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Protistology

ORIGINAL ARTICLE

Morphology and molecular phylogeny of *Euplotes japonicum* sp. n. (Ciliophora, Euplotidae) from the Peter the Great Gulf, Sea of Japan

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Summary

Euplotes japonicum sp. n. is a new species of *Euplotes* described from the Peter the Great Gulf, Sea of Japan. Traditional morphological (silver nitrate staining, Feulgen staining, DIC) and molecular (sequencing the SSU rRNA gene) methods were used in the study. *E. japonicum* characteristics: body size about 94×47 μm, a single-vannus dargyrome, 8–9 dorsolateral kineties with 17–21 cilia in central row, 10 frontoventral cirri, 5 transverse cirri, 3–5 caudal cirri, wide and deep peristome with 41–52 adoral membranelles. The best 100 hits in BLASTn analysis belong to the genus *Euplotes*, with *E. crassus* (Dujardin, 1841) Kahl, 1932 – 99.73%, and *E. vannus* (Muller, 1786) Minkjewicz, 1901 – 99.68%.

Key words: ciliates, *Euplotes*, morphological characteristics, phylogenetic analysis, SSU rRNA gene, the Sea of Japan

Introduction

Euplotes Ehrenberg, 1831 is a very diverse and ubiquitous genus of ciliates from marine, freshwater, and soil habitats (Curds, 1975; Lokhande et al., 2015; Syberg-Olsen et al., 2016; Huang et al., 2021). The latest comprehensive summary of *Euplotes* (Curds, 1975) provides information on 80 species and varieties. Nowadays, the species diversity of *Euplotes* is over 100 morphospecies: according to some reports, they are more than 150 (Pedrini et al., 2017; Liu et al., 2020; Huang et al., 2021). Most of them are described only morphologically. Traditionally, several features are distinguished for the purposes of identification of *Euplotes*: body size and shape, dorsal ridges, and location of cirri on the ventral surface (Curds, 1975; Gates and Curds, 1979). Recently, molecular analyses have been actively used to identify new and/or controversial species (Sheng et al., 2018; Lian et al., 2020; Serra et al., 2020). For example, 6 new species of *Euplotes* were recently identified within the coastal zone of the China Sea (Lian et al., 2020). In this paper, I have described a new species *Euplotes japonicum* sp. n. from the Peter the Great Gulf, Sea of Japan using combined (morphological and molecular) approach.

Material and methods

Euplotes japonicum sp. n. was collected from macrophytes of the Sea of Japan (Peter the Great

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Gulf: 42°85′ N, 132°66′ E) in July 2021, when salinity was 30‰ (Fig. 1). Monoclonal strain (EsJ) was cultured in artificial 30‰ salinity water, maintained in 18 °C incubator and fed with the green alga *Dunaliella salina* Teodoresco, 1905. The species was characterized using a combination of traditional morphological (silver nitrate and Feulgen staining (Foissner, 2014), differential interference contrast (DIC)) and molecular biology (sequencing the SSU rRNA gene) methods.

DIC microscopy was performed using a Nikon H600L microscope (Nikon, Japan) with digital camera DS-Fi3 (Nikon, Japan). Morphometric measurements were obtained with ImageJ ver.2.1.0 and were analyzed with RStudio ver.2021.09.1 + build 372 (Ferreira and Rasband, 2012). Linear dimensions of the strain EsJ were measured *in vivo*.

Approximately 30 cells of *E. japonicum* sp. n. were used for DNA extraction. The total genome DNA was extracted with guanidine isothiocyanate buffer (Maniatis et al., 1982). The primers used for PCR amplification were forward 18S F9 (5'–CTGGTTGATCCTGCCAG–3') and reverse 18S R1513 Hypo (5'–TGATCCTTCYGCAG GTTC–3') (Rosati et al., 2004). DNA amplification was done in T100 Thermal Cycler (BioRad, USA). PCR cycles were set as follows: 3 min 94 °C, 35 × (30 s 94 °C, 30 s 55 °C, 2 min 72 °C), 6 min 72 °C (Serra et al., 2020). To improve the quality of the sequences, the amplicons were cloned using InsTAclone PCR cloning kit (Fermentas, USA) following manufacturer's protocol. Colonies were tested by PCR amplification using vector-specific M13 primers. PCR products were sequenced using Sanger sequencing by ABI310 (ABI Prism, USA) with the M13 and internal primers 18S F783 (5'–GACGATCAGATACCGTC–3'), 18S R536 (5'–CTGGAATTACCGCGGCTG–3'), and 18S R1052 (5'–AACTAAGAACGGCCATGCA–3') (Rosati et al., 2004).

