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Research Article

Genetic analyses of non-native species *Oithona davisae* Ferrari F.D. & Orsi, 1984 in the Black Sea

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Abstract

A non-native species of the cyclopoid copepod in the genus *Oithona* was first recorded in the Black Sea in 2001 and was initially identified as *Oithona brevicornis* Giesbrecht, 1891. During the following years, this species became widely distributed first in the coastal then in the open areas of the Black Sea and later spread into the adjacent Sea of Azov through the Kerch Strait. Recent detailed re-examination of morphological features of this *Oithona* species indicated it is not *O. brevicornis*, but may be *O. davisae* Ferrari F.D. & Orsi, 1984 or perhaps one of the other species similar to *O. brevicornis*. This study examined the morphological features of this *Oithona* species collected from sites in the western and northeastern Black Sea, and genetic analyses were performed on specimens collected in the western Black Sea to clarify its identification. While we concluded the non-native species was not *O. brevicornis* or any of the native *Oithona* species, the lack of information on *Oithona davisae* in GenBank precludes definitive identification of taxonomic status. Additional genetic data are needed on *O. davisae* collected from its native and non-native range to allow definitive identification of specimens of this non-native species.

Key words: non-native species, cyclopoid copepod, morphology, genetic analyses

Introduction

A non-native species of cyclopoid copepod of the genus *Oithona* was first recorded in the Black Sea in 2001 and was initially identified as *Oithona brevicornis* Giesbrecht, 1891 (Zagorodnyaya 2002; Altukhov and Gubanova 2006; Gubanova and Altukhov 2007). Other authors followed this identification in subsequent studies (e.g., Selifonova et al. 2008; Selifonova 2009; Shiganova et al. 2012; Mihneva and Stefanova 2013; Timofte and Tabarcea 2012). In 2011, this species from the Black Sea was specified as the subspecies *Oithona brevicornis brevicornis* Giesbrecht, 1891 in the World Registry of Marine Species (WoRMS, cited in Shiganova et al. 2012). Identification of all previous authors was based on the examination of a few morphological characters, such as the shape of the head (rounded anteriorly) and rostrum (sharply pointed) and the numbers of spines on segments 1–3 of legs 1–4 (with the formula of 1, 1, 3; 1, 1, 3; 1, 1, 3; 1, 1, 2) in the female (Zagorodnyaya 2002; Gubanova and Altukhov 2007). However recent detailed examination of morphological features of this *Oithona* species by Temnykh and Nishida (2012) led them to the conclusion that the cyclopoid species recently recorded as new to the Black Sea is not *O. brevicornis*, but *O. davisae* Ferrari F.D. and Orsi, 1984, or include one or more species that are similar morphologically to *O. brevicornis* (Temnykh and Nishida 2012). Since this non-native species was morphologically determined as *O. davisae* and our identification supported this identification, we used that name throughout this document.

Oithona davisae spread widely around the Black Sea. Populations of the species first developed mainly in the coastal waters but now are found in waters above up to 1000 m depth in northeastern, northwestern and western Black Sea. Maximum abundance and biomass occurs during August to December (Temnykh et al. 2012; Selifonova 2009; 2011; Shiganova et al. 2012; Mikhneva and Stephanova 2013). *O. davisae* often dominates in

the mesozooplankton community, comprising 80–85% of the total abundance in late autumn (Selifonova 2011; Shiganova et al. 2012). In August 2010, *O. davisae* was recorded in Temruk Bay of the Sea of Azov and by 2013 it had spread around the whole of the western and central Sea of Azov, where it occurred in high numbers (Svistunova 2013).

In the coastal and open Black Sea, *O. davisae* occupied the niche of the now absent *O. nana* (Shiganova et al. 2012; Temnykh et al. 2012), a species that was a valuable food item for fish larvae (Tkach et al. 1998). At present, the suitability of *O. davisae* as prey for small pelagic fishes is unresolved (Mihneva and Stefanova 2013). The feeding preference on flagellates and microzooplankton by *O. davisae* (Nakamura and Turner 1997) suggests it has an importance in the food web but more detailed studies are needed. Before the role of the non-native species in the food web can be evaluated, it is important to ascertain the organisms tentatively identified as *Oithona davisae* are indeed this species or are there multiple species involved. Therefore the goal of the paper was to use molecular genetic techniques, along with more traditional morphometric means, to clarify the identification of this widely distributed non-native *Oithona* species in the Black Sea.

