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6 1 **In the Sea of Japan and the Adriatic Sea, a COX1 DNA test revealed genetic**
7 2 **similarity of Manila clams, although shell parameters, spermatogenesis**
8 3 **patterns, and sperm characteristics are area specific**
9 4

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6 **33 Abstract**

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8 34 The two Manila clam forms living in the Sea of Japan and in the Adriatic Sea were compared
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10 35 genetically and morphologically to identify traits that could be used to authenticate these
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12 36 geographic forms. COX1 DNA barcoding confirmed that both forms of these venerid bivalve
13
14 37 belong to the species *Ruditapes philippinarum* (Adams and Reeve, 1850). It has also been shown
15
16 38 that, based on COX1 analysis, it is not possible to separate these forms into different clusters
17
18 39 based on geographic origin. However, some morphological features make it possible to
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20 40 differentiate these forms. It is noted that the contour of the shells of the Adriatic and Pacific
21
22 41 molluscs is rounded and oblong, respectively. Underwater analysis of Pacific clams showed that
23
24 42 shell color may be specific to specimens found at certain collection sites and differ between
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26 43 specimens taken from different collection sites. In the spermatogenesis of Adriatic and Pacific
27
28 44 clams, a difference was found in the cellular mechanism of interaction between germplasm
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30 45 granules and mitochondria, manifested by distant contact and direct contact. Spermiogenesis has
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32 46 three similar lines of acrosome development, but differs in the number of ways in which the
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34 47 nucleus is formed. Due to the variability of the ways of sperm formation, heteromorphic morphs
35
36 48 of spermatozoa are formed, the total number of which is six for the species *R. philippinarum*. In
37
38 49 the Adriatic Manila clam, five morphs from this set are expressed. The Pacific Manila clam has
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40 50 only three sperm morphs. Only two sperm morphs are similar in both forms. In addition, each
41
42 51 form is distinguished by the presence of unique sperm morphs and each form has its own type of
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44 52 dominant spermatozoon. Thus, the contour and color of the shells, as well as cytologic markers
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46 53 that have been found in the mechanisms of differentiation of meiotic cells and the characteristics
47
48 54 of spermatozoa, can be used to distinguish the Adriatic and Pacific forms of the bivalve mollusc
49
50 55 *R. philippinarum*.

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53
54 57 **Keywords** Manila clam, *Ruditapes philippinarum*, Adriatic Sea, Japan Sea, DNA barcoding,
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56 58 COX1, shells, spermatogenesis, sperm, geo-authentication.
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64 Introduction

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

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

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

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

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6 95 2017). Despite the fact that a number of factors, as well as local hybridization, can cause
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8 96 intraspecific genetic divergence in bivalves (Skurikhina et al. 2001; Ni et al. 2015; Chetoui et al.
9
10 97 2016), this is not the case for *R. philippinarum*. According to electrophoretic studies, natural
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12 98 hybridization of the Western Pacific *R. philippinarum* with the European native *R. decussatus*
13
14 99 (Linnaeus, 1758) is impossible due to the degree of genetic dissimilarity (Fava and Meggiato
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16 100 1995). Also, experimental work on obtaining hybrids by mixing mature gametes of *R.*
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18 101 *philippinarum* and *R. decussatus* showed the impossibility of successful hybridization (Markaide
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20 102 et al. 2021).

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22 103 Considering the genetic similarity between the invasive (“North American/European”) and
23
24 104 the original (“Japanese”) *R. philippinarum*, the question arises whether it is possible to
25
26 105 distinguish the geographical forms of this species. This situation is becoming increasingly
27
28 106 important at the front of today commercial challenges. For example, in the lagoons of the
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30 107 Adriatic Sea in Northern Italy, Manila clam farming provides important socio-economic benefits,
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32 108 and local clams must be registered in the Protected Designation of Origin system. Therefore, it is
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34 109 necessary to develop tests for tracking the commercial circulation of shellfish in order to ensure
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36 110 the identification of the origin of the product with the possible prevention of fraud (Bianchini
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38 111 2021). In this regard, a comprehensive comparative study of the Adriatic Manila clam in relation
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40 112 to the original form of this species is necessary in order to find reliable criteria for identifying
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42 113 specimens.

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44 114 When looking for criteria, it should be taken into account that the genetic analysis of *R.*
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46 115 *philippinarum* is incomplete, since not all original populations of this species have been
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48 116 genetically tested. For example, the wild Manila clam living in the Russian part of the Sea of
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50 117 Japan has never been studied. It seems appropriate to study this Pacific form in order to compare
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52 118 it with the Adriatic form.

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54 119 It is not entirely clear whether shell color can be useful in identifying the geographic forms of
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56 120 the Manila clam. The shells of this species usually vary a lot in color: white, cream, yellow or
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58 121 light brown, sometimes with rays, streaks, blotches or zigzags of a darker brown/black, often
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60 122 with curved radiating darker bands or dark blotches (Yan et al. 2019; Ding et al. 2021).
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62 123 However, despite the diversity of coloration, there is probably a chance to use shell coloration to
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64 124 distinguish between geographic forms. Indeed, despite the wide variety of shell colors of the
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66 125 mactrid mollusc *Macra chinensis* (Philippi, 1846), it has been shown that shell shade

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6 126 corresponds to the shade of the seabed, and this phenomenon may help to establish the
7 127 geographical origin of this mollusc (Reunov et al. 2021a). Thus, it is worth checking whether a
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9 128 similar phenomenon can be found in the Manila clam.

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11 129 Recently, as a result of detailed re-examination of sperm, heteromorphism or plasticity of
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13 130 spermatozoa has been discovered as the normal physiological state of marine bivalves such as *C.*
14 131 *gigas* and *M. chinensis*. Moreover, it was found that because the quantitative characteristics of
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16 132 heteromorphic sets of spermatozoa correlate with local environmental conditions, this
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18 133 characteristic can serve as a geographical marker (Reunov et al. 2018, 2021a). It is interesting to
19
20 134 find out whether analysis of sperm morphs can help in establishing the geographical origin of the
21
22 135 Manila clam. In addition, the ultrastructural features of the spermatogenesis of the Adriatic *R.*
23
24 136 *philippinarum* have recently been studied (Reunov et al. 2019a, 2021b), and it would be relevant
25
26 137 to study the spermatogenesis of the Pacific *R. philippinarum* to find out if there are any
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28 138 differences suitable for determining geographic forms.

