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FIRST RECORD OF ZOANTHARIA IN THE BLACK SEA: *ISOZOANTHUS* CF. *SULCATUS* REARED FROM PLANULAE

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The first record of a species from the order Zoantharia in the Black Sea is given. Zoantharians were successfully reared from planktonic larvae collected in a single planktonic net haul conducted in shallow coastal waters of the Golubaya Bay, Gelendzhik area, Caucasus. The larvae were subsequently settled in a small glass container and resulting colonies were maintained in an aquarium with a salinity level of 18 psu for approximately nine months, but in June 2018 all colonies died due to uncontrolled bloom of filamentous algae. The presence of symbiotic algae of the family Symbiodiniacea in tissues of colonies is shown. Phylogenetic analysis using mitochondrial (COI) markers revealed a high degree of similarity between the Black Sea zoantharian and *Isozoanthus sulcatus* (Gosse 1860) from European seas.

Keywords: Parazoanthidae, shallow waters, Golubaya Bay, Caucasus

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Zoantharia is an order within hexacorallian Anthozoa (Sinniger et al., 2008), comprising primarily colonial species with polyps characterized by two rows of tentacles, a single siphonoglyph, and presence of incrustations in their body walls (Low et al., 2016). The incrustations pose challenges when applying traditional morphological identification methods. Consequently, in recent years, molecular barcoding has emerged as an approach broadly used within Zoantharia for species identification and phylogenetic reconstructions (Sinniger et al., 2008; Fujii, Reimer, 2013; Low et al., 2016).

Despite zoantharians being found in many marine ecosystems from Arctic to Antarctic (Low et al., 2016), they have never been documented in the Black Sea. In September 2017, hundreds of early cnidarian larvae were collected in a planktonic net haul taken from the pier of the Southern Branch of the Shirshov Institute of Oceanology of Russian Academy of Sciences (SO IO RAS), Golubaya (Blue) Bay, NW Black Sea. Initially regarded as juvenile actiniarians, settled larvae were left unattended in a glass container in a sea water aquarium. Within a few weeks, settled larvae unexpectedly developed into small colonies exhibiting the characteristic appearance of zoantharians (two rings of tentacles, colonial growth form). The primary objective of our work was to document the first record of Zoantharia in the Black Sea and to provide preliminary description and identification.

MATERIAL AND METHODS

Zoanthid larvae (several hundred) were collected from a haul of a simple Apstein plankton net (max diameter 50 cm, mesh size 100 μm) taken from the pier of SO IO RAS, 44°34.5’N, 37°58.7’E (15 km northwest of Gelendzhik, NW Black Sea). Twenty larvae were fixed in 2.5% glutaraldehyde/0.1 M cacodylate buffer (pH 7.2). Remaining larvae were transported alive in a Falcon tube filled with filtered seawater to the Invertebrate Zoology Department at Lomonosov Moscow State University. The larvae were kept in a glass container with mesohaline salinity water at ambient temperature (18 psu, 18 °C), where some of planulae have settled. Settled larvae in the same glass container were transferred into a sea water aquarium where they were maintained at the same conditions (18 psu, 18 °C) for several months. In April and June 2018, polyps were sampled from three largest colonies and fixed