Euplotes sequences of the SSU rRNA gene were collected from the GenBank/EMBL database. Sequences longer than 1500 nucleotides were taken into account. Data set also includes seven outgroup sequences (genera: *Aspidisca* Ehrenberg, 1830; *Euplotidium* Noland, 1937; *Stylonychia* Ehrenberg, 1830; *Phacodinium* Prowazek, 1900; *Gastrocirrhus* Lepsi, 1928). To carry out phylogenetic analysis, we used the facilities of the computing server at the Zoological Institute RAS. Mafft ver.7.490 (Katoh and Standley, 2013) was used to align the sequences and trimAl ver.1.4.1 (Capella-Gutierrez et al., 2009) was used for tree trimming. Maximum likelihood (ML) tree was calculated with the RAxML ver.8.2.12 (Stamatakis, 2014) using the GTRGAMMAI model. Bayesian interference (BI) tree was inferred with Mr.Bayes ver. 3.2.7 (Ronquist et al., 2012). Four Markov chain Monte Carlo (MCMC) were run with 10^6 generations and sampled every 100th cycle, with a burn-in of 25%.

Fig. 1. Schematic map of the sampling location. The *arrow* points to the sampling site (42°85′ N, 132°66′ E).

Results and discussion

Morphological description of *Euplotes japonicum* sp. n.

The average cell size is 93.8 ± 6.9 × 47.3 ± 4.9 µm (*in vivo*). Cell shape is oval to ellipsoid (Fig. 2, A, B), with a length/width ratio of about 1.7–2.4 (Table 1). The body ends are smoothly rounded. The peristome is wide and deep, extending for about 70% of body length. The average length of adoral zone (AZM) is 62 µm. There are 41–52 membranelles in the adoral zone that start at the top of the cell and then are descending along the left side of the body (Fig. 2, B). Paroral membrane about 8.3 × 12.4 µm in length (Fig. 2, G). The contractile vacuole is single and large, located near of transverse cirri (Fig. 2, B, D). Cytoplasm is colorless and transparent. The dorsal argyrome is of the single-vannus type, with equal row of rectangular alveoli between each pair of dorsolateral kineties (Fig. 2, A, E). Kineties are 8 or 9 (rarely) with 17-21 bristles in the mid-dorsal row, leftmost row consisting of 9–14 bristles and rightmost row – of 18–22 (Fig. 2, C, E). The dorsal ridges are not visible. There are 10 frontoventral, 5 transverse, and 3–5 caudal cirri, 1–2 of which

