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Genetic and morphological evidence of the existence of three gastropod species of the family Caecidae in Vostok Bay (Peter the Great Bay, Sea of Japan)

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Three gastropod species of the family Caecidae Gray, 1850 were found in Vostok Bay: *Caecum* (*Fartulum*) *bucerium* (Golikov, 1967), *Caecum* (*Brochina*) *derjugini* (Golikov, 1967), and one unknown species. All species were compared using 12 allozyme loci, the shell and penial morphology. *C.* (*F.*) *bucerium* and *C.* (*B.*) *derjugini* differ both genetically (genetic distance between them is 1.329) and morphologically (penial morphology and shape of the mucro). The third species is more similar to *C.* (*F.*) *bucerium* (genetic distance between them is 0.633) but it differs from the latter species in the penial morphology and the shape of the mucro.

Генетические и морфологические доказательства существования трех видов брюхоногих моллюсков семейства Caecidae в заливе Восток (залив Петра Великого, Японское море)

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В зал. Восток было обнаружено три вида брюхоногих моллюсков сем. Caecidae Gray, 1850: *Caecum* (*Fartulum*) *bucerium* (Golikov, 1967), *Caecum* (*Brochina*) *derjugini* (Golikov, 1967), и один неизвестный ранее вид. Все виды сравнили, используя генетические (12 аллозимных локусов) и морфологические (морфология раковины и пениса) признаки. *C.* (*F.*) *bucerium* и *C.* (*B.*) *derjugini* хорошо различаются как генетически (генетическое расстояние (D) между ними составляет 1.329), так и морфологически (по морфологии пениса и мукро). Третий вид генетически оказался наиболее близок к *C.* (*F.*) *bucerium* (D=0.633), но отличался от последнего по форме мукро и пениса.

Caecids, which are the small widespread marine snails (to 3 mm in length) have a confused and controversial taxonomy [Absaläo, Gomes 2001; Absaläo, Pizzini, 2002]. According to Russian authors [Golikov, Scarlato 1967; Volova et al., 1979; Adrianov, Kussakin, 1998; Gulbin, 2004], two species of caecids, *Brochina derjugini* Golikov, 1967 and *Fartulum bucerium* Golikov, 1967, occur

in Peter the Great Bay (Sea of Japan). Conchological analysis showed that *Brochina* and *Fartulum* are two subgenera of the genus *Caecum* [Absaläo, Pizzini, 2002] included to the subfamily Caecinae. Both species occur from the upper to lower intertidal zone (depths of no more than 10 m). *Caecum derjugini* lives among rocks and on sandy gravel. *Caecum bucerium* is commonly found on sand among eelgrass *Zostera marina* and on rocks among the rhizoids of *Laminaria japonica*. Both species readily differ in the shape of the mucro. *C. derjugini* has a rather high, hemispherical mucro (Fig. 1A), whereas in *C. bucerium* it is the low and a triangular in outline (Fig. 1C). Specimens with an intermediate shape of the mucro (rounded-oval) were found together with *C. derjugini* (Fig. 1B) in Vostok Bay (Sea of Japan).

Allozymes as markers of genes are widely used in systematic studies of gastropod mollusks with morphological variability [Ward, Warwick, 1980; Palmer et al., 1990; Marko, 1998; Tatarenkov, Johannesson, 1998; Zaslavskaya, 1995; Zaslavskaya, Kolotukhina, 2003]. Nevertheless, this kind of data has never been applied for solving of taxonomic problems in the family Caecidae, although there are many questions for taxonomists