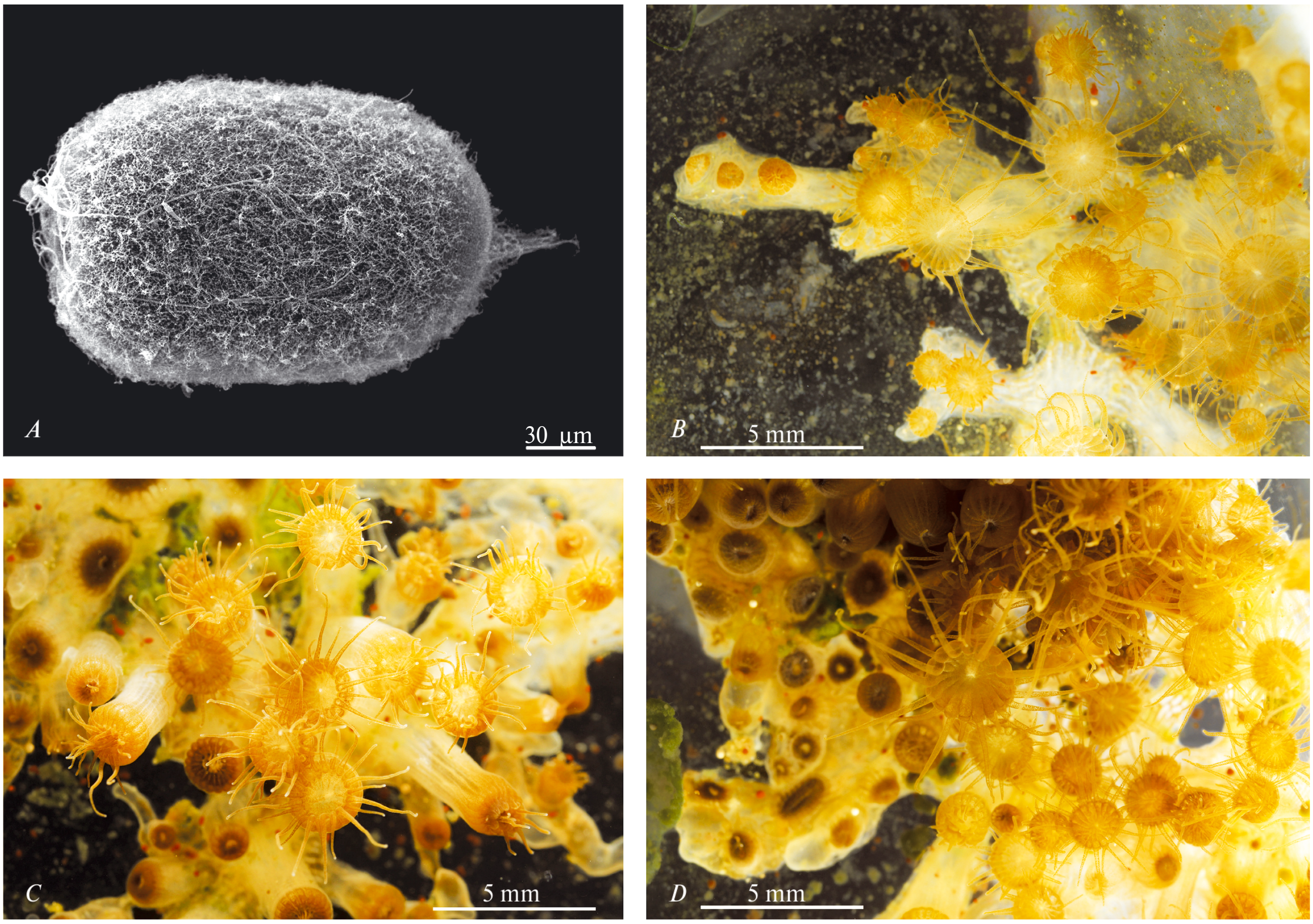

Fig. 1. *Isozoanthus* cf. *sulcatus* from the Black Sea: *A* – Planula, SEM; *B*–*D* – Close-ups of colonies reared in aquarium.

in 96% ethanol for DNA analysis. In addition, we used 96% ethanol fixed fragment of *Isozoanthus sulcatus* (Gosse 1860) from the Plymouth area (UK) ~55 km from the type locality.

For SEM (scanning electron microscopy) observations, the larvae fixed in glutaraldehyde were dehydrated in an increasing ethanol series, critical point dried with CO_2, mounted on aluminum stubs, coated with an Au-Pd mixture and examined using a CAM SCAN S-2 (Cambridge Instruments, Cambridgeshire, UK) of the Shared Facilities center "Electron Microscopy for Life Sciences" at Lomonosov Moscow State University.