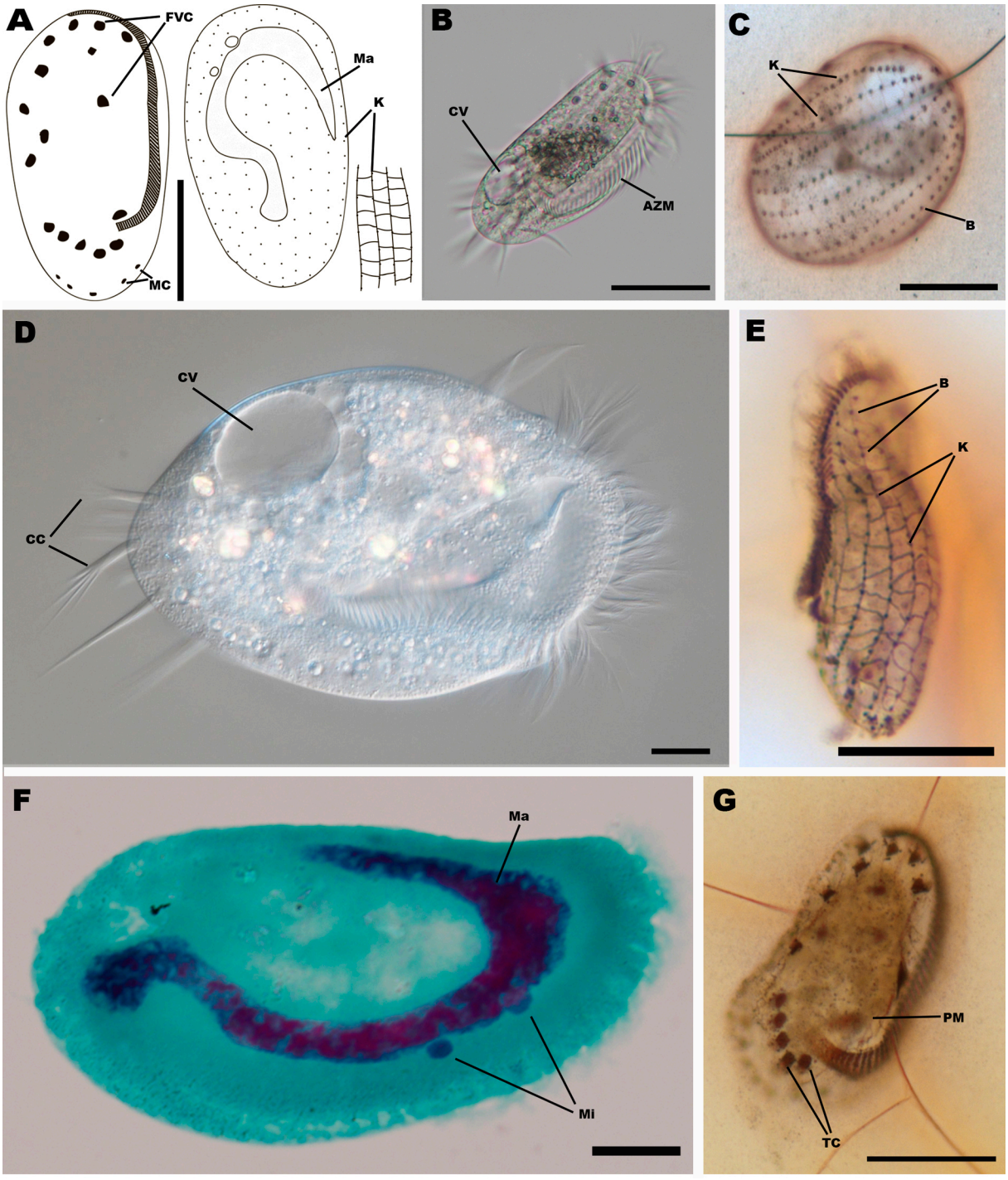

Fig. 2. Morphology of *Euplotes japonicum* sp. n. A – General view; B – ventral view of a living organism; C – dorsal argyrome features after silver staining; D – DIC microscopy; E – dorsal view of a silver-nitrate stained specimen, showing the single-vannus pattern; F – nuclear apparatus stained with the Feulgen method; G – ventral argyrome features after silver staining. *Abbreviations*: AZM – adoral zone of membranelles, B – bristles, CC – caudal cirri, CV – contractile vacuole, FVC – frontoventral cirri, K – kineties, Ma – macronucleus, MC – marginal cirri, Mi – micronucleus, PM – paroral membrane, TC – transverse cirri. Scale bars: A, B, C, E, G – 35 μm; D, F – 10 μm.

Table 1. Morphometric data of *Euplotes japonicum* sp. n.

Morphometric features	Min	Max	Median	Mean	SD	CV
Body length, μm	79.4	105.7	95.3	93.8	6.9	7.4
Body width, μm	39.6	54.8	47.6	47.3	4.9	10.4
Length of micronucleus, μm	2.5	3.3	3	2.9	0.2	7
Width of micronucleus, μm	1.7	2.5	2.1	2.1	0.2	10.6
No of micronucleus	1	2	1	1.3	0.4	36.2
Length of adoral zone, μm	54.1	70.6	61.4	62.0	5.1	8.3
Length of paroral membrane, μm	8.3	12.4	9.6	9.8	1.2	12.5
No of adoral membranelles	41	52	46	46.3	2.7	6.0
No of FVC	10	10	10	10	0	0
No of dorsolateral kineties	8	9	8	8.2	0.4	11.0
No of bristles in central row	17	21	19	19	1.4	7.4
No of bristles in leftmost dorsal kinety	9	14	12	11.6	1.7	14.7
No of bristles in rightmost dorsal kinety	18	22	20	20.1	1.3	6.6
No of transverse cirri	5	5	5	5	0	0
No of caudal cirri	3	5	3	3.5	0.6	19.2

Notes: Number of cells measured – 21; Min – minimum; Max – maximum; Mean – arithmetic mean; SD – standard deviation; CV – coefficient of variation in %; FVC – frontoventral cirri.

are usually positioned to the left of the peristome (marginal cirri) (Fig. 2, A, D, G). The macronucleus usually mirror-inverted, C-shaped, with rounded ends (Fig. 2, A, F). The micronucleus is spherical (compact type), single or double, usually localized in the cavity of the macronucleus (Fig. 2, F).