29 139 The aim of this work was to: (1) compare the COX1 sequence between the Adriatic Manila
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31 140 clam and the wild Pacific Manila clam living in the Russian part of the Sea of Japan, and to
32
33 141 compare the obtained data with already existing data for other populations of this species; (2)
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35 142 check by analysis directly on the seabed using scuba diving whether the shell color of Manila
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37 143 clams collected in different areas can be geographically specific; (3) find out if any differences in
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39 144 the development of spermatogenic cells and the structure of spermatozoa can be detected and
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41 145 used to distinguish between Atlantic and Pacific geographic forms. We hope that our study will
42
43 146 help to find new criteria for geo-authentication of specimens of the bivalve mollusc *R.*
44 147 *philippinarum*.

45 148 46 47 48 149 **Materials and methods**

49 150 50 51 151 **Sample collection**

52 152 53 54 55 153 **Adriatic Manila clam**

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59 155 Specimens were obtained from the fish market in Bologna (Italy) in May-July 2019.
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61 156 According to the supplier, the specimens were collected from Sacca di Goro (Adriatic Sea, FE,
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6 157 Italy). Geographic coordinate of the location cite is 44°49'N, 12°18'E. However, the origin of
7 158 the specimens is not exactly known, as underwater research has not been carried out.

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11 160 Pacific Manila clam

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15 162 Specimens were collected in Peter the Great Bay (Sea of Japan, Russia) by scuba diving
16
17 163 during May-July 2019 and May-July 2020 from three geographic locations (Fig. 1). At each
18
19 164 point, shellfish were collected on an area of about 10 square meters. The first collection site
20
21 165 (point # 1) was in Vostok Bay (coordinates: 42°54'13.1"N 132°43'35.7"E). The second and third
22
23 166 locations (point # 2 and point # 3) were in the Amursky Bay (coordinates: 43°12'03.3"N
24
25 167 131°55'13.2"E and 43°11'53.6"N 131°55'09.7"E, respectively). Coordinates were identified
26
27 168 using manual profiler Cast Away ctd. (SonTek, USA). To analyze the color of the shells, 30
28
29 169 individuals were collected at each collection point.

30 170

31 171 **COX1 barcoding and phylogenetic analyses**

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35 173 Five specimens (Rph m1 cox1F, Rph m2 cox1F, Rph m3 cox1F, Rph m4 cox1F, Rph m5
36
37 174 cox1F) and eight specimens (CCDB-ST03131, CCDB-ST03132, CCDB-ST03133, CCDB-
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39 175 ST03136, CCDB-ST03137, CCDB-ST03138, CCDB-ST03140, CCDB-ST03141) were analysed
40
41 176 for the Adriatic Manila clam and Pacific Manila clam, correspondingly. Extraction of DNA from
42
43 177 the samples was performed with spin-column extraction kit (DongSheng Biotech) following
44
45 178 producer instructions. For PCR amplification (with GoTaq Flexi2 kit, Promega), we used COX1
46
47 179 primer pairs designed on online available sequences to amplify the F-type mitochondrial gene
48
49 180 (Left Primer: TTTATGGGGTTGGTGTTAAAAA; Tm: 58.3 °C; GC: 31.8 %; Right Primer:
50
51 181 TAGTTAAACCCCTGCCAAA, bp Tm: 59.4 °C; GC: 45.0 %; Product Size: 732 bp). The
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53 182 PCR cycle consisted in an initial denaturation step at 94 °C for 2 min, followed by 35 cycles of
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55 183 denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 73 °C for 90 s. At the
56
57 184 end of the cycling steps, a final extension at 73 °C for 5 min was done. Both strands of
58
59 185 amplicons were sequenced at Macrogen Europe (The Netherlands).

60
61 186 To the COX1 partial sequences of the present study, we added all available *R. philippinarum*
62
63 187 COX1 sequences from the previous works of Cordero et al. (2017) and of Sekine et al. (2006).
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6 188 Since we were interested in population structures only, we considered only F-type sequences and
7
8 189 not M-type ones. Sequences were aligned with MAFFT (default parameters; Katoh and Standley
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10 190 2013) and the alignment was manually trimmed to keep only positions shared by all samples,
11
12 191 with a final length of 555 base pairs. A maximum likelihood tree was inferred from it with IQ-
13
14 192 TREE (MG+F1X4+G4 codon model, inferred as best model of evolution by ModelFinder as
15
16 193 implemented on IQ-TREE; 1000 bootstrap replicates were performed to retrieve node supports;
17
18 194 Minh et al. 2020). A phylogenetic network was built on the same alignment with PopART
19
20 195 (Leigh and Bryant 2015) using the median-joining method (default parameters).

21 22 197 **Shell coloration comparison**

23
24 198
25
26 199 For the Adriatic Manila clam, as well as for the Pacific Manila clam, the shells of genetically
27
28 200 identified specimens were dried and photographed. The images were compared.

29 30 201 31 202 **Transmission electron microscopy**

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33 203
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35 204 Some of the genetically identified specimens were selected for study. Three specimens of the
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37 205 Adriatic Manila clam and three specimens of the Pacific Manila clam were analyzed. For the
38
39 206 Pacific Manila clam, specimens collected only at point # 1 were used. The testes were removed,
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41 207 cut into small pieces and fixed overnight in primary fixative containing 2.5% glutaraldehyde in
42
43 208 0.1 M cacodylate buffer, pH 7.4) at 4 °C. Fixed tissues were washed in buffer, postfixed in 2%
44
45 209 OsO₄ in 0.1 M cacodylate buffer for 2 h, rinsed in 0.1 M cacodylate buffer and distilled water,
46
47 210 dehydrated in an ethanol series and acetone, infiltrated and embedded in Spurr resin. Ultra-thin
48
49 211 sections were mounted on slot grids that were coated with formvar film. Sections were stained
50
51 212 with 2% alcoholic uranyl acetate and aqueous lead citrate and were examined with a
52
53 213 transmission electron microscope Zeiss Libra 120 (A Carl Zeiss SMT AG Company,
54
55 214 Oberkochen, Germany) and Philips 410 Transmission Electron Microscope (Philips 123
56
57 215 Electronics, Eindhoven, The Netherlands).