Genomic DNA was extracted from 96% ethanol fixed specimens. Fragments were exsiccated at 60 °C before lysis and genomic DNA was extracted using LumiPure kit from AnySample (Lumiprobe) according to the manufacturer's recommendations. PCR amplification was accomplished for the mitochondrial COI sequenced with universal primers jgHCO2198 and jgLCO1490 (Geller et al., 2013) using HS-ScreenMix kit (Evrogen) according to the manufacturer's protocol. The resulting PCR product was sequenced with same primers using ABI PRISM® BigDye™ Terminator v. 3.1 on Applied Biosystems (Foster City, CA, USA) DNA Analyzer 3500 ABI. Sequences of *Isozoanthus sulcatus* (OR785060) and *I.* cf. *sulcatus* (OR785057, OR785058, OR785059) generated in the study were deposited in the NCBI database. Additional sequences of species representing several families of Zoantharia were obtained from NCBI (GenBank) database (Benson et al., 2012). Sequences were aligned using the MAFFT v7.450 (Katoh, Standley, 2013) with the manual check and correction.

For phylogenetic reconstructions we employed the Maximum Likelihood (ML) method implemented in the IQ-TREE 1.6.12 software (Nguyen et al., 2015). To assess branch support we used ultrafast bootstrap (Hoang et al., 2018) approximation (UFboot) and the SH-like approximate likelihood ratio test (SH-aLRT) (Guindon et al., 2010) with 10000 bootstrap replicates. The substitution model was chosen according to the Bayesian information criterion (BIC) with the help of the ModelFinder (Kalyaanamoorthy et al., 2017) implemented in the IQ-TREE software. Obtained phylogenetic trees were visualized with the help of ITOL v.6.7.5 (Letunic, Bork, 2021).

To test the presence of Symbiodiniacea in specimens of *Isozoanthus sulcatus* from Plymouth (1) and *I.* cf. *sulcatus* from the Black Sea (3) we used specific primers and protocols developed by Manning and Gates (2008) for Symbiodiniacea (*Symbiodinium* on original paper). The results of PCR were visualized with 2% agarose gel.

RESULTS AND DISCUSSION

The larvae were elongated ciliary planulae, 0.3 mm in length, with a cluster of longer cilia forming an apical tuft at the aboral pole (Fig. 1*A*). The vast majority of larvae settled in a small glass container and developed into small anemone-like polyps. The polyps were left unattended, and in few weeks settled polyps developed into small colonies few millimeters across comprised of flat horizontal stolons bearing diminutive cylindrical polyps. Each polyp had a small number (18–21) of alternating longer and shorter tentacles arranged in two rings distinguishable by their size and position. Siphonoglyph was not detected. Measured stolons were 1.2–2.0 mm in width, with 3–5 polyps per 1 cm. At thinner stolons (up to 1.5 mm) polyps were arranged in one row, and in two rows and more on thicker (1.5–2.0 mm) ones. In the middle of colonies, most of stolons were interconnected and fused, and polyps were denser set. By April–May 2018, larger irregularly shaped colonies reached a size up to 55–60 mm across.

The larger polyps were 3–4 mm high, with an oral disk up to 1.5–2.5 mm in diameter. Tentacles of undisturbed, fully expanded polyps reached up to 2.5–4 mm, with basal diameter 0.2–0.25 mm (Fig. 1*B*–1*D*). By May 2018, in the grown colonies brownish-green globular bodies were clearly visible within the polyp walls, oral disks, and tentacles, giving the tissues a brownish color (Fig. 1*B*–1*D*). Small growing polyps (<1 mm in diameter) in the distal part of stolons were almost transparent (Fig. 1*B*). Same structures have been seen observed at early developmental stages (planulae and settled larvae). Based on PCR with Symbiodiniacea-specific primers (Manning, Gates, 2008), we consider these structures to be symbiotic algae of the family Symbiodiniacea.