The species inhabits the fouling (periphyton-benthic group), where it actively crawls on the substrate. *Euplotes japonicum* sp. n. can successfully reproduce in a wide range of water salinities from 10 to 40‰. The strain EsJ was resistant to starvation period (about 2 months). Cysts were not recorded.

Molecular data and phylogenetic analysis

The 18S rRNA gene sequence of *E. japonicum* sp. n. obtained from PCR was 1977 bp long. The best 100 hits in BLASTn analysis (except for the sequences from uncultured ciliates) belonged to the genus *Euplotes*, with *Euplotes crassus* (Dujardin, 1841) Kahl, 1932 (accession number: AJ305239) – 99.73%, and *E. vannus* (Muller, 1786) Minkjewicz, 1901 (accession number: MT568588) – 99.68%. Most of the topology nodes had high support as shown in Fig. 3. *E. japonicum* sp. n. formed a well-supported clade (ML 97-100%, BI 1.00) and clustered with four close relatives: *E. crassus* (Dujardin, 1841) Kahl, 1932; *E. vannus* (Muller, 1786) Minkjewicz, 1901; *E. minuta* Yocum, 1930; and *E. cristatus* Kahl, 1932. The tree topologies (ML and BI) were similar, except for some minor differences that had a low bootstrap. The pairwise comparison of sequences (Table 2) shows that *E. japonicum* sp. n. has a high level of identity with neighboring sequences, and all of them are close to each other.

The SSU-rRNA gene sequences of *Euplotes* spp. had high similarities: 93.2% for *E. japonicum – E. crassus*; 95% for *E. japonicum – E. vannus*; 88.4% for *E. japonicum – E. minuta*, and 91.3% for *E. japonicum – E. cristatus.*

Comparison of *Euplotes japonicum* sp. nov. with similar species

As indicated earlier, *Euplotes* has a wide geographic distribution. According to C. Curds (1975) and A. Borror (1968), *Euplotes* can be identified by the