Material and method

Sampling

Zooplankton samples for genetic analyses were collected by means of integrated tows in the 0–15 m water column, using a Juday net (0.1 m^2 opening mouth and 150 μm mesh), in the coastal waters of Bulgaria at Varna Bay (43°10.710' N, 27°55.930' E) and Cape Kaliakra (43°22.000' N, 28°30.000' E) in August 2013. Immediately after collection, samples were drained of excess water and preserved by un-denatured 95% etahnol.

We selected 25 individuals of *O. davisae* for molecular analysis. A few specimens were picked out, put in a glycerin drop, and examined under a microscope (Magnification 200 or 400 x) for species identification using morphological characteristics. The total length of these individuals was measured by ocular micrometer from the anterior end of the prosome to the posterior end of the caudal ramus.

DNA amplification and sequencing

A Promega Wizard SV Genomic DNA Purification Kit (Promega Corporation, Madison, USA) was used for tissue lysis from each analyzed specimen and DNA purification, in accordance with the manufacturer's protocol. DNA concentration and purification efficiency was determined by the electrophoresis on a 1.2% agarose gel. We analyzed the fragment 18S rDNA of five O. *davisae* specimens. The 18S rDNA gene was amplified in three overlapping fragments of about 950, 900 and 850 bp each, using primer pairs 1F-5R, 3F-18Sbi and 18Sa2.0-9R, respectively (Giribet et al. 1996): 1F (TACCTGGTTGATCCTGCCAGTAG), 5R (CTTGGCAAATGCTTTCGC), 3F (GTTCGATTCCGGAGAGGGA), 18Sbi (GAGTCTCGTTCGTTATCGGA), 18Sa2.0 (ATGGTTGCAAAGCTGAAAC), 9R (GATCCTTCCGCAGGTTCACCTAC).

Loci were amplified using Encyclo PCR kit (Evrogen Joint Stock Company, Russia). Amplification was done in a total volume of 25 мl solution mix containing 1 X PCR buffer, 1 μl of 10 μM of primer pair mix, 1 μl of template, 0.2 mM of each dNTP and 0.5 units Taq polymerase. Solution mixture were heated to 94°C for 120 s, followed by 35 cycles of 15 s at 94°C, 30 s at a specific annealing temperature and 60 s at 72 °C, and then a final extension of 7 min at 72°C on Veriti® Thermal Cycler. Annealing temperature was set to 49 °C for the 18S primer pairs 1F-5R and 18Sa2.0-9R, 52 °C for the 18S primer pair 3F-18Sbi. Amplification products were purified by the Promega PCR Purification Kit protocol (Promega) and sequenced in both directions. Each sequencing reaction mixture, including 1 мl BigDye3.1 (Applied Biosystems, Perkin-Elmer Corporation, Foster City, CA), 1 мl of 1 μM primer and 1μL of DNA template, ran for 40 cycles of 96°C (15 s), 50°C (30 s) and 60°C (4 min). Sequences were purified by ethanol precipitation to remove unincorporated primers and dyes. Products were re-suspended in 12 μl formamide and electrophoresed in an ABI Prism 3500 sequencer (Applied Biosystems).

The obtained sequence of the 1729 bp was submitted to GenBank (accession number KJ814022).

Molecular data analyses

Multiple alignments for 18S ribosomal RNA gene of genus *Oithona* were made using Clustal W (Wang and Jiang 1994). Cladogramms were built by neighbor-joining method (Saitou and Nei 1987). Tree node creditability values were calculated in 1000 iterations of bootstrap analysis (Felsenstein 1985).