58 59 217 **Scanning electron microscopy**

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6 218 Some of the genetically identified specimens were selected for study. Three specimens of the
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8 219 Adriatic Manila clam and three specimens of the Pacific Manila clam were analyzed. For the
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10 220 Pacific Manila clam, specimens collected only at point # 1 were used. The testes were removed,
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12 221 cut into small pieces, and fixed for 2–3 h (in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH
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14 222 7.4). Primary fixed materials were washed gradually in the same buffer. Washed samples were
15
16 223 rinsed in buffer and distilled water, dehydrated in a graded series of ethanol solutions. Sperm
17
18 224 suspension was prepared by crushing pieces of fixed materials. The suspension was pipetted onto
19
20 225 a Thermanox coverslip (Cat. # 72280) and allowed to settle for 1 h. Coverslips with attached
21
22 226 sperm cells were transferred to acetone and critical-point-dried in CO₂. Dried materials were
23
24 227 mounted onto aluminum stubs, coated with gold, and examined with a scanning electron
25
26 228 microscope LEO-430 (Horus Tech Inc., USA).

27 229 28 230 **Quantitative analysis of sperm morphs studied by scanning electron microscopy**

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30
31 232 500 sperm cells of each clam specimen were analyzed. 1500 sperm cells were analysed for
32
33 233 the Adriatic Manila clam and 1500 sperm cells were analysed for the Pacific Manila clams.
34
35 234 Sperm phenotypes were identified and frequency of each phenotype was calculated. The results
36
37 235 were analysed by the Microsoft Excel program using Student's t-test. All values are expressed as
38
39 236 means with standard error of the mean (SEM). Differences between groups were calculated using
40
41 237 Student's t-test. $p < 0.05$ was considered statistically significant.

42 238 43 44 239 **Results**

45 240 46 47 48 241 **Partial COX1 population structure**

49 242
50
51 243 Phylogenetic analysis carried out for five specimens of the Adriatic Manila clam and eight
52
53 244 specimens of the Pacific Manila clam confirmed that all specimens belonged to the species *R.*
54
55 245 *philippinarum*. Both the maximum-likelihood phylogenetic tree (Fig. 2) and the median-joining
56
57 246 phylogenetic network of the partial F-type COX1 sequences (Fig. 3) agreed in clustering our
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59 247 sample sequences among specimens belonging to the mitochondrial haplogroup A (referring to
60
61 248 the nomenclature adopted by Cordero et al. 2017). Within this haplogroup, our samples from the

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

253 **Adriatic Manila clam shells**

254
255 These were (1) a shell, the color of which is represented by brownish squares on a yellow
256 background (Fig. 4a), (2) a shell with a gray background, on which there are several radial purple
257 stripes, and there is also a slight yellowish tint on the side (Fig. 4b), (3) a shell with many purple
258 zigzags on a gray background (Fig. 4c), (4) a shell with a slightly yellowish background without
259 stripes (Fig. 4d) and (5) a shell with a yellowish background, on which there are radial brownish
260 stripes (Fig. 4e). The shell contour is relatively round, and the top of the shell (umbo) is more
261 centered (Fig. 4a-e) than in the shells of Pacific Manila clams (Fig. 4f-m).

263 **Pacific Manila clam shells**

264
265 Three shell colors have been found among the genetically identified specimens. These were:
266 (1) gray shells (Fig. 4f-h), (2) ocher-colored shells (Fig. 4i-k) and cream-colored shells (Fig. 4l,
267 m). Each of the three collection sites (Fig. 1) corresponded to one of the three revealed colors.
268 Apart from the genetically determined specimens, the same shell color was found in each of the
269 thirty specimens collected at each collection point. Among the 90 specimens, shell colors did not
270 overlap between collection sites and were geographically specific.

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

275 **Early spermatogenic cells**

276
277 In the testes of the Manila clam collected both in the Sea of Japan and the Adriatic Sea, early
278 spermatogenic cells (spermatogonia and spermatocytes), identified according to the previously
279 described features (Reunov et al. 2019a), are tightly packed in groups (Fig. 5a). In the testes of

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280 both clam forms, spermatogonia and spermatocytes are similar in that they have the so-called
281 germplasm granules (GG), which are located in the cytoplasm. In the spermatogonia of the
282 Manila clam of the Sea of Japan, GG are irregularly shaped groups of granules (Fig. 5b). These
283 granules tend to be in contact with mitochondria (Fig. 5c, d). Due to the interaction of GG with
284 mitochondria, mitochondrial clusters arise, which are characteristic of the close contact of GG
285 with mitochondrial membranes (Fig. 5e). In the spermatogonia of the Adriatic Manila clams, GG
286 are less common, have a larger size, and never come into direct contact with mitochondria. (Fig.
287 5f, g).

288
289 Early spermiogenesis

290
291 During early spermiogenesis of both forms of Manila clam, an acrosome vesicle produced by
292 the Golgi complex (not shown) was found in the basal part of the cell near the flagellum and
293 mitochondria (Fig. 6a). It is surrounded by a membrane and filled with an electron-dense
294 material that features a more electron-light central part (Fig. 6b). Initially located in the
295 middle part of the spermatid (Fig. 6a), the acrosomal vesicle then moves in the lateral
296 part of the spermatid (Fig. 6c). Finally, it is located in the apical cellular region (Fig. 6d). In
297 spermatids of both forms of Manila clam, during the next differentiation, the apically located
298 acrosomal vesicle changes its shape from round to flat (Fig. 6e, f). Flat acrosomal vesicles
299 show an invagination filled with loose periacrosomal material (Fig. 6g). In more advanced
300 spermatids, the acrosome vesicle acquires a conical shape, and its invagination contains
301 periacrosomal material that contacts the surface of the nucleus (Fig. 6h). The next stage of
302 differentiation is characterised by elongation of the periacrosomal material body and upward
303 ascent of the acrosomal vesicle (Fig. 6i).

304
305 Late spermiogenesis

306
307 During late spermiogenesis, both the Adriatic Manila clam and the Pacific Manila clam were
308 found to have three patterns of acrosome formation. The first was distinguished by the formation
309 of a straight acrosome. This is found with a gradual elongation of the apical part of the
310 spermatid. This elongation occurs due to the expansion of the column of periacrosomal material

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311 with the gradual ascent of the cone-shaped acrosomal vesicle (Fig. 7a-c). This pattern of
312 spermiogenesis culminates in the formation of sperm with straight or slightly curved acrosomes,
313 which are located along the anteroposterior axis of the spermatozoa (Fig. 7d). The second pattern
314 is usually found with the acrosome having a strong bend during its elongation (Fig. 7e). The third
315 is a pattern recorded as acrosome folding, that occurs at the onset of acrosome elongation (Fig.
316 7f, g). During the next formation, the acrosome gradually rises upward (Fig. 7h-j). As a result of
317 this pattern, undulating acrosomes were recorded, which have a bend in the middle part (Fig. 7k).

