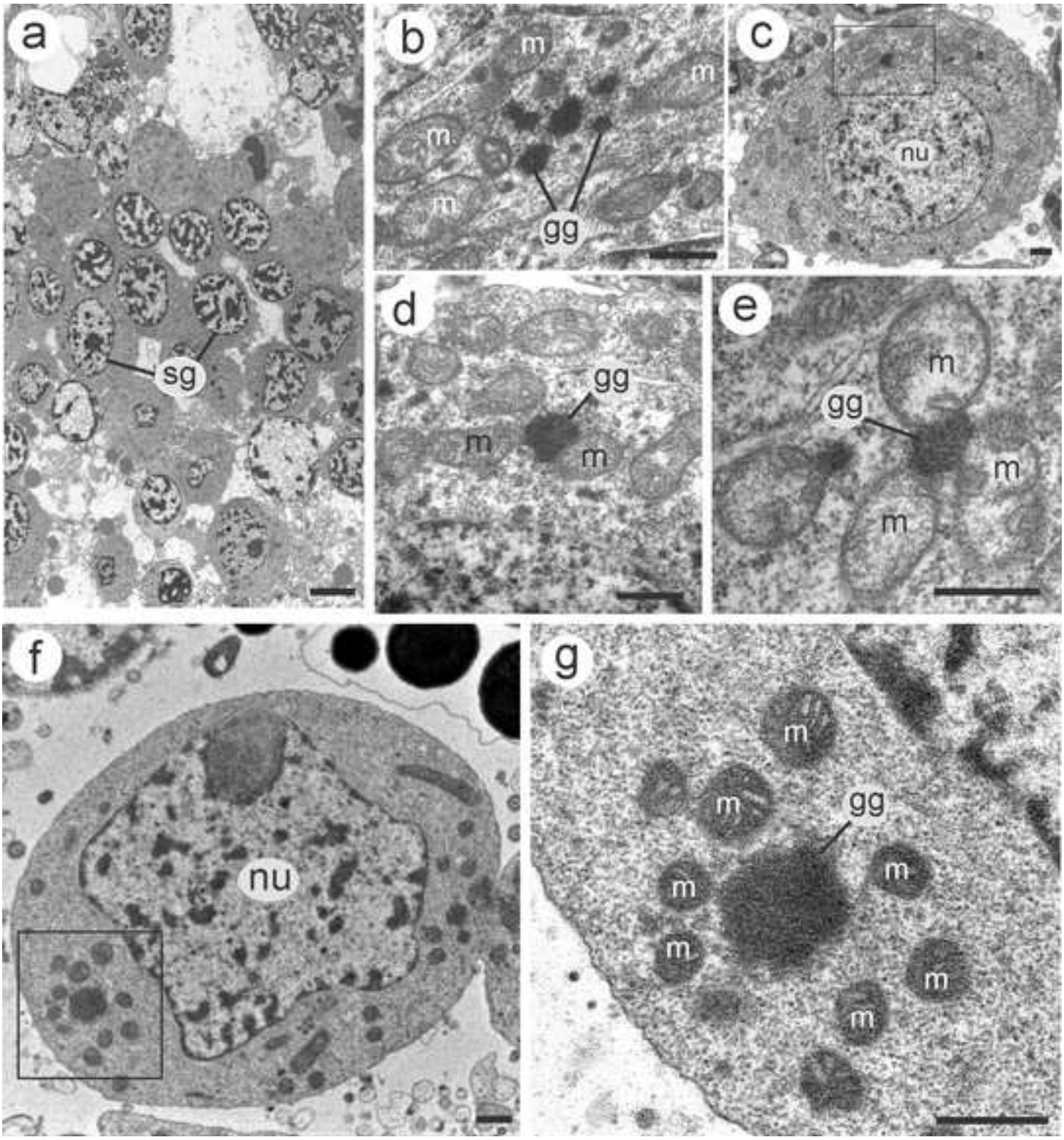

CCDB-ST03126
Rph m4 cox1F
Rph m3 cox1F
Rph m1 cox1F
CCDB-ST03136
CCDB-ST03141
CCDB-ST03138
CCDB-ST03131
CCDB-ST03133
Rph m2 cox1F
Rph m5 cox1F
CCDB-ST03140
CCDB-ST03137
CCDB-ST03132