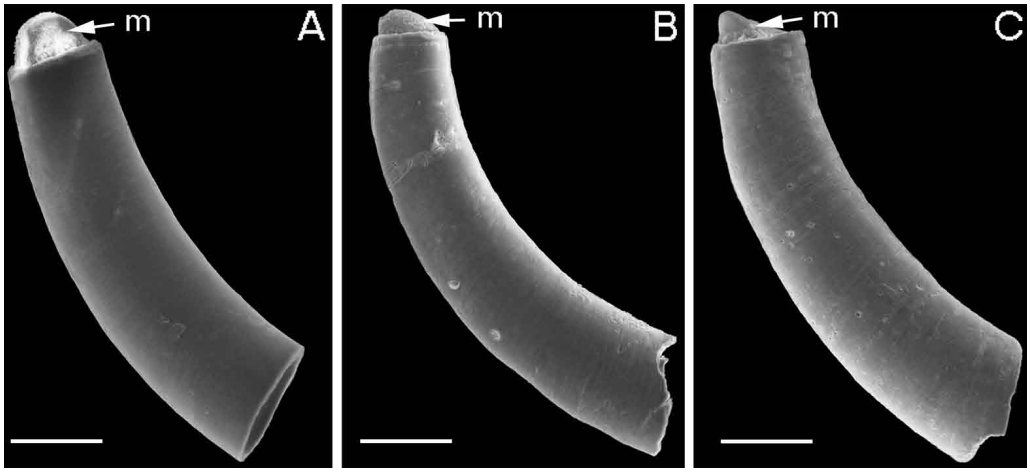

Fig. 1. Shell morphology of Caecidae (SEM). **A** – *Caecum* (*Brochina*) *derjugini*, **B** – *Caecum* sp., **C** – *Caecum* (*Fartulum*) *bucerium.* m – mucro; scale bar – 100 μm.

study of this group [Bandel, 1996; Ponder, Keyzer, 1998; Absaläo, Pizzini, 2002]. Obviously, this is due to the small size of the mollusks.

One more group of features, morphology of male and female reproductive system, successfully used for the systematics of prosobranch gastropod mollusks [Kool, 1993; Reid, 1996] previously was not used in the species descriptions in this family.

In this study, a comparative analysis of *C. derjugini*, *C. bucerium,* and the specimens with an intermediate shape of the mucro was carried out using 12 allozyme loci and the penis morphology.

Materials and methods

Sample collection

Mollusks were collected in Vostok Bay (Peter the Great Bay, Sea of Japan) at a depth of 0.5–0.8 m. *C. derjugini* was collected from sandy and rocky-gravelly sites of the bottom, *C. bucerium* was collected among rhizoids of *L. japonica* and *Costaria costata*. Specimens with an intermediate shape of mucro (hereinafter referred to as *Caecum* sp.) were collected together with *C. derjugini.*

Enzyme electrophoresis

Microelectrophoresis [Korochkin, 1977] was used for screening of genetic differentiation because of a small size of specimens. Starch gel electrophoresis was carried out using enzyme preparations derived from whole mollusks. Animals were grinded on the surface of 2x4 mm pieces of Whatman 3 MM chromatographic paper with an equal volume of distilled water. Two continuous buffer systems were used to resolve 11 enzymes: (1) TEB (tris-EDTA-boric acid, pH 8.5) [Boyer et al., 1963] for esterase D (*EstD*; EC 3.1.1…; detected with 4-methylumbelliferyl acetate as substrate), glutamate pyruvate transaminase (EC 2.6.1.2, *Gpt*), inorganic pyrophosphatase (*Ipp*; E.C.3.6.1.1), isocitrate dehydrogenase (*Idh*; EC 1.1.1.42), peptidase (*Pep-1*, *Pep-2*; EC 3.4.11; detected with gly-leu dipeptide as substrate); (2) TM (tris-maleic acid, pH 7.4) [Spencer et al., 1964] for alanopine dehydrogenase (*Aldh*; E.C.1.5.1.17), glucose phosphate isomerase (*Gpi*; EC 5.3.1.5), malate dehydrogenase (*Mdh*; EC 1.1.1.37), phosphoglucomutase (*Pgm*; EC 2.7.5.1), phosphoglycerate kinase (*Pgk*; EC 2.7.2.3), superoxide dismutase (*Sod*; EC 1.15.1.1). After electrophoresis, a gel block was sliced into 4 slices, which were then histochemically stained for specific enzymatic activities as described by Manchenko [1994].

Data analysis

Allele frequencies, Nei's [1978] unbiased genetic identity (I), and genetic distance (D) coefficients were calculated using the program BIOSYS [Swofford, Selander, 1981]. The significance of deviations of observed genotype frequencies from those expected under Hardy–Weinberg equilibrium was estimated using a pseudo-probability test [the CHIHW program by Zaykin, Pudovkin, 1993]. A phenogram was constructed by the unweighted pair group method from estimates of Nei's [1978] genetic distance using software packages NTSYS [Rohlf, 1988].

Results and discussion

Morphological variation

The shell of juvenile specimens of *Caecum* sp. is more similar to the shell of *C. bucerium*: the anterior region is significantly greater in diameter than the rest of the shell. *C. derjugini* has a terminal varix that slightly increases the shell diameter at the aperture. Adult specimens of all studied species have similar shell morphology: the shell is thin, the microsculpture is the delicate growth lines, the color varies from light-yellow to deep-brown, the septum is convex. *C. derjugini* and *C. bucerius* are

distinguished by the shape of the mucro. In *Caecum* sp., the mucro is of an intermediate shape: low, rounded, slanted to the concave side of the shell.