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

324 **Structure of spermatozoa**

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

334 Using scanning electron microscopy, three sperm morphs unique to the Adriatic Manila clam
335 were found. These were (1) spermatozoa with a straight nucleus and a straight acrosome
336 (Fig. 8a); (2) spermatozoa with a straight nucleus and curved acrosome (Fig. 8b); (3)
337 spermatozoa with a straight nucleus and wavy acrosome (Fig. 8c). Also, the Adriatic Manila
338 clam have (4) spermatozoa with a curved nucleus and a slightly curved acrosome (Fig.
339 8d), and (5) spermatozoa with a curved nucleus and a strongly curved acrosome (Fig. 8e).

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

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6 342 acrosome (Fig. 8f, g), which morphologically correspond to the sperm models 4 and 5 found in
7 343 the Adriatic Manila clam (Fig. 8d, e). Moreover, the Pacific Manila clam have (6) a sperm morph
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9 344 with a curved nucleus and undulating acrosome (Fig. 8h). Thus, the intraspecific set of
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11 345 spermatozoa of *R. philippinarum*, found on the basis of the analysis of the sperm of two
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13 346 geographic forms of this species, includes six morphs, which we designated here as SPERM1,
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15 347 SPERM2, SPERM3, SPERM4, SPERM5, and SPERM6 (Fig. 8i).

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17 348 Quantitative analysis showed that the proportions of sperm samples differ. Adriatic
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19 349 Manila clams have different percentages of sperm morphs: 12%, 22%, 17%, 40% and 9% are
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21 350 accounted for by SPERM1, SPERM2, SPERM3, SPERM4 and SPERM5, respectively (Fig.
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23 351 8j). In the Pacific Manila clams: 20%, 14%, 66% correspond to SPERM4, SPERM5 and
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25 352 SPERM6 (Fig. 8k).

27 354 **Discussion**

31 356 **Manila clam from the Sea of Japan and the Adriatic Sea belong to the same haplogroup of** 32 33 357 **COX1 genes and cannot be separated based on geographic origin**

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37 359 By analysing the COX1 partial DNA sequences of our specimens, we could confidently
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39 360 assess that they belonged to the species *R. philippinarum*. Moreover, we were also able to
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41 361 confirm the genetic similarity of such species in the Russian part of the Sea of Japan and in the
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43 362 Adriatic Sea. Indeed, all our samples belonged to haplogroup A (referring to the
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45 363 nomenclature adopted by Cordero et al. 2017). This haplogroup was characteristic of *R.*
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47 364 *philippinarum* inhabiting the Japanese part of the Sea of Japan, and from our data it now also
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49 365 includes samples from the Russian part of the Sea of Japan. The clustering of these samples
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51 366 together with those from the Adriatic Sea is coherent with the fact that the colonization of
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53 367 *R. philippinarum* in America, and subsequently in the Adriatic Sea, involved individuals
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55 368 belonging to haplogroup A (Cordero et al. 2017).

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57 370 However, the haplogroup A is separated by haplogroups B and C, that are more spread in the
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59 371 Chinese coast (Cordero et al. 2017). Indeed, the indigenous population of *R. philippinarum*
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61 372 studied in East Asia is divided into three geographic populations. These wild populations
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63 have large genetic differences, which may be due to geographic isolation (Tan et al. 2020).

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65 Thus,

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6 373 although COX1 testing is not useful to differentiate samples from the Adriatic Sea from those of
7 374 the Sea of Japan, geo-authentication of samples from south areas of China is possible.

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9 375 It should be noted that other molecular approaches work well for Manila clam geo-
10 authentication. Using a dual-labelled PNA-probe-based melting curve analysis the foreign
11 376 (Chinese) and domestic (Korean) Manila clams having similar morphology were discriminated
12 377 (Kim et al. 2015). In addition, elemental analysis coupled with isotope ratio mass
13 378 spectrometry has been used to identify isotopic fingerprints of *R. philippinarum* collected from
14 379 three Adriatic lagoons, and this method appears promising for tracking the geographic origin
15 380 of the Manila clam at a regional level (Bianchini et al. 2021).
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23 383 **The color of the shell may be promising for determining the origin of specimens**

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27 385 During this study, five color patterns were found in specimens of the Adriatic Manila clam.
28 386 Three variants of shell coloration were found in the Pacific Manila clam. Thus, both forms of *R.*
29 387 *philippinarum* are characterized by the phenomenon of intraspecific shell plasticity.

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32 388 This conclusion is consistent with previous data on the Manila clam. It is known that the
33 389 shells of this mollusc can have a variety of colors, which can be complemented by rays, stripes,
34 390 spots and zigzags (Yan et al. 2019; Ding et al. 2021). This color diversity is provided by the
35 391 differential expression of genes such as Tyr and Mitf (Yan et al. 2019), and miRNAs, which
36 392 have been shown to play a role in the production and regulation of shell pigments (Ding et
37 393 al. 2021). With regard to the role of environmental factors, it has been suggested that various
38 394 biotic and abiotic factors can influence shell color in molluscs (Sokolova and Berger 2000;
39 395 Williams 2017; Yuan et al. 2020). Certainly, the color of the substrate is very important in
40 396 determining the color of the shells (Morton 1976; Moss 1990; Sigurdson and Sundari 1990;
41 397 Reunov et al. 2021a).

