

Disproportional Heteromorphism of Male Gametes in the Bivalve Mollusk *Mactra chinensis* is Related to Genetic Divergence of This Species

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The bivalve mollusk *Mactra chinensis* Philippi, 1847 is of significant commercial importance in the Asian–Pacific area; therefore, it is studied by malacologists intensely. On the basis of morphological data, researchers suggested a divergence of this species in the Sea of Japan. As a consequence of the divergence, the southern and northern populations have already appeared which are different in the shape, color, and the shell pattern. For example, in the South Korean part of the Sea of Japan, the *Mactra* shell is described as faintly colored, yellowish, without any brown rays, while in the Russian part of the Sea of Japan, there is *Mactra* with brownish-purple shell and brown and violet-purple rays [3, 8, 9, 11, 12, 15]. Since the category of this species is of important commercial value, some additional approaches are necessary to define *Mactra* more accurately.

Along with shell morphology, analysis of the gamete structure can be also used to determine species divergence. Compared with the body as a whole, the reproductive cells were found out to undergo more rapid evolution and, hence, different morphologies of sperms in the remote geographic populations of the species is a sign of probable divergence and speciation [13].

This study is a comparative analysis of male gametes in the members of the northern and southern populations of Chinese *Mactra* in the Sea of Japan. In addition, in genetic analysis of the representatives of both populations, we used DNA barcoding, which is an acknowledged method of species identification [6]. Three specimens of reproductively active *M. chinensis* of the northern population were collected in the Vostok Bay (the Sea of Japan, Russia) in July–August

2012 on the marine biological station Vostok, Institute of Marine Biology, Far East Branch, Russian Academy of Sciences (42°53' N, 132°44' E). Two specimens of the southern population were collected in July 2012 in the South Korean aquatic area near the settlement Uljin, Kyonsanbuk province (37°4' N, 129°24' E).

Male gametes were examined under a scanning electron microscope. For this purpose, the seminal vesicle fragments were fixed for 2–4 h at 4°C in a solution of 2.5% glutaraldehyde prepared on 0.2 M cacodylate buffer (pH 7.4). Afterwards, the samples were washed out in 0.1 M cacodylate buffer for 15–20 min. Sperm suspension was pipetted on the surface of Thermanox coverslips (Cat. no. 72280). After sperm precipitation for 30 min, the preparations were dehydrated in increasing concentration of alcohol to get them gradually into acetone. Thereafter, the samples were dried completely in carbon dioxide in a critical point dryer 030 (BAL-TEC) and placed on the aluminum surface to perform carbon sputter coating. The samples were examined using a Zeiss EVO 40 microscope. The resultant images were edited using a graphic program Adobe Photoshop 7.0. Statistical processing of the quantitative data was performed by means of the Microsoft XL software using Student's *t* test.

When studying the male gametes of *M. chinensis* in the southern and northern populations of the Sea of Japan, we have found out that so-called “primitive sperm cells,” the structure of which is adapted to external insemination, is characteristic of the members of both populations [2]. These cells have a streamlined head consisting of an acrosome, a nucleus, four mitochondria, and flagella. There are three variants of this morphotype in molluscs of both populations. In one of them, the acrosome has a concave apical surface without any other structures (Fig. 1a); in the second variant, the acrosome has a small ledge (Fig. 1b), while in the third variant, the ledge is relatively extended and slightly curved (Fig. 1c). Quantitative

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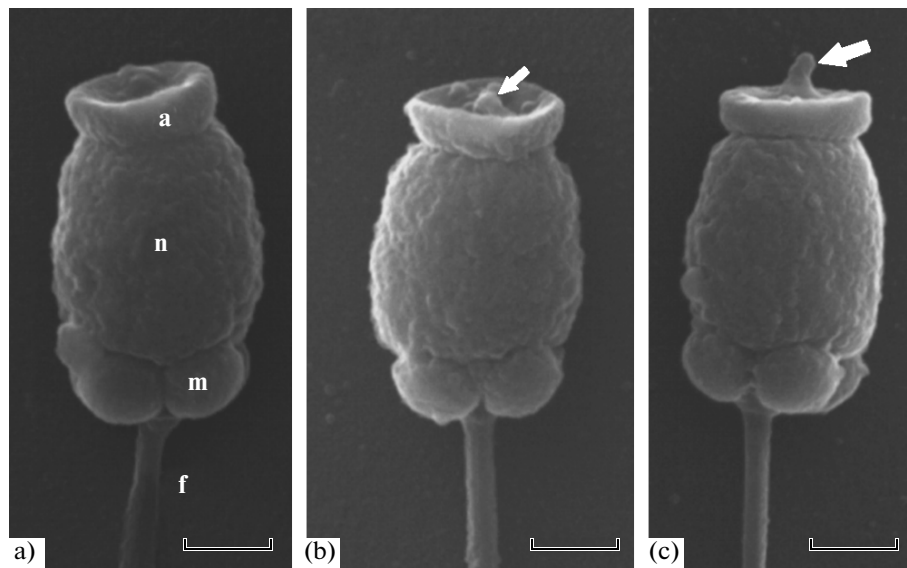