18S ribosomal RNA gene sequences from NCBI GenBank (JF288757 *O. brevicornis*, GU969179

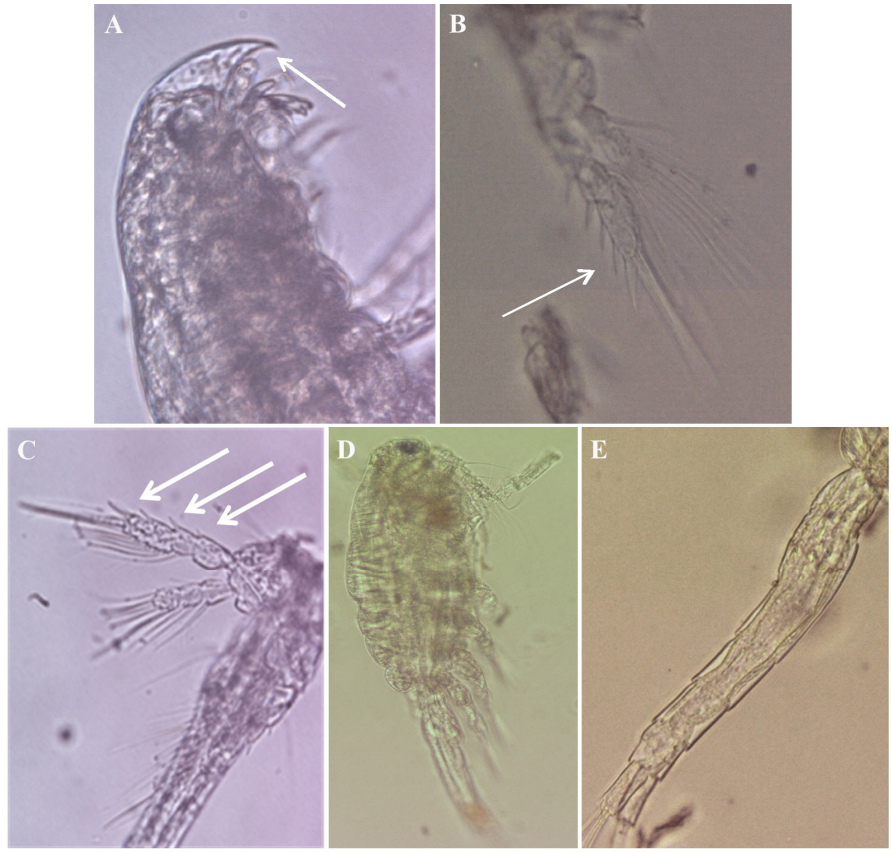

Figure 1. Photos *of Oithona davisae*: (A) laterally view of rostrum of female, (B) 2nd swimming leg, (C) 4th swimming leg with exopodite spines (3 arrows), (D) male and (E) prosome. Photographs by K.Stefanova.

O. similis, HQ008733 *O. hebes*, HQ008734 *O. nana*, JF81539 *Oithona* sp., HQ00873 *O. simplex*) were used along with the original data. The number of base differences per 100 sites between sequences was calculated (as %). Analyses were conducted using the Maximum Composite Likelihood model (Tamura et al. 2004). Eight nucleotide sequences were involved in the analyses. All positions containing gaps and missing data were eliminated. There were a total of 545 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011).

Results

Morphological analysis

The average (± SE) total length of 25 measured female specimens was 0.57 ± 0.03 mm (range 0.52 to 0.60 mm). Body form was oval with rostrum pointed ventrally (Figure 1A). The numbers of exopod spines on the 1–3 swimming legs were 1,1,3 and on the 4th – 1,1,2 (Figure 1B, C). The total length of the males ranged between 0.48 and 0.53 mm (n=20, average 0.5 ± 0.02) and the rostrum was absent (Figure 1D). Species identification was based on Ferrari and Orsi (1984) and Temnykh and Nishida (2012). We compared the morphological differences among *O. wellershausi* Ferrari F.D., 1982, *O. aruensis* Früchtl, 1923, *O. brevicornis* and *O.davisae*. The formula for number of exopod spines on swimming legs and the body shape with rostrum are similar for all of the aforementioned species. Hair rows on the dorsolateral surface of the genital double-somite and the next somite of *O. davisae* prosome were absent (Figure 1E) in contrast to *O. brevicornis* where they exist.