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50 398 For the Pacific Manila clam, we found that shell color is specific to each collection site.
51 399 Divers who collected clams for this project reported that the type of substrate was different at the
52 400 three collection sites, and the overall color tone of the seafloor was specific to each site. We
53 401 hypothesize that shell colors correlate with seafloor hue and find it reasonable to suggest
54 402 that shell color can be used as a geographically specific marker. A more detailed study
55 403 aimed at identifying the correlation between the color of the shell and the seabed is planned by
56 us for the Manila clam living in the Sea of Japan.
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6 404 Also, we have not been able to verify whether the color of the shells of the Adriatic Manila
7 405 clams is related to the specifics of the seabed, since the samples were taken at the fish market. In
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9 406 this regard, it will be useful to plan scuba diving explorations in various geographic habitats of
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11 407 the Manila clam in the Adriatic lagoons.
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15 409 **The shell shape can be useful for distinguishing the geographic forms of the Manila clam**
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18 411 Although our study did not include an analysis of shell morphology, we observed that the
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20 412 shell outline and position of the umbo were different in the Manila clams of the Adriatic and the
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22 413 Japan Seas. The oblong shape and lateral position of the umbo were more characteristic of the
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24 414 shells of the Pacific Manila clam, while the rounded shape with a more central location of the
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26 415 umbo was more characteristic of the Adriatic Manila clam. It is possible that the morphology of
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28 416 the shell can also help distinguish the geographical forms of this mollusc. Indeed, along the coast
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30 417 of Jeju Island in the Korean part of the Pacific Ocean, the shell shape of *R. philippinarum* varied
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32 418 significantly between sites (Silina 2010). Identification of phenotypic variations of shell contour
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34 419 has been proposed in order to distinguish *R. philippinarum* collected from several points on
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36 420 the Atlantic coast of France (Caill-Milly et al. 2014). In the Matsukawaura Lagoon (Japan), the
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38 421 shell shapes of wild Manila clams also varied significantly depending on the habitat
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40 422 (Tomiyaama 2021).
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44 424 **Using morphological differences observed in differentiating meiotic cells, it is possible to**
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46 425 **distinguish the geographical forms of the Manila clam**
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48 427 We recently analyzed the oogenesis and spermatogenesis of the Adriatic Manila clam,
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50 428 focusing on the study of the so-called germplasm granules (GG), cytoplasmic structures that are
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52 429 involved in the transition from mitosis to meiosis (Reunov et al. 2019a). In the present
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54 430 work, devoted to the analysis of two forms of this mollusc, we compared the morphological
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56 431 character of the meiotic differentiation of the Adriatic Manila clam with this process
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58 432 occurring in the Manila clam living in the Sea of Japan. Interestingly, a clear difference was
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60 433 found. It was figured out that instead of "remote interaction" with mitochondria, which is
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62 434 characteristic of the GG of the Adriatic Manila clam, the pre-meiotic cells of the Manila clam
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64 living in the Sea of Japan are
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6 435 characterized by direct contact of the GG with mitochondria (Fig. 9a, a'). In our opinion, this
7 436 difference can serve as a cytological marker for distinguishing the forms of *R. philippinarum*.

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9 437 It is difficult to explain the difference in the spatial localization of GG and mitochondria
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11 438 interacting in meiotic cells. Probably, these organelles can carry out the same molecular event
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13 439 both at a distance and in contact with each other. On samples of the Adriatic Manila clam, it was
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15 440 shown that the GG/mitochondrial interaction is accompanied by the penetration of the VASA
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17 441 protein from the VASA-positive GG to the mitochondria, which, in turn, release their matrix into
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19 442 the cytoplasm, as evidenced by the cytoplasmic localization of CYTB (Reunov et al. 2019a,
20 443 2021b). It remains to be clarified whether the same phenomenon takes place during the meiotic
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22 444 differentiation of the Pacific Manila clam. Probably, such interaction of GG with mitochondria is
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24 445 also possible in the Pacific form, since the cytoplasmic localization of mitochondrial content is
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26 446 considered to be universal for meiotic differentiation of various animal species. Its role appears
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28 447 to be to provide mitochondrial ribosomes for the translation of cytoplasmic proteins during the
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30 448 transition from mitosis to meiosis, as has been suggested for *Drosophila*, mouse, and
31 449 zebrafish (for review Reunov et al. 2019b).

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35 451 **The number of sperm morphs, the presence of unique sperm morphs, and the type of**
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37 452 **dominant sperm variant allow to distinguish between the geographical forms of the Manila**
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39 453 **clam**

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42 455 Interestingly, both forms of Manila clam lack the monomorphism of the last phase of
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44 456 spermatogenesis – i.e. spermiogenesis. Indeed, during spermiogenesis of both forms, three types
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46 457 of acrosomes are formed: straight, curved, and folded. In addition, the Adriatic Manila clam has
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48 458 two patterns of nucleus development (straight and curved). Thus, in *R. philippinarum*,
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50 459 spermiogenesis is heteromorphic.

51 460 Heteromorphism in the mechanisms of sperm formation is not unique to Manila
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53 461 clams. Reunov et al. (1999) found two patterns of acrosome development in
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55 462 spermiogenesis in the mussel *Perna viridis* (Linnaeus, 1758). Au et al. (1998) described four
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57 463 lines of spermiogenesis in the sea urchin *Anthocardis crassispina* (A. Agassiz, 1864).
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59 464 Considering that the number of patterns of nucleus formation is different in the two forms of
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6 465 this feature is applicable as a cytological marker for the geographical forms of this
7 466 differentiating mollusc (Fig. 9b, b').

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9 467 We also investigated whether the structure of the sperm could be used to recognize the
10 geographic forms of the Manila clam. In the Adriatic Manila clam, a bullet-shaped nucleus
11 468 cupped with a thin acrosome was found in spermatozoa (Reunov et al. 2019a, 2021b). This
12 469 general model is consistent with sperm found in samples of Manila clams collected in the Pacific
13 470 region (this article), as well as sperm of Manila clams found along the Korean coast (Kim et al.
14 471 2013; Gwo et al. 2021). However, our study provided more detailed data on the morphology
15 472 of spermatozoa and revealed their heteromorphism.
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18 474 Interestingly, sperm heteromorphism was recently found in other bivalve molluscs, such as
19 the Pacific oyster *C. gigas*, the heteromorphic set of spermatozoa of which consists of six
20 475 morphologically stable morphs (Reunov et al. 2018), and in the surf clam *M. chinensis*, in which
21 476 the set of spermatozoa consists of four morphologically stable morphs (Reunov et al. 2021a). We
22 477 believe that *R. philippinarum* can also be attributed to the group of bivalves with heteromorphic
23 478 spermatozoa.
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26 480 The phenomenon of intraspecific heteromorphism of spermatozoa is known in animals
27 (Morrow and Gage 2001). The functional parameters of heteromorphic spermatozoa can
28 481 vary, for example, the cells having higher speed may have shorter life span and the cells having
29 482 lower speed may have higher life span (Levitan 2000). Also, spermatozoa of different
30 483 phenotypes can be genetically different, ensuring different success in sperm competition
31 484 (Borowsky et al. 2018). In *C. gigas* oysters, which reproduce by external fertilization in
32 485 seawater, the causes of sperm plasticity are associated with reproductive adaptation to the
33 486 aquatic environment, which can be influenced by intense water current or turbulence,
34 487 unstable temperature and salinity, anthropogenic pollution. The dominant expression of one
35 488 or another variant of spermatozoa may be associated with a greater degree of adaptability
36 489 of this variant to a certain type of environment. Parallel production of additional sperm
37 490 variants can increase the chances of reproductive success in *C. gigas* oysters living in
38 491 unstable conditions (Reunov et al. 2018). Because *R. philippinarum* also has external
39 492 fertilization, a link between environmental conditions and sperm shape in this species
40 493 seems likely. Given that the total number of sperm morphs is higher in the Adriatic Manila
41 494 clam (Fig. 9c, c'), it can be suggested that the fertilization conditions may be more varied
42 495 in the case of the Adriatic Manila clam than those
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6 496 experienced by the Pacific Manila clam. Considering that both forms of molluscs have unique
7 sperm morphs (Fig. 9d, d'), it seems possible that unique conditions for fertilization can be found
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9 498 in both cases. Given that the dominant sperm variant of the two forms of Manila clam is different
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11 499 (Fig. 9e, e'), it would be tempting to speculate that the dominant ecological factor influencing
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13 500 fertilization is different for these two forms. In any case, we believe that parameters such as
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15 501 different numbers of sperm morphs (Fig. 9c, c'), the presence of unique sperm morphs (Fig. 9 d,
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17 502 d'), and a difference in the dominant sperm pattern (Fig. 9e, e') can be used as cytological
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19 503 markers to distinguish geographic forms of Manila clam.
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21 504

