Mar. Sci. Tech. Bull. (2020) 9(1): 23–31
e–ISSN: 2147–9666
info@masteb.com

dergipark.org.tr/en/pub/masteb
www.masteb.com
DOI: 10.33714/masteb.668529

Marine Science and Technology Bulletin

RESEARCH ARTICLE

First molecular record of the alien species Pacific oyster (*Crassostrea gigas*, Thunberg 1793) in the Marmara Sea, Turkey

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ARTICLE INFO

Article History:

Received: 31.12.2019
Received in revised form: 03.02.2020
Accepted: 03.02.2020
Available online: 03.02.2020

Keywords:
Crassostrea gigas
Invasive species
COI gene
16S mtDNA
Marmara Sea

ABSTRACT

The Pacific oyster (*Crassostrea gigas*) has a very important economic potential for aquaculture, but on the other hand, is among the highly invasive species in the world and within the Mediterranean ecosystem. In the 1960s, *C. gigas* was brought to Europe for aquaculture in the Mediterranean and Black Sea regions from Japan and Canada. The Turkish waters are the part of the Mediterranean Sea, which is the world's most invaded sea. The invasion of alien species results from marine transportation and aquaculture activities of non-native species. A heavy maritime traffic is also present in the Marmara Sea, which connects the Black Sea and Mediterranean Sea. The identification of the invasive species and their distributions is very prominent in terms of protecting natural habitat and monitoring the effects of invasive species. In this study, 30 individuals, morphologically identified as *C. gigas*, were collected from Bandırma bay. The genomic DNAs were extracted from each sample's muscle tissue using universal salt extraction method. Partial sequences of COI and 16S Mitochondrial DNA loci of the sample DNAs were obtained for species identification. The sequences were searched against the database and results were retrieved from BLAST. All the sequences obtained in this study showed significant similarity with the *C. gigas* sequences present in the database (E=0). The sample sequences resulted in 9 different haplotypes for the COI locus (hd: 0.5296 and variance: 0.01256±0.112) and 5 different haplotypes for the 16S rDNA locus (hd: 0.2529, Variance: 0.01076±0.104). The results of this study provided the first molecular evidence for the presence of non-native Pacific oyster individuals in the Marmara Sea.

Please cite this paper as follows:

Özcan Gökçek, E., Acarlı, S., Karahan, B., Vural, P., Koban Baştanlar, E. (2020). First molecular record of the alien species Pacific oyster (*Crassostrea gigas*, Thunberg 1793) in the Marmara Sea, Turkey. *Marine Science and Technology Bulletin*, 9(1): 23-31.

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Introduction

Oysters are bivalves widely distributed all around the world estuaries. They are benthic, sessile filter-feeders, and reef-builders that are playing important roles in estuary ecosystem (Ren et al., 2016). The Pacific oyster (*Crassostrea gigas*) is one of the world's 20 most cultured species with high economic values owing to their useful traits for aquaculture like efficient filter feeding, high growth rates, strong reproductive ability and tolerance to a wide range of environmental conditions (Laugen et al., 2015). However, it is also one of the most invasive species and may exert some negative impacts on native oyster species. The possible effects of the invasive species on the native species are; sharing the same area and food resources, genetic pollution due to hybridization, introgression and decrease of genetic diversity.

As human population continues to grow, the demand on seafood continuous to increase as on any other food sources. Aquaculture is important to ensure a consistent supply of aquatic species as harvesting the wild populations (fish, crustaceans and others) cannot keep up with the increasing human population's demand. For example, *C. gigas* production in 2016 by fishery was 17370 tons meanwhile its production by aquaculture was 639030 tons (FAO, 2020).

The spread of economically important, but invasive species throughout the world has been greatly facilitated by means of aquaculture, maritime transportation and the trade of aquatic organisms (Crocetta et al., 2015). The Mediterranean Sea is the world's most invaded sea. A total of 5% of the whole marine species in the Mediterranean habitat is considered non-local, 13.5% of these species are considered as invasive species and this ratio is increasing due to abovementioned human activities (Galil, 2009, Zenetos et al., 2012; Segvic-Bubic et al., 2016). These activities also lead to the transport of invasive species from the Mediterranean to the Marmara Sea (Çınar et al., 2011).

