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Phylogenetic and morphological characterization of the green alga infesting the horse mussel *Modiolus modiolus* from Vityaz Bay (Peter the Great Bay, Sea of Japan)

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ABSTRACT

In this work, the ultrastructural features and taxonomic position of the green microalga infesting the horse mussel *Modiolus modiolus* from the north-western Pacific (Vityaz Bay, Peter the Great Bay, Sea of Japan) are reported. Mussels were collected monthly from May to September of 2009. In different months, the prevalence of mussels with green tissues was 16.6–62.5% (mean 43%). The most affected organs were the mantle, digestive gland and gonad. Histological analysis revealed severe infiltration of the connective tissue by hemocytes containing the alga cells. Electron microscopy showed that the alga was morphologically similar to the green algae from the genus *Coccomyxa* (Chlorophyta: Chlorococcales). Two new primers were designed to generate partial small subunit (SSU) rRNA sequences of the green alga from *M. modiolus*. Phylogenetic analysis based on the comparison of the SSU rRNA sequences of the trebouxiophyceans confirmed an affiliation of the green alga with the genus *Coccomyxa*. The sequence (1296 bases) of the green alga from *M. modiolus* was most closely related to the sequence CPCC 508 (AM981206) (identity 100%), obtained from an acid-tolerant, free-living chlorophyte microalga *Coccomyxa* sp. and to the sequences EU127470 (identity 99.3%) and EU127471 (identity 99.7%) of the green alga, presumably the true *Coccomyxa parasitica*, infecting the blue mussel *Mytilus edulis* from the Flensburg Fjord (North Atlantic).

1. Introduction

The phenomenon of the symbiosis of unicellular green algae with organisms of different phyla is well known. In Bivalvia, infestation by green microalgae has been described for several species from Atlantic coastal waters: the horse mussel *Modiolus modiolus* (Wiborg, 1946, as cited in Mortensen et al. (2005), the giant scallop *Placopecten magellanicus* (Naidu and South, 1970; Naidu, 1971; Stevenson and South, 1974, 1975), the mussels *Mytilus edulis chilensis* (Gray et al., 1999), *M. edulis* (Mortensen et al., 2005; Rodríguez et al., 2008) and *M. galloprovincialis* (Crespo et al., 2009), and the geoduck *Panopea abbreviata* (Vázquez et al., 2010). Recently, a microalga-infected horse mussel, *M. modiolus*, from the north-western Pacific (Peter the Great Bay, Sea of Japan) was first reported (Syasina, 2011). Based on the morphological features, most researchers have referred green microalgae associated with the tissues of marine bivalve mollusks to the genus *Coccomyxa* (Chlorophyta: Chlorococcales). The genus *Coccomyxa* represents a heterogeneous group of microalgae comprising free-living forms (Verma et al., 2009), photobionts of protozoans, lichens and higher plants (Lohtander et al., 2003; Trémouillaux-Guiller and Huss, 2007; Hoshina and Imamura, 2008), and parasitic species affecting several marine invertebrate taxa (Stevenson and South, 1974). The members of the genus *Coccomyxa* have a generally similar morphology; moreover, as shown in *Coccomyxa parasitica* isolated from *P. magellanicus* and grown under different conditions, the alga may exhibit high variability in cell shape and size (Stevenson and South, 1974). Thus, the morphology-based identification of *Coccomyxa* species is extremely difficult and necessitates the use of molecular phylogenetic analysis to identify these species (Rodríguez et al., 2008). To date, there are only two studies employing small subunit (SSU) rRNA sequence analysis to determine the phylogenetic position of the green microalgae infesting bivalve species from the North and South Atlantic, *M. edulis* (Rodríguez et al., 2008) and *P. abbreviata* (Vázquez et al., 2010).

