Journal of Species Research 11(4):278-286, 2022

New record of *Pleuronema marinum* Dujardin, 1841 (Protozoa, Ciliophora) from South Korea

Jeong Hyeon Yeo[1], Ji Hye Choi[1], Atef Omar[2,3] and Jae-Ho Jung[1,*]

[1]*Department of Biology, Gangneung-Wonju National University, Gangneung 25457, Republic of Korea*
[2]*Department of Zoology, Al Azhar University, Assiut, Egypt*
[3]*Industry Academy Cooperation Group, Gangneung-Wonju National University, Gangneung 25457, Republic of Korea*

**Correspondent: jhjung@gwnu.ac.kr*

During a field survey of Korean marine ciliates, we collected *Pleuronema marinum* from a brackish water sample. It is characterized by the presence of a contractile vacuole in mid-body, rather than the subterminal/terminal contractile vacuole as in other congeners. The cells were examined *in vivo* and based on protargol and 'wet' silver nitrate impregnation. In addition, the nuclear 18S rRNA gene was sequenced using a single cell. The Korean population morphologically and molecularly resembles a Chinese population of *P. marinum*. Historical review of the species concludes that 1) two or more species have been assigned into *P. marinum*, 2) the position of contractile vacuole (e.g., in mid-body) is a valid character state, and 3) *P. marinum* is probably a rare species. Here we provide a monographic treatment of *P. marinum* to clarify the issue and for further studies of relevant species. Considering there are about 40 nominal species and complex nomenclatural acts in the genus *Pleuronema*, further studies should provide descriptions based on protargol and 'wet' silver impregnation with marker gene(s).

Keywords: 18S rRNA gene, brackish water, ciliate, Oligohymenophorea, silver staining

DOI:10.12651/JSR.2022.11.4.278

Introduction

The genus *Pleuronema* Dujardin, 1841 is characterized by a very large and prominent paroral membrane, and consists of about 40 nominal species (Agamaliev and Suleimanova, 2004; Lynn, 2008; Liu *et al*., 2022). Of them, *Pleuronema marinum* Dujardin, 1841, which was established, together with the type species (e.g., *P. crassum* Dujardin), about 180 years ago. Since the original description, there has been a confusion about the validity of *P. marinum*. This issue seemed to be clarified by Kahl (1931; 1933), but it is still in progress (Carey, 1992; Kadhim and Mahmood, 2014; Alekperov and Tahirova, 2021). Kahl (1931) diagnosed *P. marinum* mainly by having a contractile vacuole in mid-body (vs. terminal/subterminal in other congeners). However, Kahl's diagnosis had been abandoned so that several morphospecies have been assigned into *P. marinum* (Noland, 1937; Dragesco, 1960; Borror, 1963).

Recently, Pan *et al*. (2016) described a Chinese population of *P. marinum*, which corresponds well with Kahl's populations, based on protargol-impregnated specimens and 18S rDNA sequence. In addition, Liu *et al*. (2022) reported four new species of *Pleuronema*. The speciose genus *Pleuronema* together with the complex nomenclatural acts of *P. marinum* lead us to this monographic treatment of *P. marinum* using the Korean population. During a field survey, we collected a brackish water population, and it was examined *in vivo* and based on protargol and 'wet' silver nitrate impregnation. In addition, nuclear 18S rRNA gene was sequenced using a single cell.

Materials and Methods

Sample collection and identification

The ciliate species was found in estuarine area (salinity of 12.6‰) of Namdaecheon Stream in Gangneung-si, South Korea. After stirring the bottom of the estuary, we collected the water sample including organic/inorganic debris. The sample was immediately transferred to the laboratory and kept at room temperature for about two weeks. Morphology and behavior were observed using a stereomicroscope (Olympus SZ11, Japan) and an optical

microscope (Olympus BX53, Japan) at low (×40–200) to high (×400–1,000) magnifications. Micrographs were captured using a digital camera (Olympus DP74). Protargol powder was synthesized using the method of Pan *et al*. (2013) and Kim and Jung (2017). The protargol slides were prepared using the 'procedure A' of Foissner (2014) with acetone developer. The 'wet' silver nitrate impregnation was also conducted using the method of Foissner (2014). Sequential through-focal micrographs of stained specimens were merged using the software of Helicon Focus 6.8.0 (Helicon Soft Ltd.). The basic terminology and taxonomic classification follow Lynn (2008). The specific terminology of each taxon follows the previous study (Pan *et al*., 2016).