Mollusks show an important native distribution in the eastern and middle Mediterranean. The European flat oyster (*Ostrea edulis*) is a native oyster species in the Mediterranean region. This species live in muddy, muddy sandy, rocky, muddy pebbly and dense alluvium. They feed on microalgae and they either live freely or by fixing themselves with their right shells in coastal waters (Tebble, 1966). In economic and food quality terms, *O. edulis* is a very valuable species in the markets (Yildiz et al., 2011; Acarli et al., 2015; Smyth et al., 2018). Unlike *O. edulis*, *C. gigas* is not a native species in Mediterranean region. On the contrary, it is a black-listed invasive species in conservation programs prepared for its non-native Mediterranean ecosystem (DAISIE, 2016).

The Pacific oyster is a particularly euryhaline and eurythermal species. Its salinity and temperature tolerances vary widely (Miossec et al., 2009). It attaches to rocks, debris and shells and found from the lower intertidal zone to depths of 40 m. It is naturally found in the northeastern Asia and had been widely distributed in the tropical seas (Zibrowius, 1992; Galil, 2000). It has become a popular species for aquaculture in Europe in the second half of the 20th century (Lallias et al., 2015). The aquaculture trials of *C. gigas* started in the south of France using the imported breeding populations from Japan and Canada in the late 1960s (Grizel and Heral, 1991). Then, they were found in Adriatic and soon, their distribution expanded from Cyprus to Tunisia (Dridi et al., 2006) including most regions of the Mediterranean. In 1991, an aquaculture study was conducted in Homa lagoon area in Izmir using the juvenile samples obtained from France (Özden et al., 1993). The breeding practices have resulted in the establishment of wild *C. gigas* populations in the Black Sea, the Mediterranean Sea and along the Atlantic European coasts (Nehring, 2011; Angles d'Auriac et al., 2017).

Oysters are easily affected by environmental changes and show a wide variety of morphological traits such as shell formation and color, and these factors make the accurate identification of the oyster species very difficult (Galvão et al., 2017) and may lead to taxonomic misclassifications and misidentifications (Lam and Morton, 2006; Liu et al., 2011; Pagenkopp Lohan et al., 2015; Ren et al., 2016). Therefore, besides the morphometric measurements, the use of genetic markers (e.g. SNP, RAPD, RFLP, microsatellites, etc.) is inevitable. The use of genetic markers is also very valuable in studies with different aims (Işık, 2019; Işık and Bilgen, 2019; Özdil et al., 2019). In the last few decades, the developments in the molecular science have provided better results for species identification employing suitable molecular tools (Reece et al., 2008; Salvi et al., 2014; Pagenkopp Lohan et al., 2015). DNA barcoding analysis provides high accuracy in identifying species with high morphological plasticity, based on a standard mitochondrial cytochrome c oxidase subunit I (COI) and 16SrDNA fragments (Lapègue et al., 2002; Boudry et al., 2003; Hebert et al., 2003; Varela et al., 2007; Lazoski, 2011; Keskin and Atar, 2013; Crocetta et al., 2015; Segvic-Bubic et al., 2016; Galvão et al., 2017).

The first records about the existence of *C. gigas* in Turkey was reported in (i) Marmara Island, Southern Marmara Sea, by Yüksek (1989); (ii) Tuzla, Levantine Sea by Çevik et al. (2001) (ii) Çeşme, Aegean Sea by Doğan et al. (2007), and (iv) Marmara Sea (Acarlı et al., 2017). These studies were based on morphologic investigations. However, Özcan Gökçek et al. (2017) identified oysters from *Crassostrea* genus among the

samples collected from the north Aegean Sea by using RAPD technique. The present study aimed to genetic identification of the morphologically identified non-native oysters found in the southern Marmara Sea based on two molecular markers; partial COI and 16S rDNA sequences.

Material and Methods

Sampling

A total of 30 individuals were collected from Bandırma Bay, the Marmara Sea (40°22'03.43"N, 27°55'29.47"E) (Figure 1). The individuals were selected as they all had Pacific oyster (*C. gigas*) shell characteristics (Figure 2).