Fig. 1. Sperm cells of the bivalve mollusk *M. chinensis*: (a) the variant having an acrosome without a ledge; (b) the variant having an acrosome with a short ledge (indicated by a short arrow); (c) the variant having an acrosome with elongated ledge (indicated by a long arrow). a, acrosome; n, nucleus; m, mitochondria; f, flagellum; length range is 0.5 μ m.

analysis revealed different proportional ratios of these morphotypes in molluscs of the northern and southern populations. The morphotype having acrosome without a ledge is typical of the northern population (76%); the morphotypes with short and elongated ledges on the acrosome surface accounted for about 16% and 8%, respectively (Fig. 2a). In molluscs of the southern population, only 12% of the morphotypes have no any acrosomal ledge; in 19% of morphotypes, gametes with a short acrosomal ledge are observed, and the major part of the morphotypes, 69%, are those with gametes having an elongated acrosomal ledge (Fig. 2b).

Thus, in addition to previous data that describe *M. chinensis* as the monogametic species [4, 5, 10], we have found out that heteromorphism is characteristic of *Macra*, which is manifested in that they have three variants of the sperm cells. Note that heteromorphism of male gametes has been recently found out in some other bivalve mollusks that were earlier considered monogametic [1], and this phenomenon has no explanation so far. Of interest is the fact that, in the northern and southern *M. chinensis* populations, heteromorphism of sperm cells is disproportional. Predomination of one of the three morphotypes is characteristic of the southern population, while another variant predominates in the northern population; the third morphotype seems to be a reserve one in both cases because of a small number of these gametes. The reasons for quantitative predomination of a certain morphotype remain still unclear. It may well be that the species divergence that has been suggested on the basis of differences in shell coloration is the cause of a shift to a certain variant of sperm cells [12]. To verify this

hypothesis, we compared the nucleotide sequences of the *COI* mitochondrial gene in the specimens studied. DNA was isolated, and the amplified fragments of the first half of *COI* gene (658 bp) were analyzed using a ABI3730xl automated sequencer. As a result, we have obtained five sequences. The metadata for all speci-

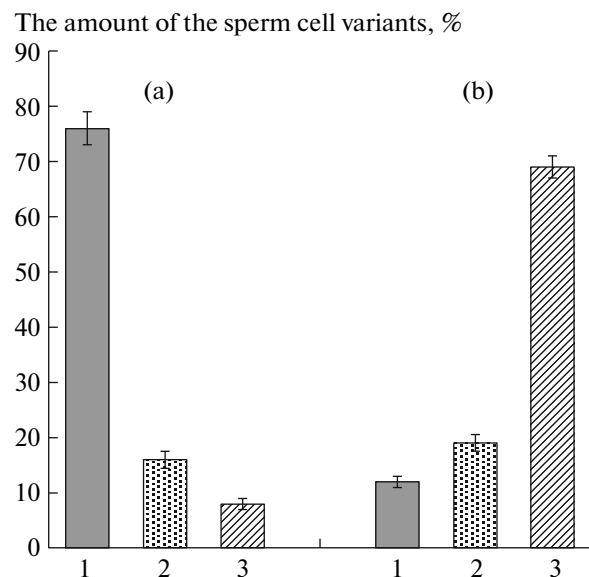


Fig. 2. Graphic presentation of the proportional ratio of three sperm cell variants in the northern (a) and southern (b) populations of *M. chinensis*. 1, the variant having an acrosome without a ledge; 2, the variant having an acrosome with a short ledge; 3, the variant having an acrosome with elongated ledge (means and 95% confidence intervals).