DNA extraction, PCR amplification, and sequencing

Two cells were isolated from the raw culture using microcapillary under the stereomicroscope. The cells were washed using the 0.22 μm-filtered culture water at least five times to remove other eukaryotes and then transferred to a 1.5 mL centrifuge tube each with a minimum volume of water. Genomic DNA was extracted using a RED-Extract-N-Amp Tissue PCR Kit (Sigma, St. Louis, MO, USA). Slightly modified versions of the primers New Euk A and LSU rev2 were used to cover 18S rRNA gene (Medlin *et al*., 1988; Sonnenberg *et al*., 2007). The PCR conditions were as follows: denaturation at 94°C for 1 min 30 s, followed by 40 cycles of denaturation at 98°C for 10 s, annealing at 58.5°C for 30 s, and extension at 72°C for 3 min, and a final extension step at 72°C for 7 min. For purification of the PCR products, MEGAquickspin Total Fragment DNA Purification Kit (iNtRON, South Korea) was used. DNA sequencing was performed using the PCR and three internal primers [18SF790v2, 18SR300, and 18SF1470 (Park *et al*., 2017; Jung *et al*., 2018)] and an ABI 3700 sequencer (Applied Biosystems, Foster City, CA, USA). Because about 800 base pairs sequenced by New Euk A were identical between the two cells, only one cell was completely sequenced and assembled by Geneious Prime 2019.2.3 (Kearse *et al*., 2012).

Phylogenetic analyses

The phylogenetic relationship of *Pleuronema marinum* was inferred using the most relevant tree by Liu *et al*. (2022). All *Pleuronema* sequences and some closely related species in the tree were retrieved from the GenBank database. They were aligned using ClustalW (Thompson *et al*., 1994) implemented in Geneious Prime 2019.2.3. After trimming both ends of the alignment to construct blunt ends using Geneious, it showed a final matrix of 1,625 columns. Of the alignment, sequences with more than one ambiguous nucleotide were discarded. A total of 40 sequences including the new sequence of *P. marinum* were analyzed. The best-fit substitution model of the alignment was selected as GTR + I (0.4860) + G (0.5840) based on the Akaike information criterion (AIC) using jModelTest 2.1.10 (Guindon and Gascuel, 2003; Darriba *et al*., 2012). A maximum likelihood consensus tree of 1,000 bootstrap replicates was inferred and constructed using IQ-TREE 1.6.12 (Nguyen *et al*., 2015). The tree was visualized using FigTree 1.4.4 (Rambaut, 2012).

Results and Discussion

Pleuronema marinum Dujardin, 1841 (Figs. 1, 2)

1841 ***Pleuronema marina*** - Dujardin, Histoire Naturelle zoophytes, p. 475, Planche XIV, Fig. 3 (original description).

1858 ***Pleuronema chrysalis*** **Perty** - Claparède & Lachmann, Mémoires de l'Institut National Genevois, 5: 274, Planche XIV, Fig. 8 (revision).

1866 ***Pleuronema chrysalis*** **Perty** - Diesing, Sitzungsberichte Akademie der Wissenschaften in Wien, Mathematisch-Naturwissenschaftliche Klasse Abteilung I, 5: 85 (revision).

1881 ***Pleuronema marina*** **Dujardin** - Kent, A manual of the infusoria: including a description of all known flagellate, ciliate, and tentaculiferous protozoa, British and foreign, and an account of the organization and affinities of the sponges. Volume II, p. 543 (revision).

1876 ***Pleuronema chrysalis*** - Fromentel, Études sur les microzoaires ou infusoires proprement dits comprenant de nouvelles recherches sur leur organisation, leur classification et la description des espèces nouvelles ou peu connues, p. 301, Planche XXI, Fig. 10, XXII, Fig. 16 (revision).

1885 ***Pleuronema marina*** **(Dujardin)** - Fabre-Domergue, Journal of Physiology, Paris, 21: 558, Planche XXIV Figs. 4, 5 (redescription, misidentification).

1931 ***Pleuronema marinum*** **Dujardin, 1841** - Kahl, Tierwelt Deutschlands, 21: 389, Fig. 65_{26} (redescription).

1933 ***Pleuronema marinum*** **Dujardin, 1841** - Kahl, Tierwelt der Nord- und Ostsee, 23: 84, Fig. 10_{41} (redescription, guide to marine ciliates).