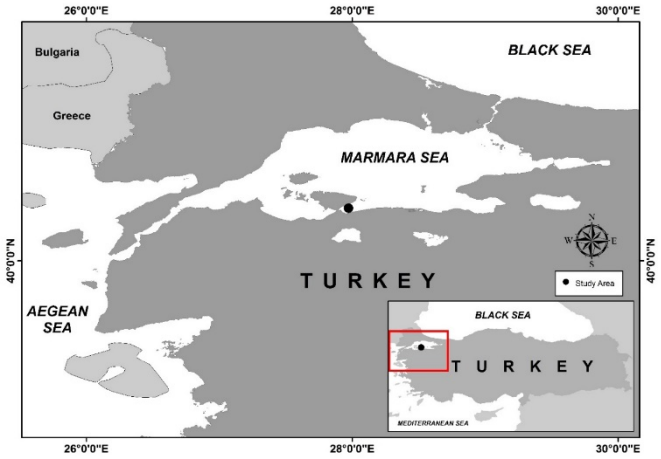

Figure 1. Sampling location of the study

Figure 2. Oyster samples collected from Bandırma Bay, Marmara Sea

DNA extraction, PCR amplification and sequencing

The adductor muscles were taken from live samples and stored at -20°C until DNA extraction. Genomic DNAs of the samples were extracted using Universal-Salt Method (Aljanabi and Martinez, 1997). The quality and quantity of the extracted DNA were checked by both agarose gel electrophoresis and spectrophotometry techniques. The RedSafe (Intron-Korea) dye was used to stain and visualize the DNA bands under UV light.

For the PCR amplification of the COI gene, the universal primers (LCO1490 and HCO2198) designed by Folmer et al. (1994) were employed. In addition, the primers (16S.AR and 16S.BR) designed by Palumbi (1996) were employed for the PCR amplification of the 16S gene. The 30 µL PCR volume contained: 50-100 ng genomic DNA, 0.4 µM of each primer, 1×PCR Buffer, 200µM dNTP, 2.5mM $MgCl_2$ and 0.6U of Taq DNA polymerase (i-Star Taq, Intron- Korea). The cycling protocol was 1 min at 94°C, 30 cycles of 94°C for 45 s annealing temperature (50°C for COI and 55°C for 16S gene) for 90 s, 72°C for 60 s with a final extension at 72°C for 10 min (Liu et al., 2011) annealing.

Having checked the PCR amplicons by electrophoresis, all the quality PCR amplicons were sent to Medsantek (Istanbul, Turkey) for sequencing by an automated capillary electrophoresis system (Applied Biosystems, 3500xL Genetic Analyzer, Thermo Fisher Scientific, UK). The electropherograms were carefully checked by Chromas Pro v1.42 (Technelysium Pty. Ltd. Australia) for miscalls and base spacing. Afterward, the contigs were formed for each sample individually by aligning its forward and reverse sequences, and a final data file consisting of consensus sequences for each sample was obtained. These sequences were deposited in the NCBI GenBank database (MN862563, MN862564, MN862565, MN862566, MN862567, MN862568, MN862569, MN862570, MN862571, MN862572, MN862573 MN862574, MN862575, MN862576).

Data analysis

The BIOEDIT software (Thompson et al., 1994) was used for multiple sequence alignment of the consensus sequences and trimming of both ends to prepare the data file for further statistical analysis. Later, the trimmed file consisting of COI and 16S gene nucleotide sequences was analyzed by the software DnaSP v5. (Librado and Rozas, 2009) for estimating the haplotype and nucleotide diversity parameters. Afterwards, the sequence data obtained for the COI and 16S regions and the reference sequences taken from GenBank were used in reconstruction of the phylogenetic tree based on Maximum Likelihood (ML) method applying HKY nucleotide substitution model for COI and T92 nucleotide substitution model for 16S rDNA by MEGA (Molecular Evolutionary Genetics Analysis) software version 7 (Kumar et al., 2016). The nucleotide substitution models were selected based on the results obtained from ModelTest implemented in the software MEGA. In order to test the reliability of the tree topology, bootstrapping (×1000) was performed.

Results

A total of 60 DNA sequences from 30 individuals and two loci were obtained. The partial mtDNA COI sequence (655 bp long) revealed 11 polymorphic sites leading to 9 different haplotypes (hd: 0.5296 and variance: 0.01256±0.112). One of these 9 haplotypes had a very high frequency (20/30).The 492 bp long partial 16S rDNA sequence revealed 4 mutations leading to 5 different haplotypes (hd: 0.2529, Variance: 0.01076±0.104). One common haplotype was observed in 26 individuals. The nucleotide sequences of the COI and 16S rDNA were found to be 98-99% identical with *C. gigas*'s mt genome when searched against the database using BLAST.