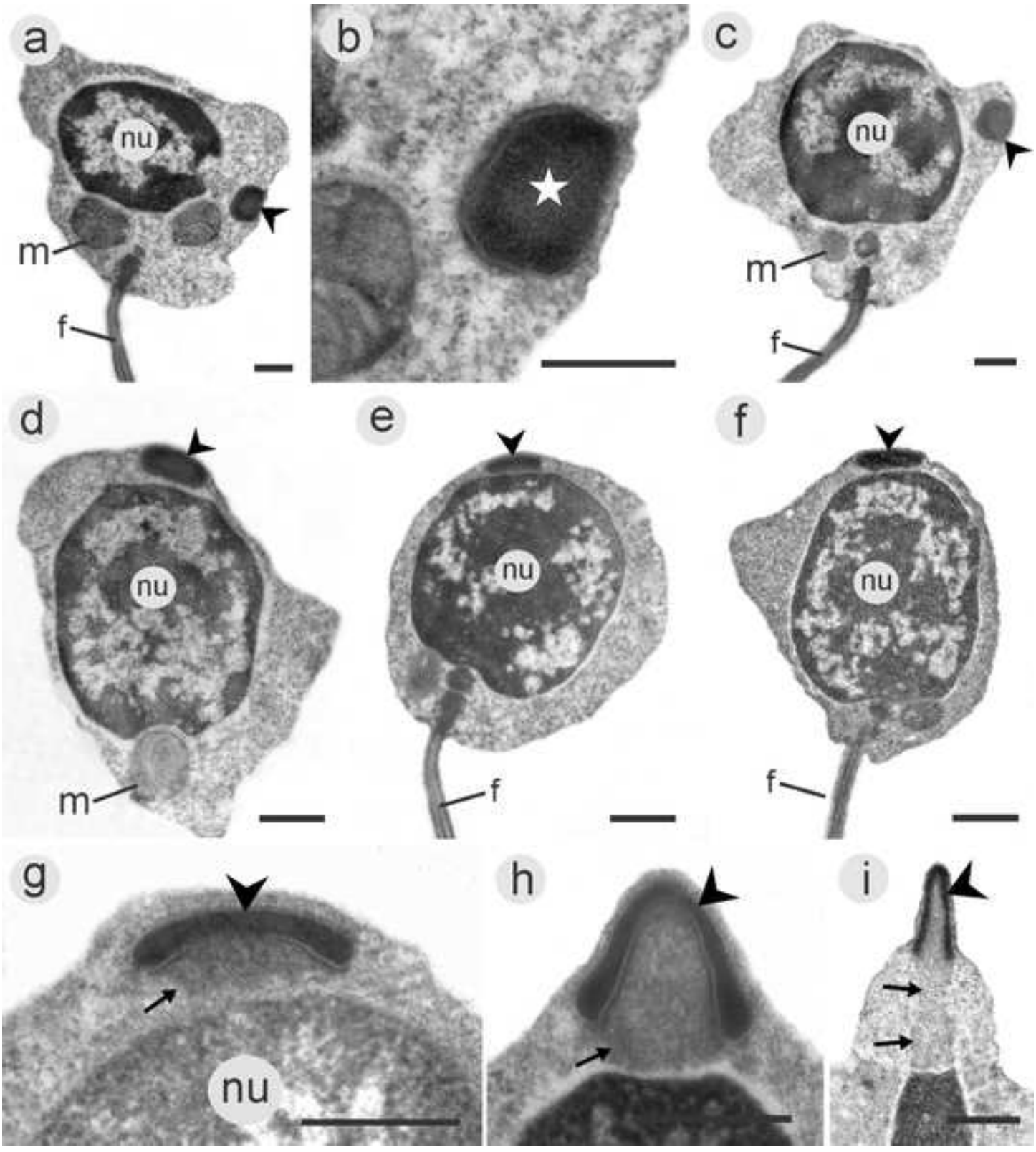
B

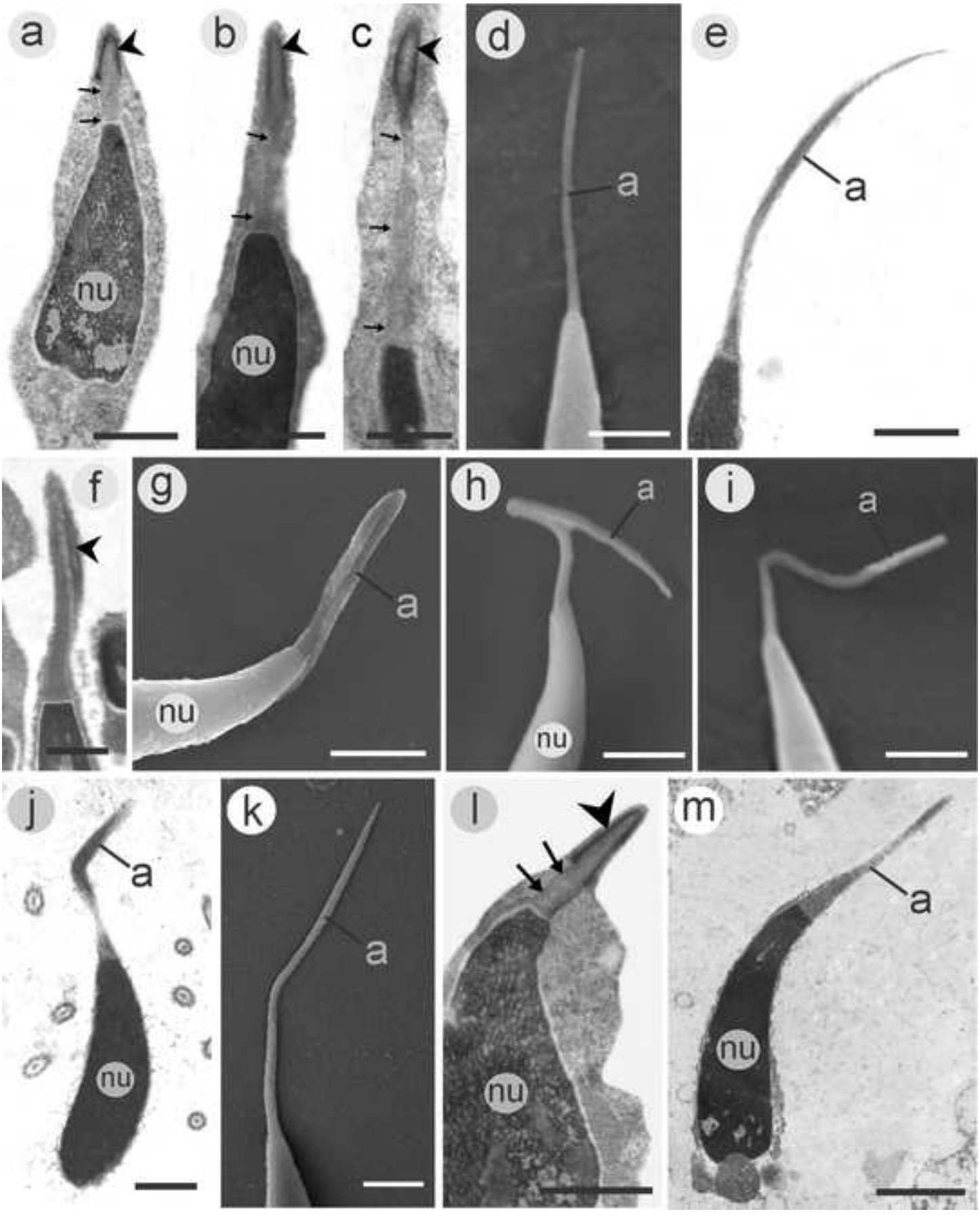
C

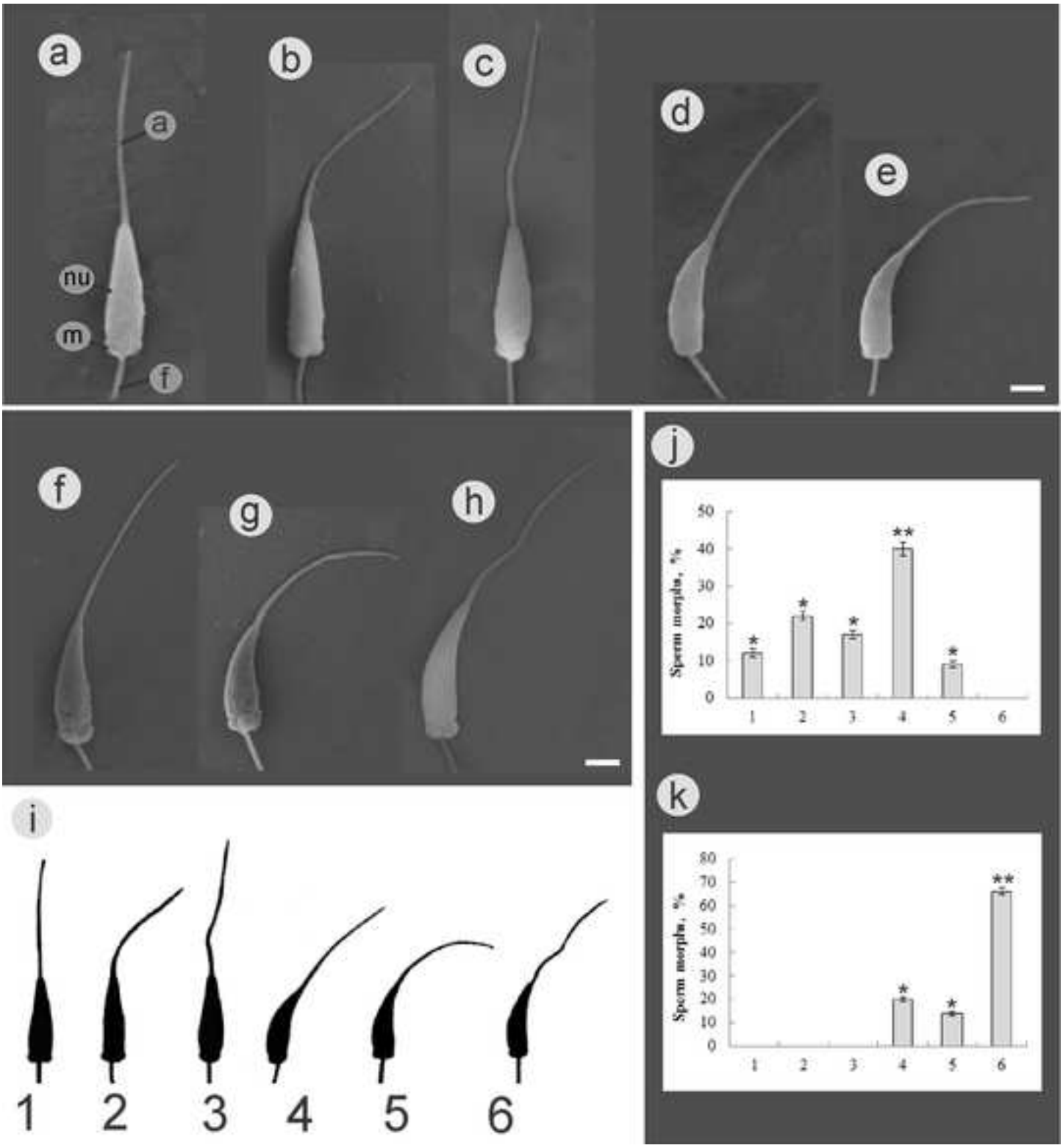
A