1937 ***Pleuronema marinum*** **Duj.** - Noland, Transactions of the American Microscopical Society, 56: 169, Fig. 5D (redescription, misidentification).

1960 ***Pleuronema marinum*** **Dujardin** - Dragesco, Travaux de la Station Biologique de Roscoff, 12: 271, Figs. 140A, 142a (redescription, misidentification).

1963 ***Pleuronema marina*** **Dujardin, 1841** - Borror, Ar-

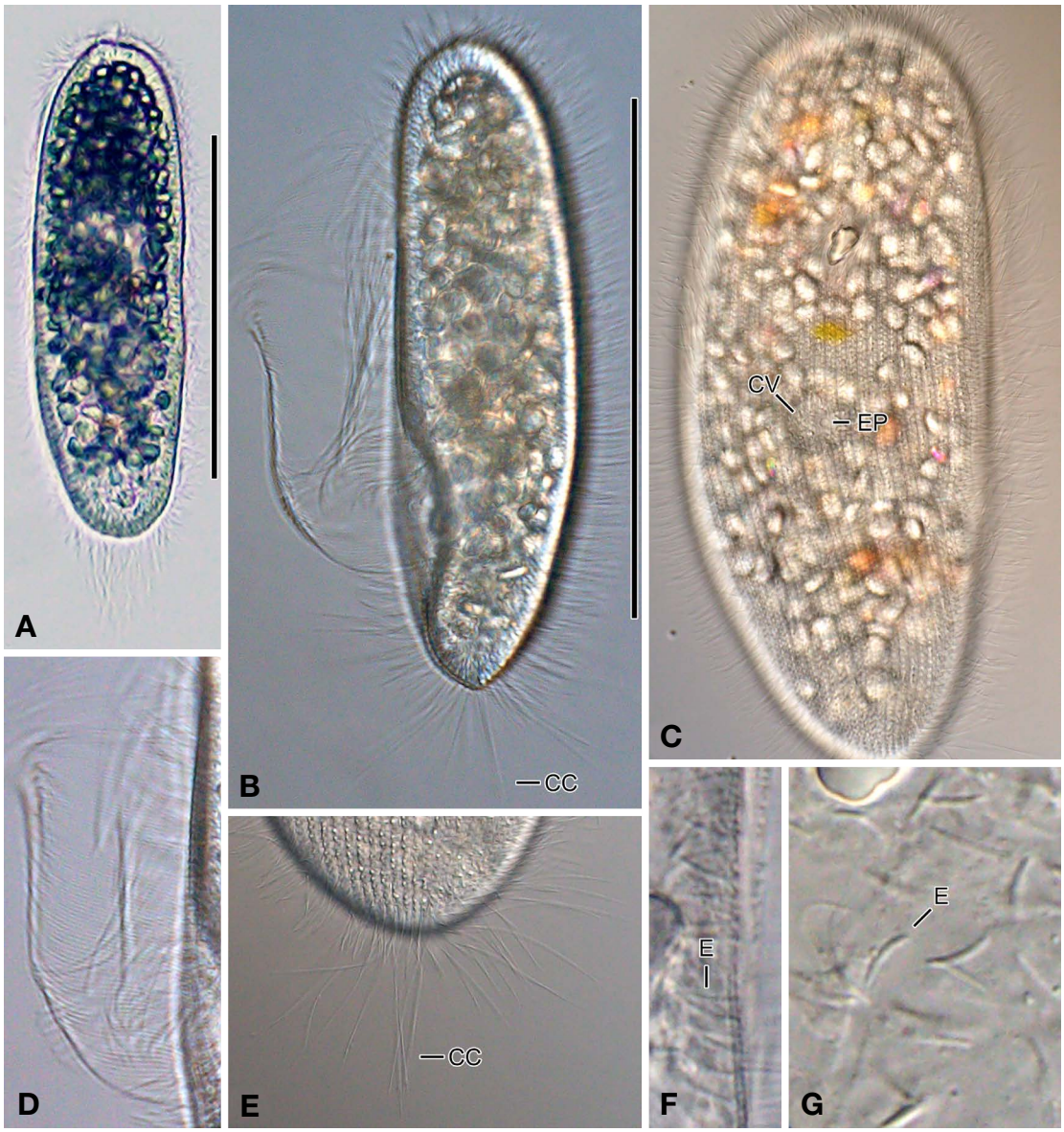

Fig. 1. *Pleuronema marinum in vivo*. A. Dorsal view of a freely motile cell. B. Lateral view of an immobile cell spreading paroral membrane. C. Lateral view showing a contractile vacuole with excretory pore. D. Paroral cilia. E. Elongated caudal cilia. F, G. Extrusomes perpendicularly arranged to body margin (F) and from a squashed cell (G). CC, caudal cilia; CV, contractile vacuole; E. extrusomes; EP, excretory pore. Scale bars = 100 μm.

chiv für Protistenkunde, 106: 496, Figs. 82, 83, 86 (redescription, misidentification).