For the phylogenetic reconstruction based on the 9 different mtDNA COI sequences (representing the 9 different haplotypes), some reference sequences were retrieved from the database initially. These sequences belonged to *C. gigas* (KJ855241, AF177226, HM626169, FJ717608, KJ855242-KJ855245, AF280608), *Crassostrea angulata* (LC383459) and *O. edulis* (JF274008) species. The Maximum Likelihood tree based on HKY nucleotide change model revealed one clade containing the *C. gigas* sequences from the database as well as all of the nine sequences of the present study (Figure 3). All the samples of the *Crassostrea* genus were separated from the *O. edulis* sample.

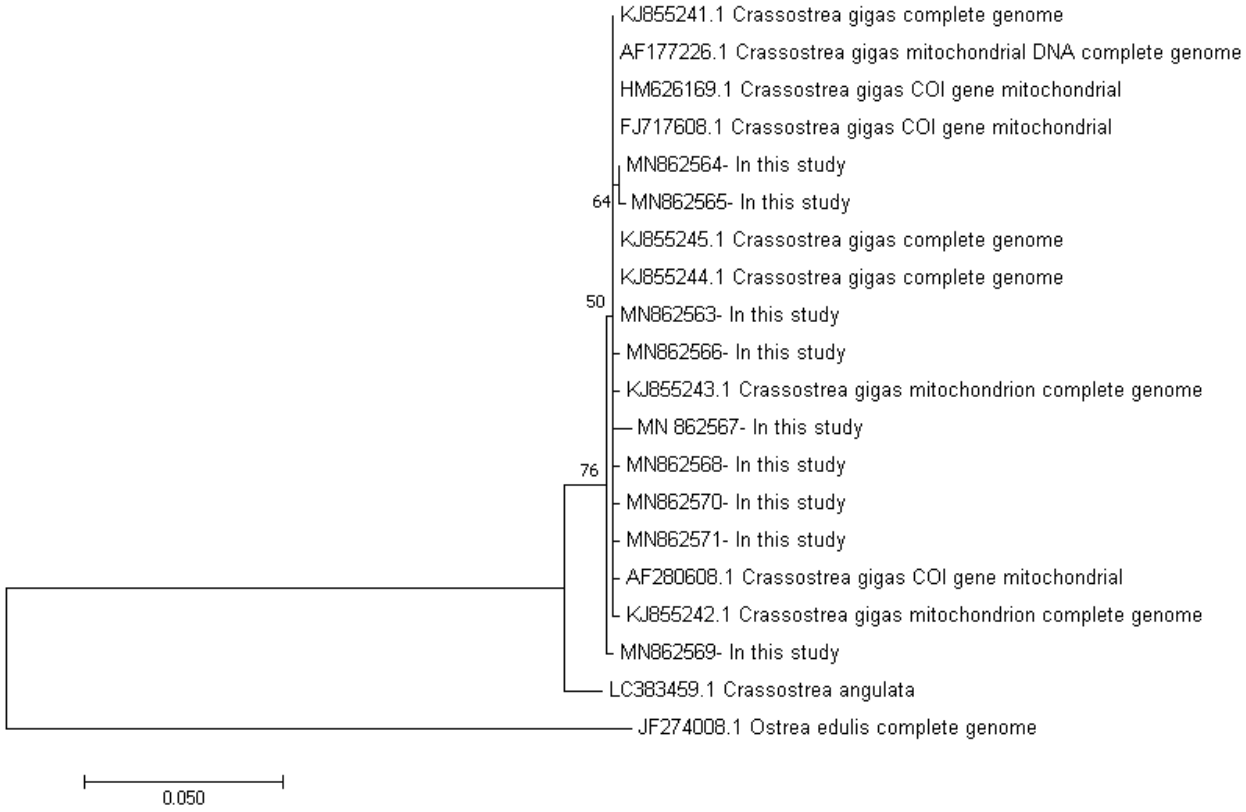

Figure 3. The ML tree was reconstructed based on the partial mtDNA COI sequences representing 9 haplotypes (MN862563-MN862571) of the present study and included the sequences of the species *O .edulis, C. gigas and C. angulata* that were retrieved from the GenBank. For this phylogenetic reconstruction MEGA 7 software was employed. Numbers at the nodes represent bootstrap supports.