22 505 **Conclusion**

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26 507 Commercial certification of the Manila clam *R. philippinarum* requires reliable methods to
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28 508 trace the geographic origin of this mollusc specimens. The study of the mitochondrial COX1
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30 509 gene showed that the Adriatic Manila clam and Manila clam collected in the Russian part of the
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32 510 Sea of Japan are genetically indistinguishable. However, shell color and morphology can be used
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34 511 to determine specimen origin. Besides, spermatogenesis of Adriatic and Pacific Manila clams
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36 512 differs in the spatial distribution of germ plasm granules and mitochondria. Moreover, spermatid
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38 513 development differs in the number of nuclear formation patterns. Manila clam forms also differ
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40 514 in the number of sperm morphs, the presence of unique sperm morphs, and the type of dominant
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42 515 sperm morphs. Although an electron microscopy laboratory is required to compare reproductive
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44 516 cells, their differences can be used as cytological markers to determine the geographical origin of
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46 517 Manila clam.
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61
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529

Author contributions

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

538

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542

Availability of data and material

544 The datasets generated and analysed during the current study are available from the
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547

Conflict of interest

549 The authors declare that they have no conflict of interest.

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Ethics approval

552 Not applicable.

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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 *Macra 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).

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

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

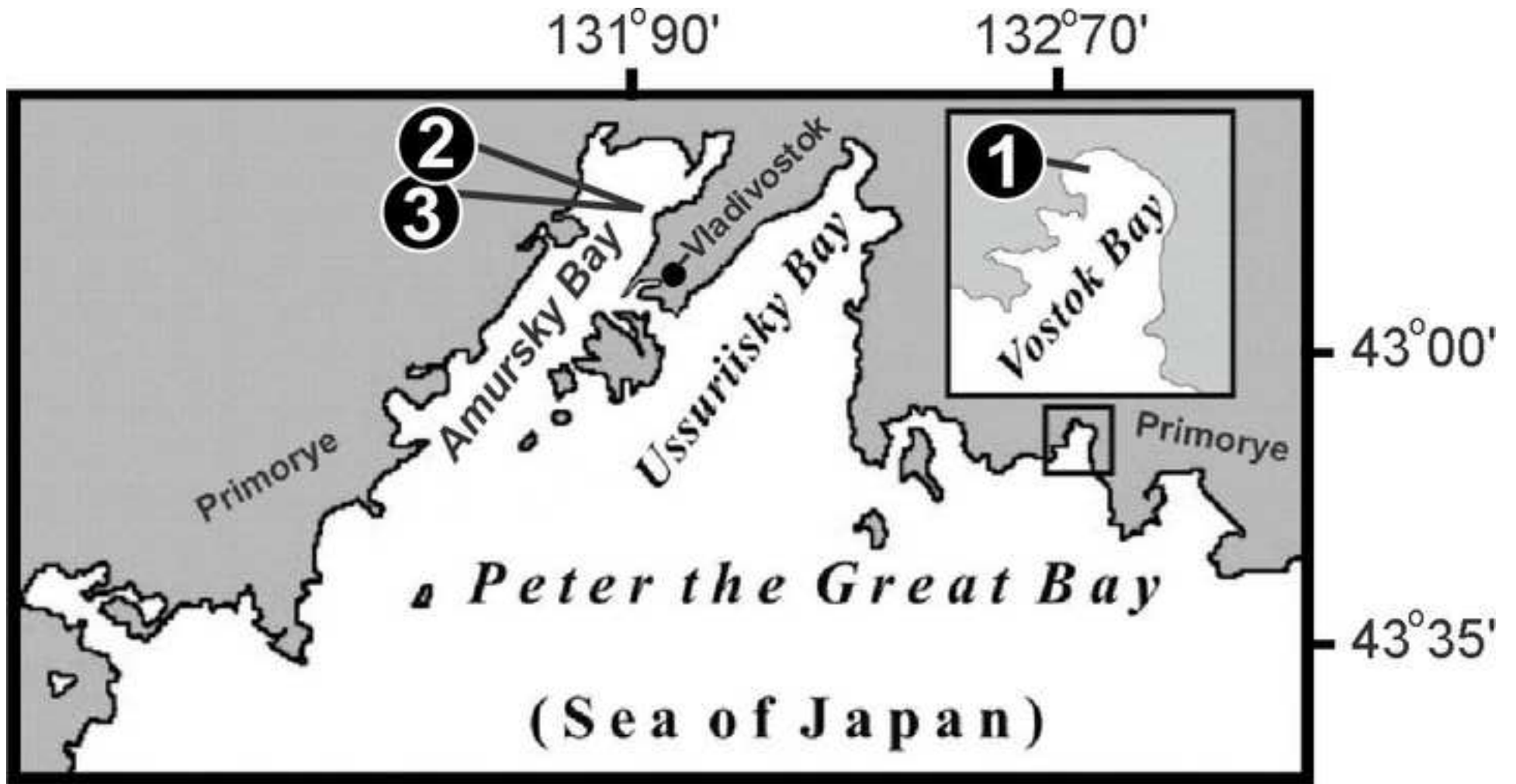
754 **Fig. 6** Early spermiogenesis of the Manila clam *Ruditapes philippinarum* by transmission
755 electron microscopy. The cell ultrastructure is the same in both forms of the Manila clam and is
756 shown here mainly on the example of the Adriatic Manila clam. **a, b** The acrosomal vesicle in
757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