1964 *Pleuronema marinum* Dujardin, 1841 - Small, Dissertation, p. 3 (revision).

1968 *Pleuronema marinum* Dujardin, 1841 - Dragesco, Protistologica, 4: 86, Figs. 1A, 2, 4A (redescription, misidentification).

1970 *Pleuronema marinum* Dujardin, 1841 - Burkovsky, Acta Protozoologica, 7: 483, Fig. 9 (redescription, misidentification).

1971 *Pleuronema marinum* Dujardin, 1841 - Agamaliev, Acta Protozoologica, 8: 386, Figs. 2, 3A−C (redescription, misidentification).

1985 *Pleuronema marinum* Dujardin, 1841 - Aladro Lubel, Anales del Instituto de Biología, Universidad Nacional Autónoma de México. Serie Zoología, 55 (year 1984): 18, Lámina 9, Fig. 1 (redescription, misidentification).

1986 *Pleuronema marinum* Dujardin, 1836 - Dragesco & Dragesco-Kernéis, Faune tropicale (Éditions de l' ORSTOM), 26: 360, 95E−H (redescription, misidentifica-

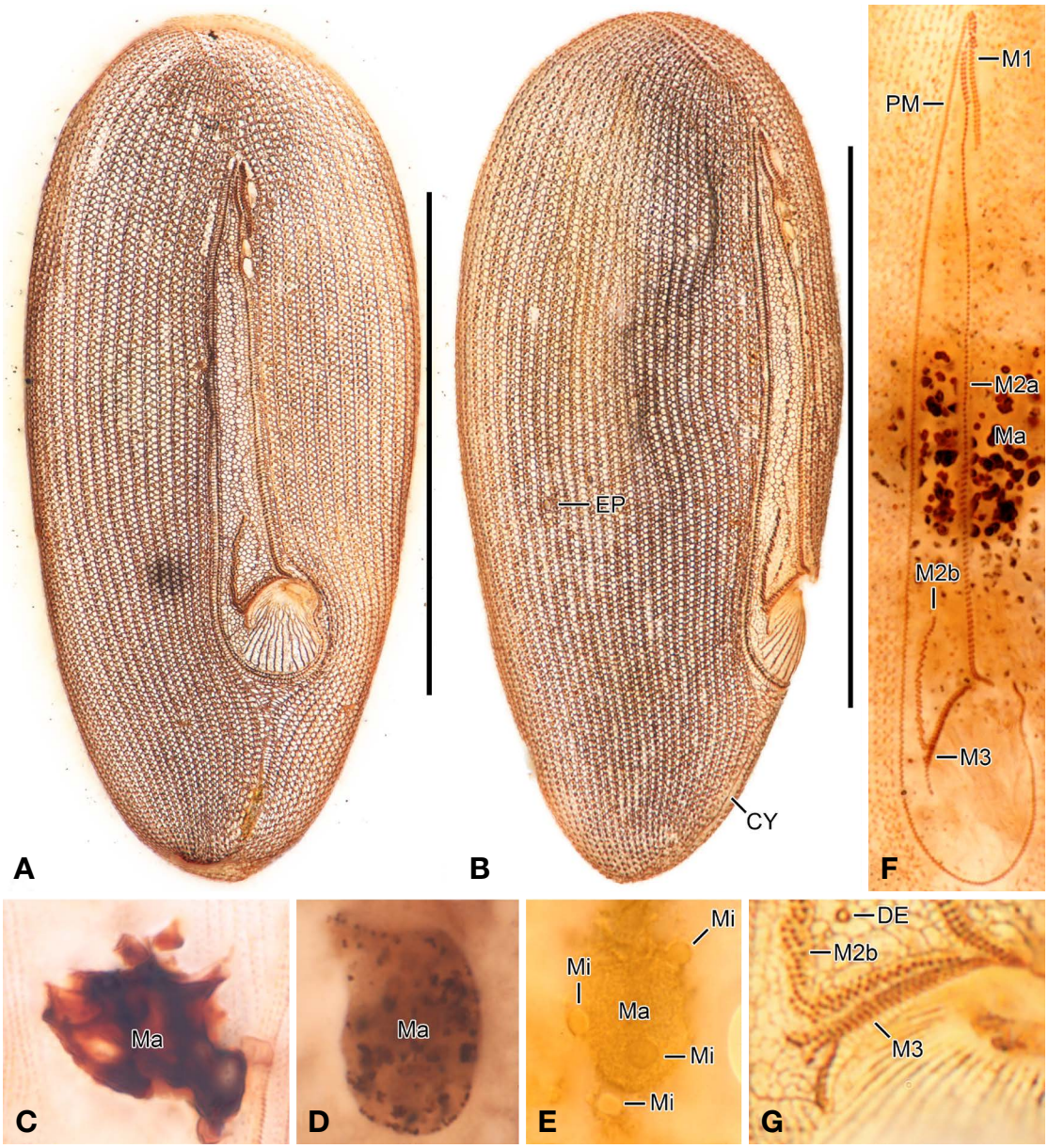

Fig. 2. *Pleuronema marinum* after wet silver nitrate (A, B, E, G) and protargol impregnation (C, D, F). A. Ventral view of a typical individual showing oral apparatus and silver line system. B. Lateral view showing