In order to infer evolutionary relationship of the sequences obtained from the present study with the other Oyster species based on the partial 16S rDNA sequence, some reference sequences were also retrieved from the GenBank. These sequences belonged to *C. gigas* (AJ553903-AJ553905, KX34620, AF280611, MF663018, LC005445), *C. angulata* (AJ553901, AJ553902, KY446769), *Crassostrea virginica* (KC429253) and *O. edulis* (KX394616, KX394618) species. The ML tree based on 16S rDNA sequences and T92 nucleotide substitution model revealed one clade containing all the haplotypes of the present study together with the *C. gigas* sequences and *C. angulata* sequences from the database (Figure 4). Yet, the *C. angulata* sequences grouped together with a 58% node support. All these sequences separated from the *C. virginica* sample with a 99% bootstrap support. Furthermore, all the samples of the *Crassostrea* genus were separated from the *O. edulis* samples.

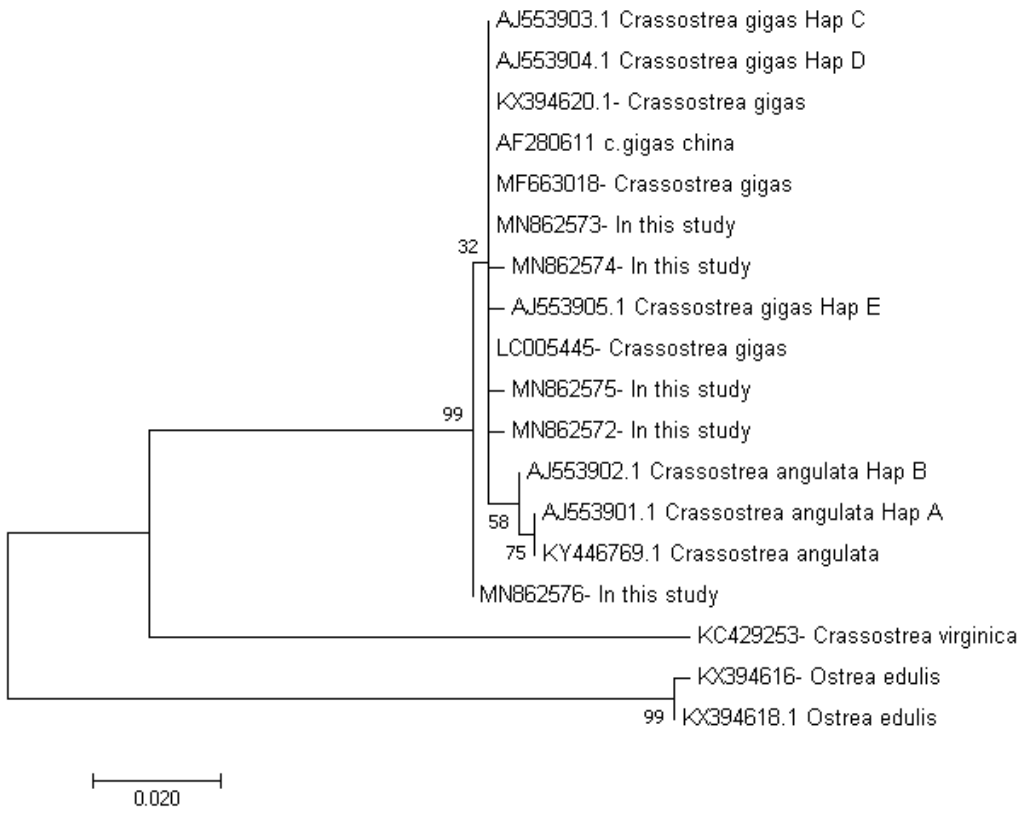

Figure 4. The ML tree was reconstructed based on the partial 16S rDNA sequences representing 5 haplotypes (MN862572-MN862576) of the present study and included the sequences of the species *O.edulis, C. gigas, C. angulata* and *C. virginica* that were retrieved from the GenBank. For this phylogenetic reconstruction MEGA 7 software was employed. Numbers at the nodes represent bootstrap supports.

Discussion

The sequences obtained in the present study clustered with the *C. gigas* samples obtained from the database. The two DNA markers employed in the study provided different resolutions when discriminating between the two closely related species: *C. gigas* and *C. angulata*. The partial mtDNA COI sequences revealed more haplotypes and separated species from each other statistical support (76%). Nonetheless, the16S rDNA sequences could not differentiate between these two species. Therefore, it can be suggested that the mtDNA COI gene provides better information in barcoding studies. Yet, it should be noted that the length of the sequences was different. The mtDNA COI sequences were 655 bp long, and the 16S rDNA sequences were 492 bp long. Increasing the sequence length may increase the discrimination power of the sequences.