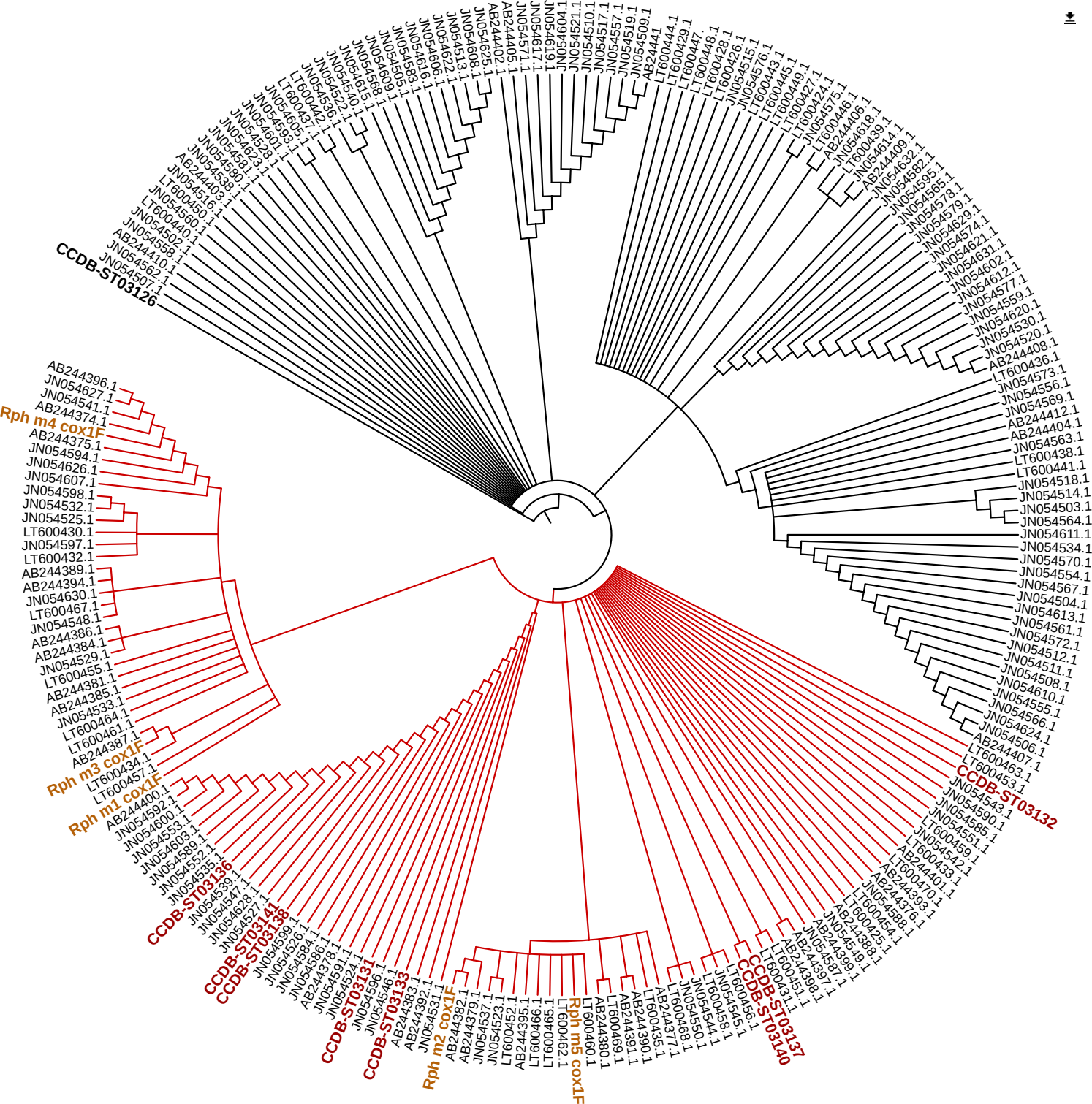
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Fig. 7 Late spermiogenesis, of the Manila clam *Ruditapes philippinarum*. The data were obtained by transmission electron microscopy (**a-c, e, f, j, l, m**) and scanning electron microscopy (**d, g-i, k**). **a-d** Development of a straight acrosome; **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 μ m.