the excretory pore near mid-body. C–E. Nuclear apparatus fixed in Bouin's solution (C), concentrated mercury chloride (D), and Champy's fixative (E). F, G. Oral apparatus. CY, cytopyge; DE, docked extrusome; EP, excretory pore; M1, 3, membranelles 1, 3; M2a, anterior part of membranelle 2; M2b, posterior part of membranelle 2; Ma, macronucleus; Mi, micronuclei; PM, paroral membrane. Scale bars = 100 μm.

tion).

1992 *Pleuronema marinum* Dujardin, 1841 - Carey, Marine interstitial ciliates. An illustrated key, p. 144, Fig. 531 (misidentification, guide to marine interstitial ciliates).

1999 *Pleuronema marinum* Dujardin, 1836 - Alekperov & Asadullayeva, Turkish Journal of Zoology, 23: 218, Fig. 4 (redescription, misidentification).

2005 *Pleuronema marinum* Dujardin, 1836 - Alekperov, An atlas of the free living ciliates (classes Kinetofragminophora, Colpodea, Oligohymenophora, Polyhymenophora), p. 162, Drawing 49.4, Fig. 15.2 (redescription, misidentification).

2014 *Pleuronema marinum* Dujardin, 1836 - Kadhim & Mahmood, Iraqi Journal of Science, 55: 661, Fig. 12 (misidentification).