All Caecinae are dioecious [Götze, 1938; Ponder, Keyzer, 1998; Kolbin, Kulikova, 2005]. The males have a well-developed penis. We found some differences in the penial morphology of the studied species. The basis of the sucker stipe of *C. derjugini* is slightly expanded (Fig. 2A), whereas two other species have a wider basis of the sucker (Fig. 2B, C). Penis of *C. bucerium* has a medial penial lobe (Fig. 2C), whereas *C. derjugini* and *Caecum* sp. have none.

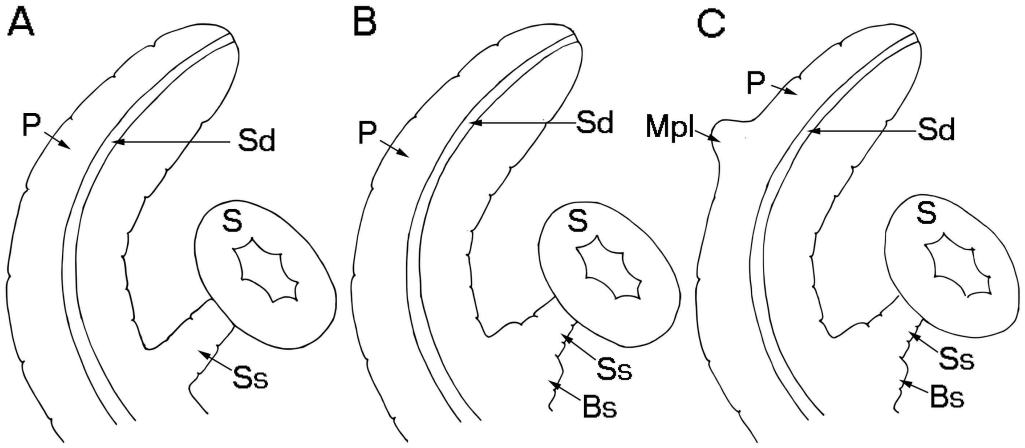

Fig. 2. Schemes of penes of Caecidae. **A** – *Caecum* (*Brochina*) *derjugini*, **B** – *Caecum* sp., **C** – *Caecum* (*Fartulum*) *bucerium*; P – penis, Sd – spermaduct, S – sucker, Ss – stipe of sucker, Bs – basis of sucker, Mpl – medial penial lobe.

Allozyme variation

Allele frequencies at the twelve examined loci are presented in Table 1. Significant genetic differences between *C. bucerium* and *C. derjugini* were found (D=1.346; I=0.262; Table 2). Fixed differences between these species were observed at three loci (*Pep-1*, *Sod* и *Mdh*), common alleles were absent at two loci (*Est*, *Gpt*) and five loci (*Idh*, *Ipp*, *Pgm*, *Gpi*, *Aldh*) had different most common alleles.

Genetic distance-values found in this study are comparable to those obtained in electrophoretic studies of different subgenera *Neritrema* and *Littorina* of the genus *Littorina* [Zaslavskaya et al., 1992]. It was earlier shown that these two species have a similar spawning period, rates of development, morphology of larvae, and sculpture of proto- and teleoconch, which indicates their close relationship [Kolbin, Kulikova 2005].

These data are in good agreement with the suggestion of Bandel [1996] and Absalão and Pizzini [2002] to consider *Brochina* and *Fartulum* as two subgenera included in the genus *Caecum*. *Caecum* sp. significantly differs from both *C. derjugini* and *C. bucerium* (D=2.034 and D=0.641, respectively). Evidently this species is closer to *C. bucerium* (Fig. 3). However, specific validity of *Caecum* sp. is beyond question: fixed differences between these species were observed at two loci (*Mdh*, *Pep-1*), and two loci (*Gpi*, *Gpt*)