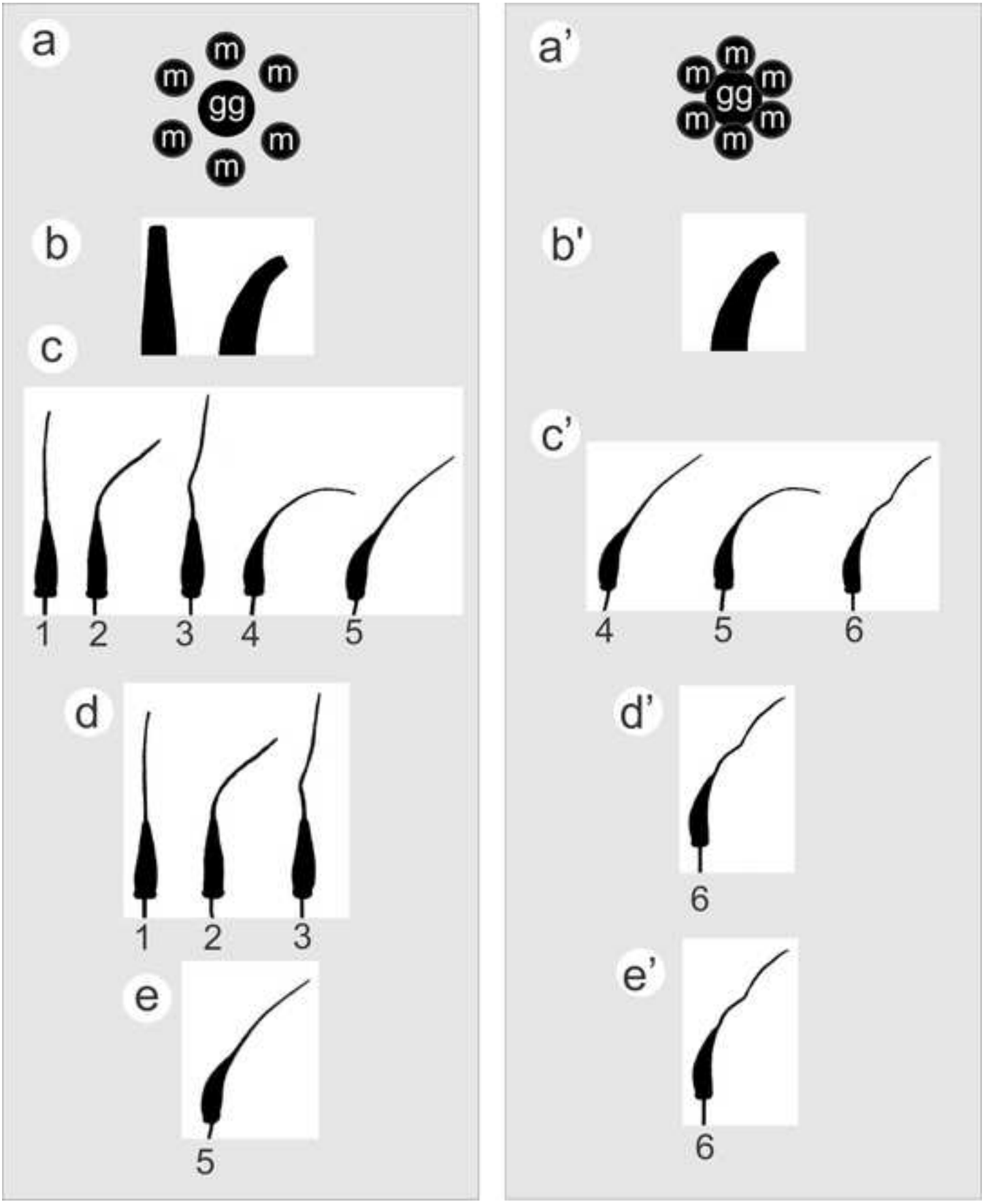

a
b
c
d
e
f
g
h
i
j
k
l
m


a
sg
b
m
m
m
m
gg
c
nu
d
gg
m
m
e
m
gg
m
m
f
nu
g
m
m
gg
m
m
m
m
m


a
nu
m
f
b
c
nu
m
f
d
nu
m
e
nu
f
f
nu
f
g
nu
h
i


a
b
c
d
e
f
g
h
i
j
k
l
m
nu
a


a
b
c
d
e
a
nu
m
f
f
g
h
i
1
2
3
4
5
6
j
Sperm morpha, %
50
40
30
20
10
0
1
2
3
4
5
6
k
Sperm morpha, %
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6


a
m
m
m
gg
m
m
m
b
c
1
2
3
4
5
d
1
2
3
e
5
a'
m
m
m
gg
m
m
m
b'
c'
4
5
6
d'
6
e'
6