2016 *Pleuronema marinum* Dujardin, 1841 - Pan, Hu,

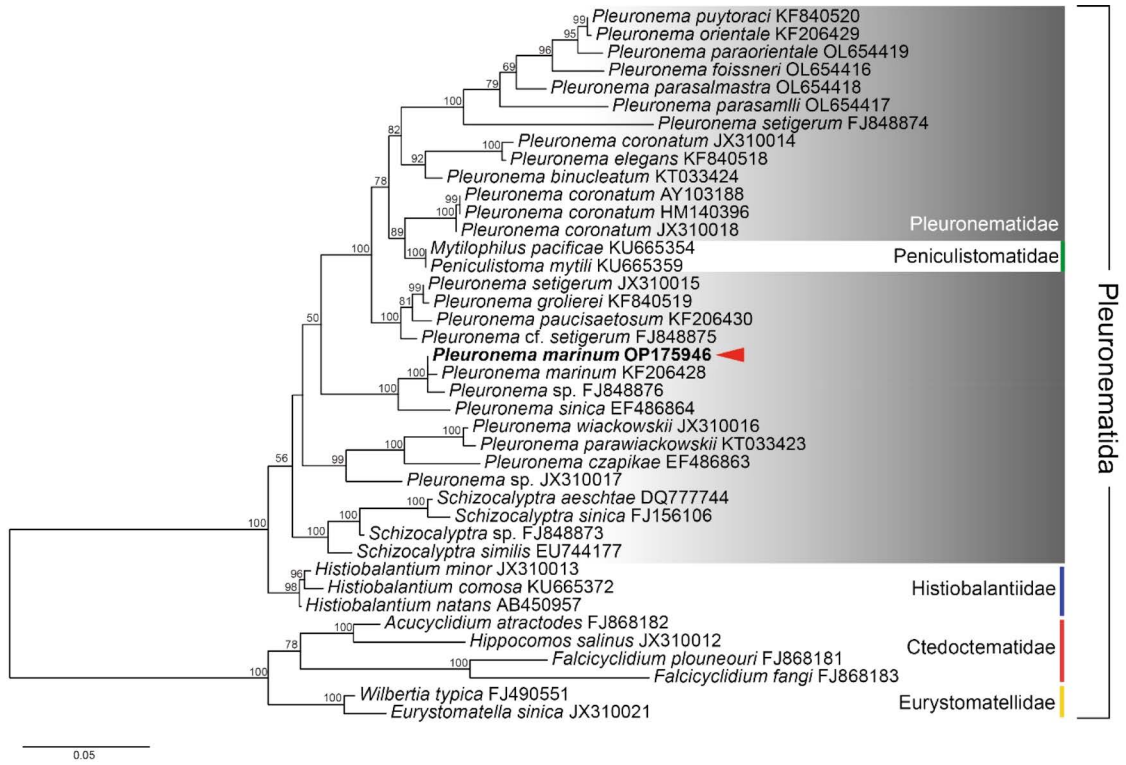

Fig. 3. Maximum likelihood tree of 18S rRNA gene sequences. Bootstrap values are shown on the branches, while the values ≤50% were not shown. The scale bar represents five nucleotide substitution per 100 nt.

Jiang, Wang & Hu, Journal of Eukaryotic Microbiology, 63: 290, Figs. 3, 4, Table 1 (redescription based on stained specimens with 18S rRNA gene).

2021 *Pleuronema marinum* Dujardin, 1841 - Alekperov & Tahirova, Amurian Zoological Journal, 13: 492, Figs. 5, 6 (no description, but a micrograph of stained specimen available; misidentification).

Material examined. Estuarine water sample (12.6‰, 11.4°C) collected from Gyeonso-dong, Gangneung-si, Gangwon-do, Republic of Korea (37°46′12.8″N 128°56′57.2″E) on 24 January 2022.

Diagnosis (n = 7). Cell size 100−145 × 30−45 μm *in vivo* (127−172 × 61−80 μm after wet silver nitrate impregnation), outline narrowly elliptical (obovate in wet silver nitrate preparations), posterior body end slightly narrower than anterior end, dorsoventrally flattened at posterior portion of body. One globular to irregularly-shaped macronucleus (irregularity more distinct in Bouin-fixed cells), 20.4−27.3 × 13.5−24.4 μm in size; two to four globular to elliptical micronuclei attached to macronucleus, 3.3−4.8 × 2.6−3.6 μm in size; one or two preoral kineties; one contractile vacuole at right margin slightly below mid-body with one excretory pore; extrusomes filiform, straight or distinctly curved, forming cortical seam because perpendicularly arranged to pellicle; 57−64 somatic kineties, 10−31 prolonged caudal cilia; buccal area occupying 59.9−70.4% (64.9% on average) of body length; paroral membrane conspicuous, hook or '6'-shaped, proximal end slightly concave (looks like '3'); M1 2-rowed, but 3-rowed at distal end (rarely 2-rowed throughout); M2a more or less straight, proximal end curved leftward, 56.1−87.0 μm long, M2a two-rowed at distal and proximal end, one-rowed at middle portion; M1 : M2a length ratio about 1 : 5; M2b V-shaped, ratio of two arms 1.2−1.8 : 1 (right : left), zigzag-pattern of basal bodies in right arm; M3 three-rowed.

18S rRNA gene. The SSU rDNA sequence of *Pleuronema marinum* is 1,605 base pairs long and has a GC content of 42.3% (OP175946). The genus *Pleuronema* is monophyletic with poor support (< 50 bootstrap value; Fig. 3), except for *Peniculistoma mytili* and *Mytilophilus pacificae* which are nested inside. Conspecific pairwise similarity of *P. marinum* is 99.69% and inter-specific similarities range from 91.11% (*P. setigerum* FJ848874) to 97.38% (*P. sinica* EF486864). The sequence of the unidentified *Pleuronema* sp. (FJ868876) clusters with and shows higher similarity (99.13%, 99.44%) to both *P. marinum* populations than to other congeners. The sequence of *P. sinica* shows a sister relationship with the two sequences of *P. marinum* and the unidentified *Pleuronema* sp.

World distribution. Mediterranean Sea, Germany, China, and South Korea. Probably rare species (see below).