The horse mussel *M. modiolus* is a boreal species widely distributed throughout the North Atlantic (Brown, 1984; Dinesen and Ockelmann, 2005), the Barents and White Seas (Flyachinskaya and Naumov, 2003; Sokolov and Shtrik, 2004), and in the Pacific

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(Bering Sea, Sea of Japan and west coast of North America) (Skarlato, 1981). It is a common subtidal species in Peter the Great Bay, which is located in the north-western part of the Sea of Japan. In the course of the studies of the reproductive cycle of *M. modiolus* in the bay, we found mussels with tissues that had a green color and suggested that these specimens were infected by green microalga. In this work, we studied the morphological characteristics and SSU rRNA sequence phylogeny of the green microalga infesting the horse mussel *M. modiolus* from Vityaz Bay (Peter the Great Bay, Sea of Japan).

2. Materials and methods

2.1. Mussel sampling

Approximately 30 specimens of *M. modiolus* were collected by a diver monthly from May to September 2009 in Vityaz Bay (Peter the Great Bay, Sea of Japan, 42°35′5″N, 131°9′55″E) from a depth of 2–5 m. The shell length was measured by calliper, the muscle-contractor was cut by scalpel, and a sample of the gonad tissue was excised and fixed for light and electron microscopy. The mussel shell length ranged from 77.0 to 126.0 mm, with a mean (SE) of 107.7 (0.7) mm; a total of 148 individuals were studied.

2.2. Histology and electron microscopy

Pieces of approximately 0.5–0.8 cm^3 in volume were cut from the middle part of the gonad, fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.5), embedded in paraffin, sectioned 5–6 μm thick and stained with hematoxylin and eosin. The sections were examined under a light microscope (Olympus BX41, Japan) equipped with a digital camera (C5060-ADU, Olympus, Japan). Small pieces of gonads of approximately 1 mm^3 in volume were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 12 h at 4 °C and post-fixed in 2% osmium tetroxide for 1 h. The fragments were then washed with the same buffer, dehydrated in ethanol and acetone, stained *en block* with uranyl acetate and embedded in SPUR resin (EMS, USA) in accordance with the manufacturer's protocol. Thin sections were stained with lead citrate and examined using a Carl Zeiss LIBRA transmission electron microscope (TEM).

2.3. DNA extraction and phylogenetic analysis

Pieces of the mantle from seven mussels showing green coloration were cut off and preserved in 96% ethanol. Total DNA was extracted from 0.1 to 0.3 g tissue using a standard proteinase K/phenol/chloroform procedure with subsequent ethanol precipitation (Sambrook et al., 1989). For amplification, we used two *Coccomyxa*-specific oligonucleotide primers, SSUF (5′-CCGACTCGC GGTGAATCA-3′) and SSUR (5′-GGCCAGAGTCCTATCGTG-3′) (Vázquez et al., 2010). The procedure for the PCR reaction was 3 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 1 min at 56 °C, 1 min 40 s at 72 °C, and a final extension of 5 min at 72 °C. The purified PCR products of the SSU rRNA genes were used as a matrix for sequencing. The sequencing was performed using BigDye Terminator v3.1 (Applied Biosystems, USA) under conditions recommended by the manufacturer. The PCR products were precipitated in ethanol and dried in a vacuum. The sequences were analyzed by an ABI Prism 3130 automatic sequencer (Applied Biosystems, USA) using a 50-cm capillary array and POP-7 polymer. The sequences were aligned with SeqScape Software v2.6 (Applied Biosystems, USA). After removing the primer-binding sites from the sequences, we obtained a sequence with a length of 558 bases, identical for all seven samples.