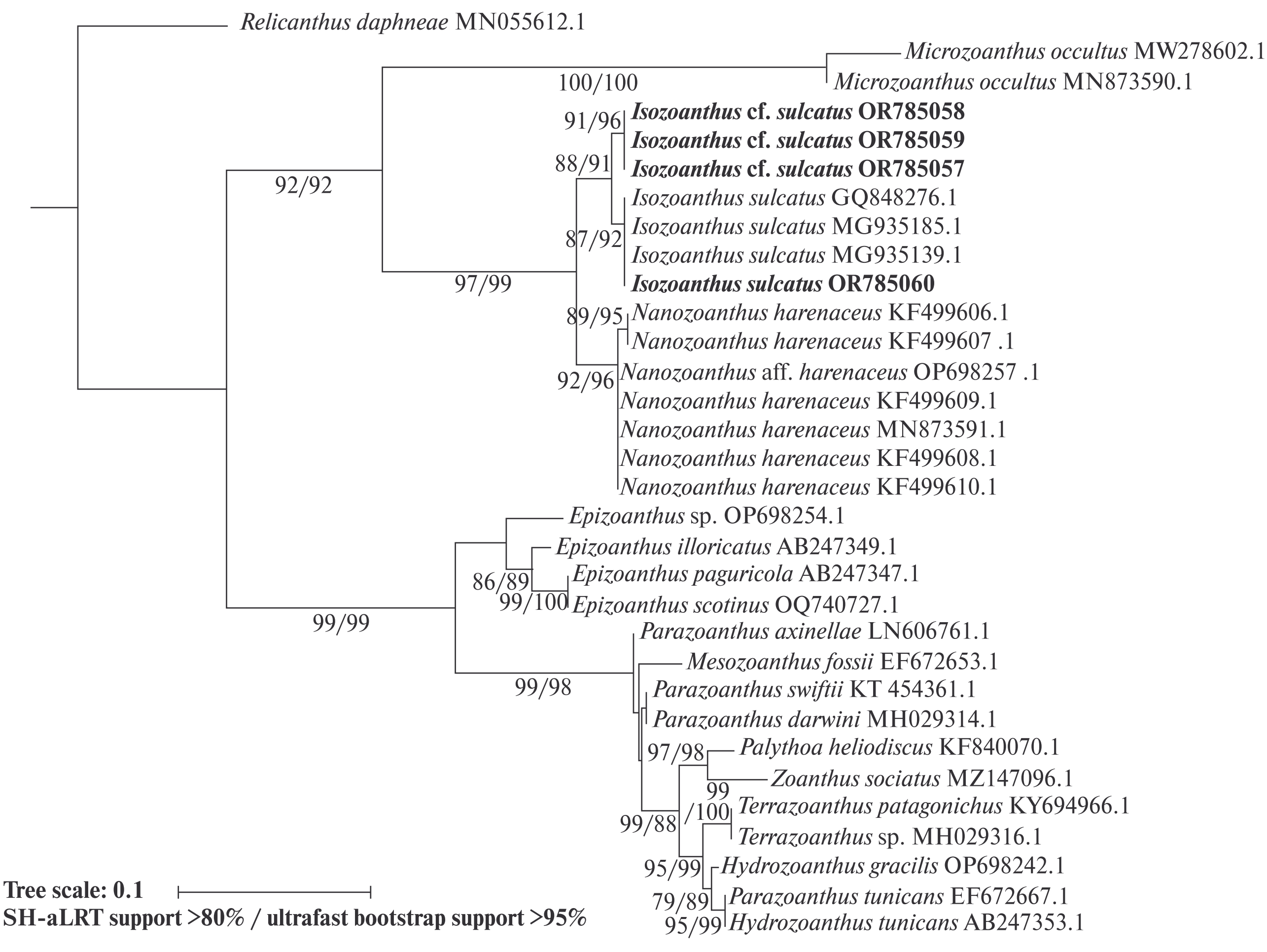

Fig 2. The ML Phylogenetic tree COI gene (658 bp). Only reliable supports values (SH–aLRT/UFboot greater than or equal to 80/95%) are shown. Data generated in our study are marked in bold.