Remarks. Dujardin (1841) originally described two *Pleuronema* species, *P. crassa* and *P. marina* (now *P. crassum* and *P. marinum*), and distinguished the two species by the body shape (more elongated in *P. marinum*) and habitat (freshwater vs. seawater). Type locality of *P. marinum* is the Mediterranean Sea. Since the original description, there has been confusion about the validity of *P. marinum*. The issue seemed to be clarified by Kahl (1931), but it is still in progress (Carey, 1992; Kadhim and Mahmood, 2014; Alekperov and Tahirova, 2021). Pan *et al*. (2016) described a Chinese population of *P. marinum* based on protargol-impregnated specimens and 18S rDNA sequence so that it allows us, as 'good' reference, to resolve that issue. In addition, Liu *et al*. (2022) reported four new species of *Pleuronema*. These new findings lead us to this monographic treatment of *P. marinum* as follows.

Perty (1852) synonymized *P. crassum* with *P. chrysalis* sensu Ehrenberg (1838), while synonymizing *P. chrysalis* Müller, 1786 was not decided as mentioned on page 146. Later, Claparède and Lachmann (1858) synonymized *P. crassum* and *P. marinum* with *P. chrysalis* sensu Ehrenberg (1838) because Ehrenberg described *Paramecium chrysalis* in 1830 and 1838, which are possibly different species, and the latter was considered as *Pleuronema crassa* by Dujardin (1841). Considering either *P. chrysalis* Müller, 1786 or *P. chrysalis* sensu Ehrenberg (1838), which were considered as the same species with *P. marinum*, the synonym is rather complex as reviewed by Small (1964). Diesing (1866) accepted the synonym and additionally included *P. chrysalis* Müller, 1786 in the list of synonyms, while Fromentel (1876) synonymized *P. chrysalis* sensu Ehrenberg (1838) and *P. marina* with *P. chrysalis* (not *P. chrysalis* Müller, 1786) and considered *P. crassa* as a valid species. Unfortunately, we did not find that Fromentel considered *P. chrysalis* Müller, 1786 as a valid species either of *Paramecium* or *Pleuronema*. Kent (1881) did not accept the synonyms because *P. marinum* has larger and longer body size than *P. chrysalis* and found in marine (vs. freshwater) habitat. Fabre-Domergue (1885) reported a wider form with a subterminal contractile vacuole. Considering the elongated body shape and the contractile vacuole in mid-body of *P. marinum*, however, the identification/synonyms cannot be accepted. For designating type species of the genus *Pleuronema*, it is also rather complex because the type is *P. crassum* (see above for the synonyms). For details, see Small (1964), Foissner *et al*. (1994), and Aescht (2001).

Kahl (1931) redescribed a German (Sylt) population of *P. marinum*, which was slightly longer than the type population (120−180 μm vs. 100 μm), and provided additional diagnosis for the species: spherical macronucleus in anterior half of cell; contractile vacuole in right of mid-body; 30−35 somatic kineties on dorsal side. Kahl (1933) briefly described *P. marinum* collected from Kiel and Sylt. Unfortunately, the key diagnosis (e.g., the position of contractile vacuole) had been abandoned because it is probably that *Pleuronema* species usually have a terminal/subterminal contractile vacuole.

Noland (1937) described a population called *P. marinum* with the subterminal contractile vacuole and wider body, while he considered *P. marinum* sensu Kahl (1931) as a misidentification of *Histiobalantium semisetatum* Noland, 1937 which has multiple contractile vacuoles. Borror (1963) probably followed Noland and described a Floridan population based on stained specimens. As the Noland's population, it had the subterminal contractile vacuole and wider body. Carey (1992) probably followed Borror's classification and an incorrect year 1965 for 'Borror (1963)' was given in the guide to marine interstitial ciliates. Burkovsky (1970) described a population from the White Sea that differs from Borror's population and other populations mentioned above in having a slender body and subterminal contractile vacuole as in *P. sinica* Wang *et al*., 2009. However, *P. marinum* sensu Burkovsky (1970) can be distinguished from *P. sinica* by the number of somatic kineties (30−34 vs. 41−52) and preoral kineties (2 vs. 1).