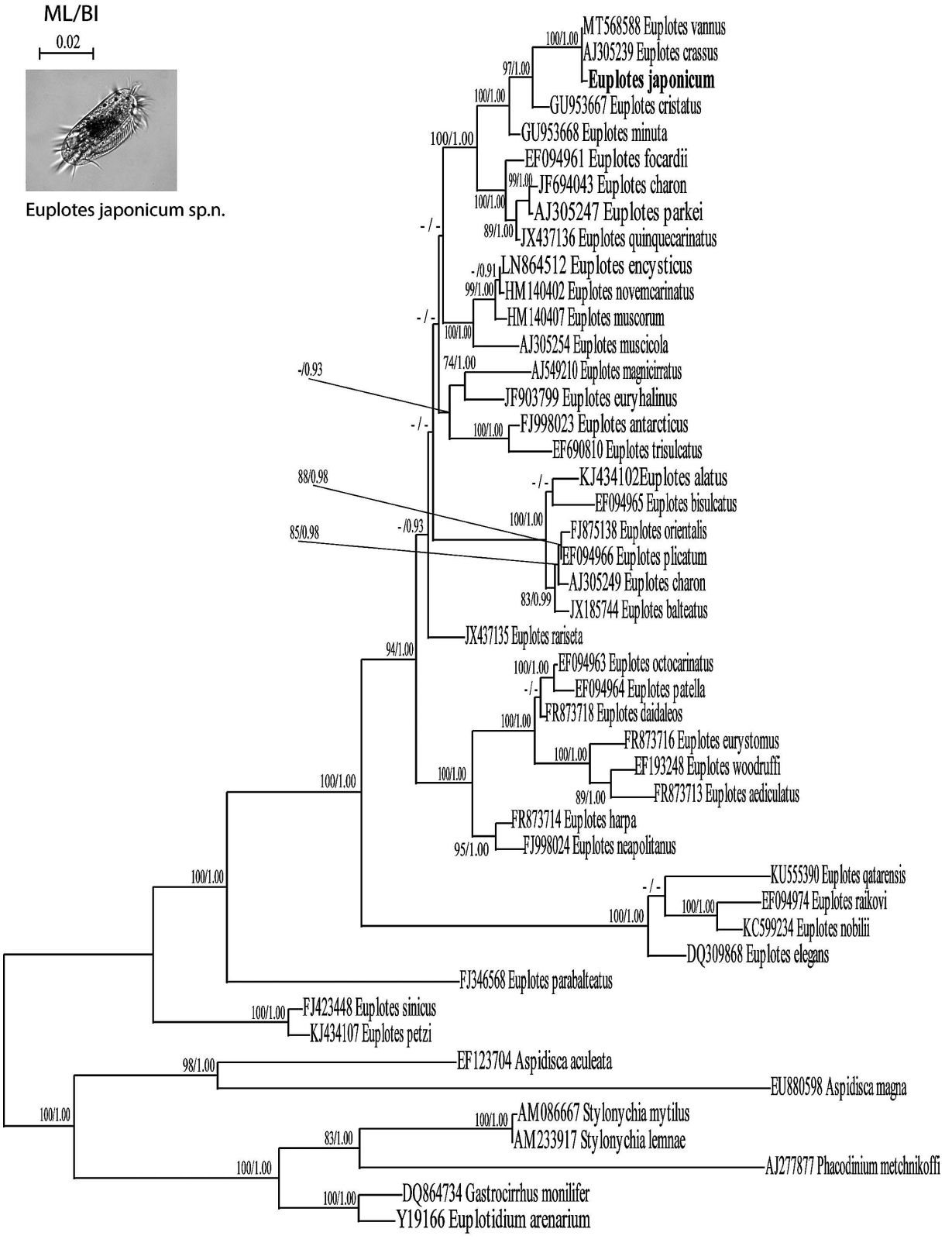

Fig. 3. Phylogenetic position of *E. japonicum* based on SSU rRNA gene sequence data. Numbers at nodes represent the bootstrap values of the ML analysis and the posterior probability of the Bayesian inference (BI) analysis. Values below 70/0.90 are not shown.

Table 2. The pairwise comparison of sequences belonging to the clade containing *Euplotes japonicum* sp. n. Values indicate the percent of sequence identity.

Species	*E. japonicum* sp. n.	*E. vannus*	*E. crassus*	*E. cristatus*	*E. minuta*
E. japonicum sp. n. (ON387646)		99.68	99.73	97.65	95.81
E. vannus (MT568588)			99.95	97.91	96.22
E. crassus (AJ305239)				97.79	96.29
E. cristatus (GU953667)					97.38
E. minuta (GU953668)					

following features: a type of dargyrome; number of frontoventral, transversal and caudal cirri; number of dorsal ridges; body size and shape; shape of the macronucleus; number of membranelles in the adoral zone; their habitat. However, according to M. Syberg-Olsen et al. (2016), a number of these parameters cannot be viewed as unique and have a low taxonomic value for species identification. For example, only *E. woodruffi* Gaw, 1939 has a macronucleus shape different from «C» or «3» shapes, and the number of frontoventral cirri is usually equal to 10.

Among all the above parameters, the most morphologically significant is a type of dargyrome. M. Tuffrau (1960) first introduced this feature for taxonomic identification and described three general types (muscicola, eurystomus, vannus) named after the species for which it was first reported. According to previous revisions of *Euplotes* (Yan et al., 2018; Lian et al., 2020), *E. crassus, E. vannus, E. minuta*, and *E. cristatus* that have the same type of dargyrome (single-vannus), are closely related. *Euplotes japonicum* has the same dorsal side pattern with the aforementioned four species and falls within a fully supported group. It is also consistent with the morphological similarity of the species and indicates that the dargyrome type is an important morphological and evolutionary characteristic in this subclade. Morphological differences with closely related species are shown in Table 3.