To increase the length of the SSU rRNA sequence analyzed in the present study, we developed two new primers. For this purpose, we used the *Coccomyxa* sequences deposited in GenBank, which were the most similar to the above described sequence. These sequences were *Coccomyxa* sp. CPCC 508 (AM981206), with a completely identical sequence of 558 bases, and *Coccomyxa* sp. Flensburg fjord 2 (EU127471) and *Coccomyxa* sp. Flensburg fjord 1 (EU127470), with the sequences differing from the first one by two and four nucleotides, respectively. In addition, a sequence of the *M. modiolus* SSU rRNA gene (EF526454) was taken from GenBank. The alignment was performed using the ClustalW program to find DNA sequences of the *Coccomyxa* SSU rRNA gene exhibiting the most dissimilarity with those of the *M. modiolus* gene. Finally, two new primers (F: 5′-ATCCCGACTTCTGGAAGGGACGTA-3′ and R: 5′-TCTAGGTGGGAGGGTTTAACGAA-3′) were designed for these sequences. The conditions for amplification and sequencing were as described above. After removing the primer-binding sites from the sequences, we obtained a sequence with a length of 1521 nucleotides, identical for all seven samples. The sequence limited by the primers SSUF + SSUR (Vázquez et al., 2010) was completely identical to the sequence of 558 bases obtained in the first step. The sequence of the SSU rRNA gene of *Coccomyxa* sp., the green alga from *M. modiolus*, consisting of 1521 nucleotides was deposited in GenBank under accession number JQ717057.

The phylogenetic analysis was performed in two steps. At the first stage, to reveal the species that were phylogenetically close to the green alga from *M. modiolus*, we analyzed 36 sequences of the SSU rRNA gene of trebouxiophycean microalgae from GenBank, which exhibited high homology in the nucleotide sequence (identity not less than 92.2%). Among them, there was the SSU rRNA sequence from the green alga culture CCAP 216/18 (EU127469) denoted in NCBI (National Center for Biotechnology Information) as *Coccomyxa parasitica*. The length of the alignment (ClustalW algorithm) was 1530 nucleotides. The phylogenetic analysis placed the sequence JQ717057 obtained in our study in the clade *Coccomyxa* (inter-sequence identity not less than 97.2%), whereas the sequence EU127469 was very far apart in the tree, in a clade together with *Nannochloris bacillaris* (AB080300), *Nannochloris* sp. RCC 011 (AJ131691) and *Picochlorum oculatum* (AY422075) (identity between the sequences JQ717057 and EU127469 of 93.9%) (Fig. 1). The position of the sequence EU127469 was almost identical to that in the tree obtained by Rodríguez et al. (2008). As the sequence EU127469 does not belong to an original species *C. parasitica* (Rodríguez et al., 2008), it was excluded from our further phylogenetic analysis. The sequence JQ717057 obtained in our study also had a similarity with SSU rRNA sequences of several free living algae from the genus *Prasiola*: *P. meridionalis* (EF200528, identity 94.5%), *P. calophylla* (EF200521, identity 94.5%), *P. stipitata* (EF200524, identity 94.7%), *P. crispa* (EF200532, identity 95.1%) as well as with the SSU rRNA sequence of *Prasiococcus calcarius* (EF200527, identity 94.7%). As the similarity was relatively low and the species mentioned do not belong to the symbionts of marine organisms, they were also excluded from further phylogenetic analysis.

At the second stage, the phylogenetic analysis included 30 partial SSU rRNA sequences (1296 bases) of the representatives of *Coccomyxa* from lichens (HQ287293, HQ287295, AM167525), the tree *Ginkgo biloba* (AJ302339, AJ302340), and the blue mussel *Mytilus edulis* (EU127470, EU127471), and several sequences from other classified and unclassified trebouxiophycean species (FJ648512, FJ648513, FJ648514) with an identity not less than 97.2%. The SSU rRNA sequences of the green alga *Elliptochloris marina* (Trebouxiophyceae, Chlorophyta), a symbiont of the Pacific sea anemones *Anthopleura elegantissima* and *Anthopleura xanthogrammica* (Anthozoa, Cnidaria) (FJ217358–FJ217366), were also included. Two outgroup sequences, *Trebouxia impressa* (Z21552) and *T. asymmetrica* (Z21553), were used to root the trees.