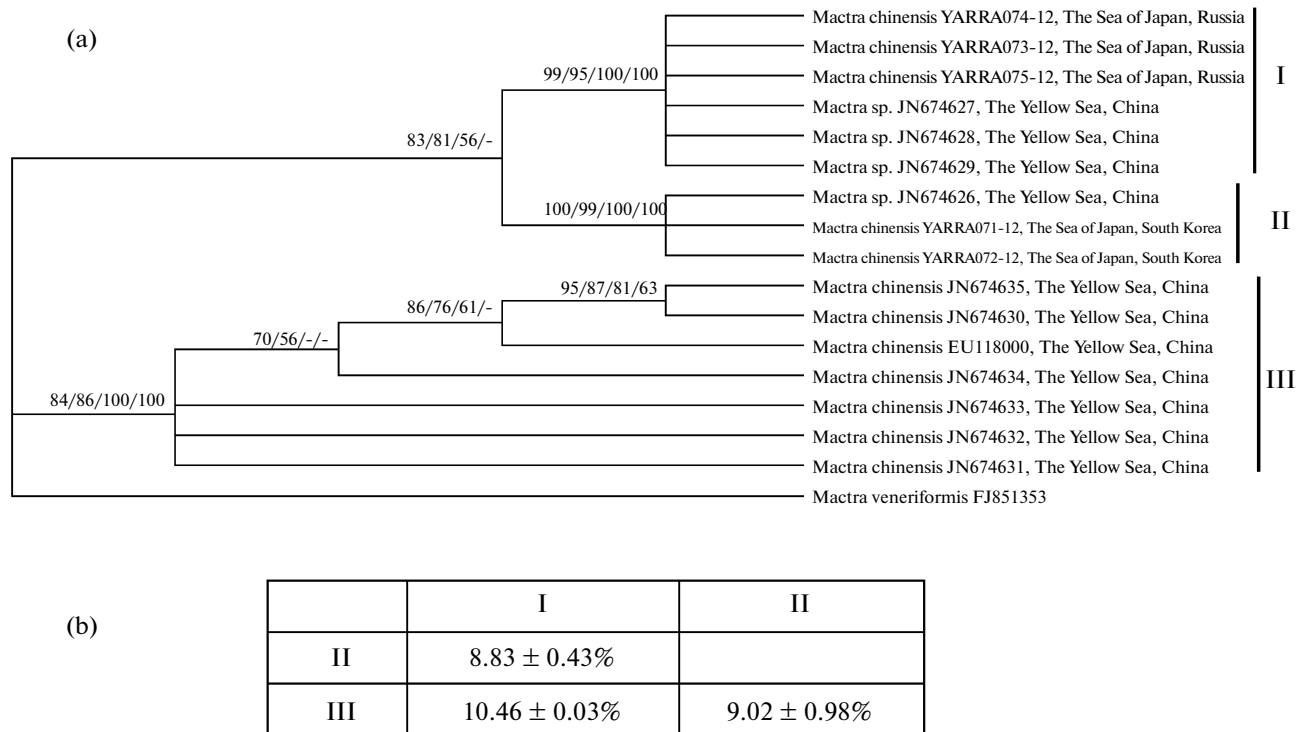


Fig. 3. Genetic analysis of *M. chinensis* for the *COI* gene. (a) Bayesian consensus tree that reflects the ratio between *M. chinensis* specimens included in *COI* gene analysis. The supports obtained according to the Bayesian method as well as the maximum likelihood, joining the nearest neighbor, and the maximum parsimony approaches (BA/ML/NJ/MP) are indicated in the nodes. The number of generations for the BA tree is 1 000 000; the bootstrap replicas according to ML and NJ is 1000, and to MP—500. Note that the members of the northern population of Chinese *Maetra* in the Sea of Japan (YARRA074-12; YARRA073-12; YARRA075-12) belong to the clade I, while the members of the southern population of the Sea of Japan (YARRA071-12; YARRA072-12) are assigned to the clade II. (b) The distances between clades on the tree (the average ± standard error) as calculated according to Kimura two parameter model (K2P).

mens, their sequences, and chromatograms are stored in the database BOLD (Barcode of Life Data System) (<http://boldsystems.org>). For more complete identification of the *Maetra* species status, we used also the data on *M. chinensis* and *Maetra* sp. collected along the coast of China [14]. In total, 16 sequences of the species *M. chinensis* and *Maetra* sp. were used and one sequence of *M. veneriformes* served as an outgroup. The length of sequences after alignment and reduction to a certain length was 591 bp. The number of the variable sites was 126 bp, of which 78 bp were parsimoniously informative. The phylogenetic trees were constructed on the basis of the aligned set of sequences. According to the Hierarchical Likelihood Ratio Test, the model TN + I (Tamura–Nei model) has been chosen as the best one for the set of sequences of *COI* gene. The following approaches were used to construct the phylogenetic tree: the Bayesian (BA), maximum parsimony (MP), maximum likelihood (ML), and the neighbor-joining (NJ) methods.

The nucleotide sequence analysis of the mitochondrial gene *COI* has demonstrated that there are three clades of *M. chinensis* in the Yellow Sea and the Sea of Japan (Fig. 3a). The average distances within clades I,

II, and III were the following: 0.18% ± 0.03%, 0%, and 0.65% ± 0.08%, respectively (the average distance ± standard error). Hence, according to average distances (Fig. 3b), the three clades representing *M. chinensis* are assumed to constitute separate species taxa with a high significance (Fig. 3b), if we take into account the Rule 10-fold that has been proposed earlier in the reports on DNA-barcoding of species [5, 6]. Thus, *M. chinensis* species is likely to be revised as a complex that consists of three divergent species.

Since the members of the northern and southern populations in the Sea of Japan belong to different clades (clades I and II, respectively) (Fig. 3a), the assumption on *M. chinensis* divergence in the Sea of Japan is most probably correct [10, 11]. Thus, the fact that different variants of sperm cells predominate in the northern and southern populations is probably related to genetic divergence, which is accompanied by optimization of the species-specific pattern of male gametes in arising subspecies of Chinese *Maetra*. It may well be that the gamete heteromorphism is a specific phenomenon characteristic of the diverging species, whose genome produces “a set for choice.” Since three clades of the Chinese *Maetra* appeared due to

genetic divergence, the members of the third clade should be studied for possible predomination of the third morphotype of sperm cells, which is the reserve variant in the members of two other clades. We are going to study this issue in the near future.

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