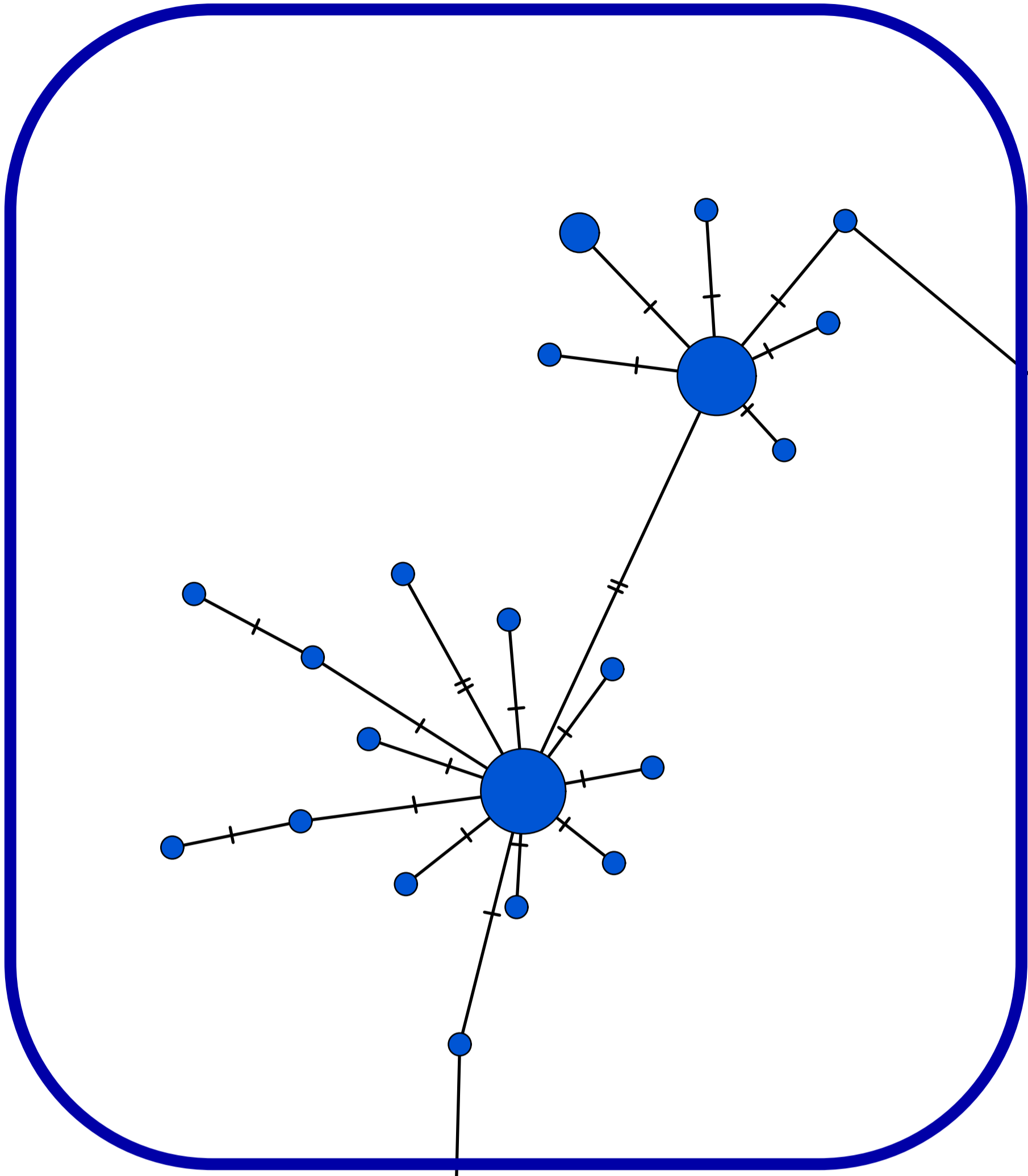
Fig. 8 Intraspecific diversity of spermatozoa of the Manila clam *Ruditapes philippinarum* revealed by the analysis of external cell morphology by scanning electron microscopy. **a-e** Sperm morphs characteristic of the Adriatic Manila clam; **f-h** sperm morphs characteristic of the Pacific Manila clam. **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 μ m.

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'**).

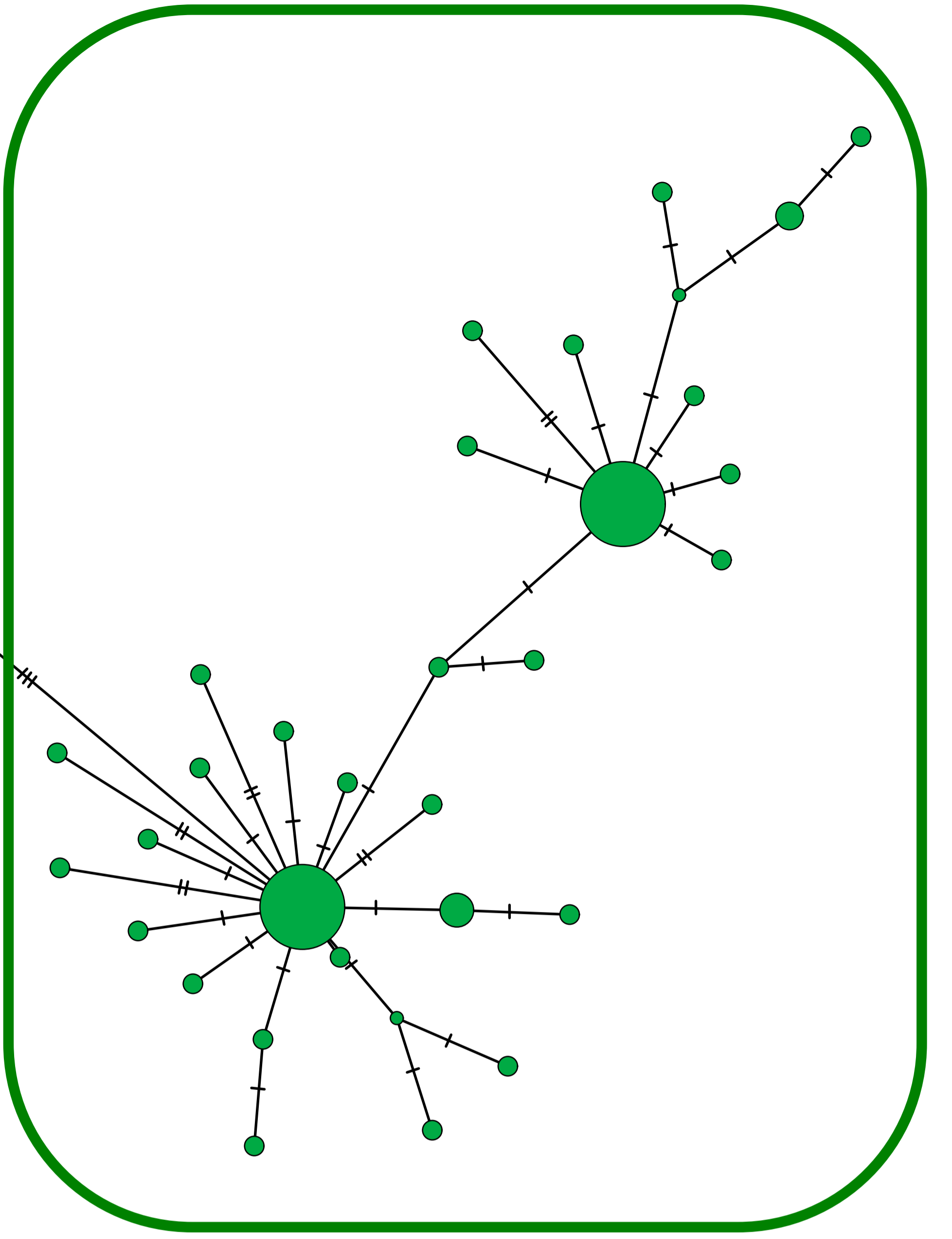




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