A specific PCR product was obtained for two of three zoantharian specimens from the Black Sea and *Isozoanthus sulcatus* from the Plymouth area. The latter is reported to harbor "zooxanthellae" (Williams, 2000). Genetic identification of symbionts from *Isozoanthus* cf. *sulcatus* from the Black Sea is currently under way.

The colonies survived in the aquarium for approximately nine months, but in June 2018 they all died due to algal bloom.

The resulting ML reconstruction using COI sequences (Fig. 2) supported the monophyly of specimens from the Black Sea with *Isozoanthus sulcatus* (Gosse 1860) from the North Atlantic Ocean (Mediterranean Sea, Sweden and Plymouth area). Three COI sequences of the Black Sea specimens were identical to each other. Sequences of *I. sulcatus* from the Plymouth area were identical to those of GenBank *I. sulcatus* from the Medditerranean Sea and Skagerrak (Sweden). The similarity between the two *Isozoanthus* groups of specimens was 98.8%. Variability of COI in Zoantharia is rather low, thus, specimens from the Black Sea may represent a new species closely related to *I. sulcatus*.

In absence of material for morphological analysis, we were not able to formally describe a new species. In resulting phylogenetic tree, the clade comprised of *I. sulcatus* and *I.* cf. *sulcatus* was closely related to *Nanozoanthus* Fujii & Reimer 2013 and less to *Microzoanthus* Fujii & Reimer 2013. The lack of COI sequences for type species of the genus, *Isozoanthus giganteus* Carlgren in Chun 1903, does not allow us to discuss further phylogenetic relationships of the genus *Isozoanthus* Carlgren in Chun 1903. More genetic data is required to address this question.

Information on reproductive strategies in Zoantharia is limited (Ryland, 1997; Previati et al., 2010). Nevertheless, the majority of Macrocnemina (Previati et al., 2010) and particularly *I. sulcatus* (Carlgren, 1913; Williams, 2000) are reported to have separate sexes. Thus, the discovery of hundreds of competent planulae suggests the presence of a reproductive population.

The question of whether this species is invasive or native remains unresolved. *Isozoanthus* cf. *sulcatus,* from the Golubaya Bay demonstrates remarkable tolerance for low (17.5–18 psu) salinity. The invasiveness of the new species could explain this, but there is no direct indication of where it might have come from. The genus *Isozoanthus* has never been reported from the Black Sea neither it been listed from the Sea of Marmara (Cinar et al., 2014). However, the small size of polyps (1.0–2.5 mm in diameter in studied colonies), and the lack of focused research may contribute to diminutive zoantharians remaining undetected (e.g. Reimer et al., 2017). Further targeted investigations of cryptic habitats, such as shallow sedimented benches, cracks and crevices, hold the potential to enhance our understanding of the Black Sea biodiversity.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work does not include any human or animal studies that meet the criteria of Directive 2010/63/EU.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

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ПЕРВАЯ НАХОДКА ЗОАНТАРИИ В ЧЕРНОМ МОРЕ: *ISOZOANTHUS* CF. *SULCATUS*, ВЫРАЩЕННЫЕ ИЗ ПЛАНУЛ

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Приведены данные о первой находке представителя отряда Zoantharia в Черном море. Зоантарии были успешно выращены из планктонных личинок, собранных в прибрежном мелководье Голубой бухты (район Геленджика). Личинки были отсажены в небольшой стеклянный контейнер, и образовавшиеся колонии содержались в аквариуме с соленостью 18 psu в течение примерно 9 месяцев, пока не погибли из-за неконтролируемого роста нитчатых водорослей в аквариуме. Показано присутствие в тканях колоний симбиотических водорослей семейства Symbiodiniaceae. Филогенетический анализ с использованием митохондриальных (COI) маркеров выявил высокую степень сходства черноморской зоантарии с *Isozoanthus sulcatus* (Gosse 1860) из морей Европы.

Ключевые слова: Parazoanthidae, мелководье, Голубая бухта, Кавказ