Euplotes minuta differs from *E. japonicum* in its body size (54 × 28 μm vs. 94 × 47 μm), fewer membranelles in AZM (30–40 vs. 41–52), number of caudal cirri (4 vs. 3–5), and fewer dorsal bristles in central row (12–13 vs. 17–21). *Euplotes cristatus* is also a small *Euplotes*. It differs from *E. japonicum* in its body size (60 × 45 μm vs. 94 × 47 μm), fewer dorsal bristles in central row (11–15 vs. 17–21), and number of caudal cirri (3 vs. 3–5). Also, *E. cristatus* has 6 dorsal ridges. *Euplotes crassus* differs from *E. japonicum* in having more dorsal kineties (10 vs. 8–9) and more dorsal bristles in central row (26 vs. 17–21). In addition, *E. crassus* has 8 dorsal ridges. *Euplotes vannus* differs from *E. japonicum* in its body size (103 × 60 μm vs. 94 × 47 μm), more membranelles in AZM (57–74 vs. 41–52), and the number of their caudal cirri is more diverse (4–7 vs. 3–5).

Based on morphological and molecular differences with the known species of *Euplotes, E. japonicum* has been described as a new species.

Taxonomic summary

(Classification of ciliates according to Lynn, 2008)

Phylum Ciliophora Doflein, 1901
Class Spirotrichea Butschli, 1889
Subclass Hypotrichia Stein, 1859
Order Euplotida Small et Lynn, 1985
Family Euplotidae Ehrenberg, 1838
Genus *Euplotes* Ehrenberg, 1831
Euplotes japonicum sp. n.

Diagnosis: Large-sized marine *Euplotes*, 93.8 × 47.3 μm (*in vivo*). The cell shape oval or ellipsoidal and the body ends are smoothly rounded. The per-istome is wide and deep, extending for about 70% of body length. There are 41–52 adoral membranelles, which starting at the top of the cell, then they descending along the left side of the body. On the ventral side, 10 frontoventral cirri (FVC), 5 transverse cirri and 3–5 caudal cirri (1–2 marginal cirri). The contractile vacuole is single and large, located near the transverse cirri. Macronucleus «C-shaped». Micronucleus is spherical (compact type), single or double, usually localized in the cavity of the macronucleus. Dargyrome of single-vannus type. Dorsal kineties are 8 or 9 (rarely), the central row contains 17–21 bristles, the leftmost row – 9–14, and the rightmost row – 18-22. The dorsal ridges are not visible.

Euplotes japonicum was collected from macro-

Table 3. Morphological comparisons between *Euplotes japonicum* sp. n. and congeneric species.

Features	*E. japonicum* sp. n.	*E. minuta*	*E. crassus*	*E. vannus*	*E. cristatus*
Size, μm (mean)	94x47	54x28	80x50	103x60	60x45
Shape	Elongated, oval	Oval	Elongated, oval	Oval	Oval
Peristome	2/3 of the body length	2/3 of the body length	2/3 of the body length	2/3 of the body length	1/2-2/3 of the body length
Membranelles in adoral zone	41-52	30-40	~ 50	57-74	35-47
No of dorsolateral kineties	8-9	9	10	9-10	8
Dorsal ridges	Not visible	Not described	8	Not described	6
No of dorsal bristles in central row	17-21	12-13	26	15-22	11-15
Caudal cirri	3-5	4	5-6	4-7	3
Reference	This work	Curds (1975)	Curds (1975) Valbonesi et al. (1988)	Kwon et al. (2007)	Curds (1975)

phytes of the Sea of Japan (Peter the Great Gulf: 42°85′ N, 132°66′ E) in July 2021.

Etymology: the species name *E. japonicum* refers to the area (Sea of Japan, Peter the Great Gulf, Russia) where the sample was collected.

Type material: holotype consists of the type culture (accession No EsJ), DNA samples (accession No C01, C 01.1), slides containing silver nitrate-impregnated (accession No SI-C01.1 – SI-C01.3), and Feulgen staining (accession No F-C01.1). Paratype consists of the silver nitrate-impregnated slide (accession No P.SI-C01.4) and the slide with Feulgen staining (accession No P.F-C01.2).

Data availability: slides containing silver nitrate-impregnated and Feulgen staining of the type strain EsJ are available from Zoological Institute RAS (Laboratory of Cellular and Molecular Protistology). DNA sample is available upon request from the author's laboratory.

18S rRNA gene sequence of the type strain: GenBank number – ON387646.

Zoobank LSID of the publication: urn:lsid:zoobank.org:pub: C2A6B783-7D36-424F-BBD4-332D2FCB7936.

Zoobank LSID of the species: urn:lsid:zoobank.org:act: 2A32B442-E90B-4BFC-BB05-CAE400793C3F.

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