Table1

Allele frequencies at 12 loci in three species of Caecidae

Loci	*Caecum* (*Fartulum*) *bucerium*	*Caecum* (*Brochina*) *derjugini*	*Caecum* sp.
Aldh (N)	121	121	142
1*	0.000	0.017	0.000
2	0.008	0.707	0.000
3	0.277	0.000	0.042
4	0.000	0.264	0.000
5	0.698	0.012	0.937
6	0.017	0.000	0.021
Mdh (N)	127	137	147
1	0.000	0.000	1.000
2	1.000	0.000	0.000
3	0.000	1.000	0.000
Pgi (N)	73	80	66
1	0.007	0.000	0.000
2	0.465	0.000	0.000
3	0.000	0.187	0.030
4	0.493	0.000	0.000
5	0.007	0.356	0.113
6	0.021	0.000	0.129
7	0.007	0.444	0.023
8	0.000	0.000	0.667
9	0.000	0.013	0.038
Gpt (N)	43	66	72
1	0.000	0.068	0.000
2	0.047	0.000	0.000
3	0.000	0.894	0.014
4	0.593	0.000	0.000
5	0.000	0.038	0.000
6	0.360	0.000	0.069
7	0.000	0.000	0.861
8	0.000	0.000	0.056
Pgm (N)	49	53	35
1	0.000	0.255	0.014
2	0.133	0.670	0.214
3	0.327	0.075	0.286
4	0.378	0.000	0.300
5	0.162	0.000	0.157
6	0.000	0.000	0.029

Table 1 (continued)

Loci	*Caecum* (*Fartulum*) *bucerium*	*Caecum* (*Brochina*) *derjugini*	*Caecum* sp.
Ipp (N)	50	63	54
1	0.000	0.976	0.000
2	1.000	0.024	0.991
3	0.000	0.000	0.009
Pgk (N)	26	32	42
1	0.000	0.016	0.024
2	1.000	0.984	0.952
3	0.000	0.000	0.024
Sod (N)	23	30	33
1	0.000	1.000	0.000
2	1.000	0.000	1.000
Idh (N)	13	20	15
1	0.038	0.000	0.067
2	0.308	0.000	0.300
3	0.423	0.000	0.623
4	0.231	1.000	0.000
Pep-1 (N)	24	17	23
1	1.000	0.000	0.000
2	0.000	0.000	1.000
3	0.000	1.000	0.000
Pep-2 (N)	24	17	23
1	0.000	0.000	0.957
2	1.000	1.000	0.000
3	0.000	0.000	0.043
Est (N)	9	8	15
1	0.000	0.250	0.000
2	0.000	0.750	0.033
3	0.389	0.000	0.367
4	0.389	0.000	0.567
5	0.222	0.000	0.033

* Detected alleles are labelled by figures in decreasing order of electrophoretic mobility (1 – the fastest).
Note. N – number of the individuals studied.

Table 2

Nei's [1978] genetic similarity (above diagonal) and distance (below diagonal) among the three caecid species

Species	1	2	3
1. *Caecum* (*Fartulum*) *bucerium*	0.000	0.262	0.531
2. *Caecum* (*Brochina*) *derjugini*	1.329	0.000	0.131
3. *Caecum* sp.	0.633	2.029	0.000

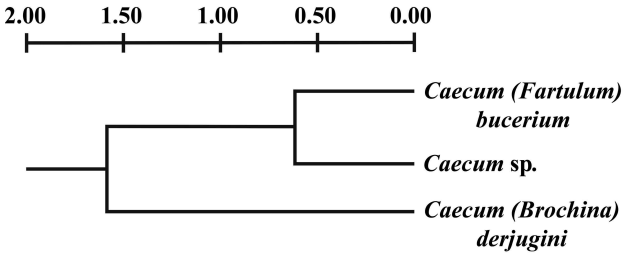

Fig. 3. Phenetic tree of genetic relationships among three species of Caecidae based on UPGMA clustering of Nei's [1978] genetic distances.

had the different most common alleles. Richardson et al., [1986] suppose that two fixed genetically determined electrophoretic differences between sympatric populations of a diploid sexually reproducing species is sufficient to both recognize and characterize two co-existing cryptic species. *C. bucerium* and unknown species are not really sympatric species (it is true only for *Caecum* sp. and *C. derjugini*). Even if it is granted that these species are different populations of the same species, a small distance between sample sites (1–2 m) and floating larvae should be to grade the genetic differences between them. Genetic distance between *C. bucerium* and *Caecum* sp. (0.641) is similar with the mean value estimated by Thorpe [1982] for congeneric species (D=0.616). Unknown species is similar in shell morphology to *Caecum* (*Brochina*) *glabella* (A. Adams, 1968), which occurs at the Japanese coast of the Sea of Japan and Pacific Ocean from south Kyushu Island to northern Hokkaido [Higo et al., 1999]. However, genetic comparison carried out in this study suggests that the unknown species from Peter the Great Bay is a *Caecum* (*Fartulum*). In order to define more exactly the taxonomic status of *Caecum* sp. (whether this species is *C.* (*B.*) *glabella* or it is a new species), these species need to be compared using allozyme markers.

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