Dragesco (1960, 1968) described *P. marinum* based on silver staining. However, the French populations had the wider body and the terminal contractile vacuole so that it obviously differs from the original and Kahl's population. Later, Dragesco and Dragesco-Kernéis (1986) considered that *P. roscoffensis* Dragesco, 1968 might be a synonym of *P. marinum*. However, the main key to differentiate between *P. marinum* sensu Dragesco and *P. roscoffensis* is the presence vs. absence of the curvature at the proximal end of the paroral membrane. The curvature is 3-shaped and is apparent in both the Chinese (Pan *et al*., 2016) and the present population of *P. marinum*.

Agamaliev (1971) described two populations from the Caspian Sea based on stained specimens: the first population is slender with 7−10 macronuclear nodules arranged longitudinally; the second population is characterized by the broad body, the 3 macronuclear nodules in mid-body, and the absence of membranelle 1. Both populations differ from all *P. marinum* populations mentioned above so that Agamaliev and Suleimanova (2004) established them as new species *P. multinucleatum* and *P. ovatum*, respectively. The new species, together with *P. ovata* Fernandez Leborans and Novillo, 1994, seem to be not widely known for taxonomists because they are not mentioned in recent/relevant publications (Wang *et al*., 2008a; 2008b; 2009; Pan *et al*., 2016; Liu *et al*., 2022) and include a homonym (e.g., *ovata*, *ovatum*). *Pleuronema ovata* sensu Fernandez Leborans and Novillo (1994) differs from *P. ovatum* sensu Agamaliev and Suleimanova (2004) mainly by the presence (vs. absence) of membranelle 1 and the

number of macronuclear nodules (1 vs. 3). For the validity of these species, further study is necessary.

Alekperov and Asadullayeva (1999) reported another population of *P. marinum* from the Caspian Sea. Further, they considered the Caspian Sea populations described by Agamaliev (1971) as exconjugants because of the nuclear apparatus (Alekperov, 2005). In terms of the number of somatic kineties (35−38), they considered that it is similar to the White Sea population of *P. marinum* described by Burkovsky (1970). Even though they did not mention the position of the contractile vacuole, we confirm that the Alekperov and Asadullayeva's population had the terminal/subterminal contractile vacuole because they did not denote the position when comparing with the populations with terminal/subterminal contractile vacuole. However, Alekperov and Asadullayeva's population had shorter body length (60−75 μm *in vivo*). Alekperov (2005) considered that the Agamaliev and Suleimanova's species (e.g., *P. multinucleatum* and *P. ovatum*) are exconjugants so that the validity of the two new species was doubted. He also provided a drawing and micrograph of *P. marinum* (wider form), but the latter might be another species of *Pleuronema* because the proximal end of membranelle 2 is curved to right.

Aladro Lubel (1985) reported a Mexican population with a brief description and illustration. It had rather slender body, but with a terminal contractile vacuole. Kadhim and Mahmood (2014) reported an Iraqi population collected from the Tigris River (Baghdad city) and provided a brief description with a single micrograph. However, it had a smaller body length (about 50 μm). Alekperov and Tahirova (2021) provided a micrograph of stained specimen collected from North-East Azerbaijan. They did not provide a description, but based on the stained specimen, we can conclude that it differs from *P. marinum* mainly by the wider body shape and the hook-shape of membranelle 2a.

The Korean population morphologically and genetically resembles the Chinese population reported by Pan *et al.* (2016) except for the nuclear apparatus (1 macronucleus with globular or irregular shape vs. usually 4−7 macronuclear nodules gathered slightly above mid-body or sometimes only 1 globular or irregular macronucleus) and the extrusomes (straight or slightly curved vs. only straight). Considering the number of macronuclear nodules, one of the main diagnostic features to identify ciliates, the Chinese population is peculiar and differs from the authoritative redescription by Kahl (1931) (Wang *et al.*, 2009). The high variation, however, might result from the sexual cycle in *P. marinum* (e.g., autogamy for the 4−7 nodules, interphasic for the 1 nodule) rather than co-occurrence of another congener (Lynn, 2008).

Voucher slides. One slide with wet silver nitrate-impregnated specimens was deposited at the National Institute of Biological Resources (NIBRPR0000111138). Six slides (GUC005709 - wet silver nitrate-impregnated; GUC00 5713−5717 - protargol-impregnated) have been deposited in the Jung-lab (J.-H. Jung) in Gangneung-Wonju National University.

Acknowledgements

This work was supported by grants from the National Institute of Biological Resources (NIBR), funded by the Ministry of Environment (MOE) of the Republic of Korea (NIBR202002204, NIBR202231206).

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Submitted: July 13, 2022
Revised: August 6, 2022
Accepted: August 9, 2022