Although there is a study (Albayrak et al., 2004) mentioning the existence of *C. gigas* in the Marmara Sea; this is the first study investigating the presence of this species in this region based on molecular markers. Since oysters have high levels of morphological plasticity, it can be misleading to make identification only based on the morphological characters (Boudry, 2003). For instance; Segvic-Bubic et al. (2016) reported that some of the oyster specimens classified as *Crassostrea* clade according to the morphological investigations were actually *O. edulis* based on the 16s mitochondrial DNA marker. Therefore, it is important to use molecular markers as well as morphological measurements for species identification in oysters.

There has been no record of aquaculture practices for the Pacific oyster in Marmara Sea. It is known that *C. gigas* is capable of long-distance transport in the planktonic phase of 20-30 days (Schmidt et al., 2008). They are found around aquaculture areas and they can attach to the vessels. It is highly likely that human activities may induce their spread to non-native ecosystems (Pecarevic et al., 2013). Therefore, it can be concluded that the transportation and spread of *C. gigas* to the Marmara Sea have probably occurred via vessels or water currents (Albayrak, 2011); the international maritime traffic being probably the main factor.

Considering the habitat preferences of *C. gigas*, Marmara Sea may provide a very suitable habitat for this invasive species due to its proper environmental conditions. Acarli et al. (2017) reported that the meat yield (AFNOR index-oyster quality) of *C. gigas* has changed from “fine” to “special” in the Bandırma Bay population. In this study, the oysters sampled for

sequencing had an average length of 88.02±22.26 mm. These large individuals observed in the area and the DNA sequencing data obtained in this study provide support for the existence of a self-sustaining population of *C. gigas* in the southern Sea of Marmara. These results suggest that oysters had adapted to environmental conditions in Bandırma Bay such as temperature, salinity, etc., and showed good development performance when evaluated commercially. Furthermore, the large individuals in the study area indicate that the oysters have adapted and reproductive activity was performed. Similarly, Segvic-Bubic et al. (2016) provided the evidence of self-sustaining *C. gigas* populations in Adriatic Sea based on the mt 16S rRNA sequence analysis.

C. gigas is listed in the Delivering Alien Invasive Species Inventories for Europe (DAISIE, 2016). Due to the high physiological capacity and adaptation ability of Pacific oyster, the competition risk with other indigenous species is a very important issue (Laugen et al., 2015). *C. gigas* prefers similar habitats to the native blue mussel (*Mytilus edulis*) and *Mytilus galloprovincialis* found in different areas of Mediterranean and Atlantic coasts as reported by different studies (Diederich et al., 2005; Crocetta, 2011; Lipej et al., 2012; Dolmer et al., 2014; Angles d'Auriac et al., 2017). There are some negative impacts exerted by *C. gigas* on these native species such as competition for food and space (Nehls et al., 2006; Nehring, 2011). In addition, cross-fertilization may occur and hybridizations may be observed. During the sampling work of this study, it was observed that *C. gigas* shared the same beds with *O. edulis* (the native species) at the sampling site (Bandırma Bay, Marmara Sea) possibly causing competition for space and food between the two species.

Conclusion

The Pacific oyster has been reported to cause a decline in natural populations of native oyster and mussel species, with which it shares the habitat and resources (Markert et al., 2009; Wilkie et al., 2012). As the presence of this species was confirmed for the first time based on molecular markers by this study, it can be a start signal for monitoring studies employing both molecular markers and morphological markers when assessing the status of both invasive and native species. Molecular markers are especially important when the species of interest has high phenotypic plasticity.

The native oyster species are part of their natural habitat and they have an economic value. However, they are under threat by invasive species. The invasive Pacific oyster *C. gigas* species have already established populations in the Mediterranean Sea. Considering the reports from Turkish waters based on morphology and the results of this study, it can be suggested that this species has already established populations in Turkish waters, too. Moreover, this species has a high economic value on its own, too. Immediate programs on monitoring the possible effects of Pacific oyster on *O. edulis* and the other bivalve species sharing the same habitat should be started in Bandırma Bay as well as in the other areas of the Marmara Sea. The results to be obtained from monitoring studies should aid in the development of accurate action plans for the sustainable protection of the ecosystem. In addition, even though currently it is not cultured/harvested for economic purposes, monitoring studies may help in the consideration of this invasive species economically.

Acknowledgments

This work was supported by the Scientific Research Projects Coordination Unit of Çanakkale Onsekiz Mart University (Project No: FBA 2014-331, granted to Sefa Acarlı).

Conflict of Interest

The authors declare that there are no conflicts of interest to disclose.

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