Molecular data analyses

No information was available for *Oithona davisae* Ferrari and Orsi DNA sequences in open access species data bases; therefore the first task was to determine whether the new species belonged to *O. brevicornis*, as it had first been identified. There were 18S and 28S ribosomal RNA genes

Table 1. Phylogeny of the genus *Oithona* based on 18S ribosomal RNA gene sequences with *Limnoithona tetraspina* is as the out group. Estimates of Evolutionary Divergence between Sequences (%). The unidentified species was presumed to be *O. davisae* (based on morphology).

	O. davisae	*L. tetraspina*	*O. brevicornis*	*O. hebes*	*O. nana*	*O. similis*	*O. simplex*
Limnoithona tetraspina	6.5						
Oithona brevicornis	8.0	6.2					
Oithona hebes	8.2	6.1	5.0				
Oithona nana	10.0	7.7	7.8	6,4			
Oithona similis	6.5	4.8	1.3	3.8	6.5		
Oithona simplex	7.7	4.3	5.6	4.2	7.5	4.2	
Oithona sp.	5.9	4.8	4.0	3.6	6.3	2.6	3.2

primers available for *O. brevicornis* and primer 18S was chosen because it was the longest (1600 pairs of nucleoids). In addition, available data of all representatives of genus *Oithona* 18S ribosomal RNA gene primers were used.

No polymorphisms were found in the 18S rDNA fragments of the five specimens of "*O. davisae*" that we analyzed. Comparative analyses of 18S rRNA sequences showed that the "*O. davisae*" samples did not match any *Oithona* species from GenBank data base. The genetic distance between analyzed specimens and *Oithona* representatives found in Genbank were comparable with the mean genetic distance between the different *Oithona* species (Table 1). The nucleotide differences from *O. brevicornis* was large (8%) while the least distance (5.9%) was for *Oithona* sp. from New Caledonia (Table 1**).**

Thus we may conclude that in accordance to conducted analyses this new species does not belong to *O. brevicornis* and also does not belong to any of the native Black Sea *Oithona* species, such as *Oithona nana* or *O. similis*. These data supported our hypothesis that the analyzed specimens belong to a species which is not yet in the GenBank database.

Discussion

According to review of Temnykh and Nishida (2012), *Oithona davisae* has been reported from the coastal waters of Japan (e.g. Nishida et al. 1977), as "*O. brevicornis* f. *minor*" (Nishida and Ferrari 1983). It has been reported as "*O. aruensis*"; (Nishida 1985; Ohtsuka et al. 2008) from Korea (Lee et al. 2001; Orui-Sakaguchi et al. 2011), California (Ferrari and Orsi 1984), Chile (Hirakawa 1988), and the northwestern Mediterranean (reference from Temnykh and Nishida 2012 on Nishida's unpublished observation cited by Saiz et al. 2003).

Oithona davisae was also found in the ballast water of ships that arrived in Vladivostok from Chinese coastal areas (Kasyan 2010). Therefore, *O. davisae* has been considered as endemic of the temperate coastal waters of East Asia (Ferrari and Orsi 1984; Nishida 1985; Hirakawa 1988), and its occurrence in other regions is likely a result of introduction, mainly through ship's ballast waters (e.g. Carlton 1987; Hooff and Bollens 2004; Cordell et al. 2008). In Puget Sound, USA, *O. davisae* has been reported as one of the most common and abundant non-native species found in ships ballast waters (Lawrence and Cordell 2010).

Based on previously cited observations, and on our morphological and genetic results, we can conclude that the non-native species *Oithona* in the study area is not *O. brevicornis*. However, we cannot fully support the identification as *O. davisae* by Temnykh and Nishida (2012) due to the lack of information in GenBank, although it is the correct identification based on our limited examination of morphology of the species. Clearly, a full evaluation of the taxonomic status, including additional molecular genetics analyses, of this species is warranted and should include specimens from different areas of the Black Sea, north western Mediterranean, and the Pacific Ocean - areas where the occurrence of this species (both native and non-native) has been reported.

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