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Research Article

First record, DNA identification and morphometric characterization of Pacific oyster, *Crassostrea gigas* (Thunberg, 1793) in the southern Black Sea

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Abstract

This paper reports on the first record of the Pacific oyster, *Crassostrea gigas* (Thunberg, 1793), in the southern Black Sea, based on a sample of 235 specimens collected from rocky shores 23 km west of the city of Ordu, northern Turkey. Species identification was confirmed by nucleotide sequencing of two mitochondrial gene fragments, viz. COI and 16S rRNA in five individual oysters. Analyses of mitochondrial cytochrome oxidase I (COI) sequences from southern Black Sea coastlines suggest that all samples were Pacific oyster, *Crassostrea gigas*. In addition, this study provides the first analysis of length-weight relationships (LWR) for *C. gigas* collected from the southern Black Sea. The relationship between mean shell length (SL) and mean total weight (W) was $SL = 0.0143W^{1.6662}$ ($r^2 = 0.6589$). The specimens were morphometrically characterized as follows: mean shell length (SL) 59.57 ± 13.65 mm (range: 24.09–98.17 mm), mean shell width (SWi) 28.05 ± 6.91 mm (range: 10.50–50.87 mm), mean total weight (W), 13.62 ± 5.03 g (range: 0.78–36.89 g), and mean meat weight of 1.5 ± 0.90 g (range: 0.01–5.83 g). The relationships between the morphometric parameters suggested negative allometric growth. According to the results, *C. gigas* has created breeding populations on the Turkish Coasts, becoming the dominant species on some hard substrate, including rocky bottoms and large rocks used as fill locally to gain more land in the coastal area.

Key words: invasive species, bivalve, introduced, morphology

Introduction

The Pacific oyster, *Crassostrea gigas* (Thunberg, 1793), is an important commercial species (Angell 1986; Wolff and Reise 2002; Mizuta et al. 2012; Jiang et al. 2013; Chávez-Villalba 2014; Treviño et al. 2020) and has become the leading species for shellfish culture with a world production of over 600,000 tons in 2017 (FAO 2019). As such, it also accounts for 97% of the world oyster production (Tidwell 2012). The species is currently specifically distinguished from the Portuguese oyster, *Crassostrea angulata* (Lamarck, 1819) (WoRMS: http://www.marinespecies.org/aphia.php?p=

taxdetails&id=836032, accessed on May 10th 2020). Biologically, it may be correct to treat these two taxa within the "gray zone" of species delimitation, where their taxonomic interpretation is a matter of the species concepts considered and, the level of decision applied (De Queiroz 2007; Bayne 2017). This issue is well discussed for *C. gigas* and *C. angulata* by Bayne (2017).

Morphological characters such as length-weight (L-W) and length-length (L-L) relationships are important parameters to assess the biology of molluscs for providing information about the conditions of the region where molluscs live (Agboola and Anetekhai 2008). Negative allometric growth means that an organism's weight increases slower relative to its growth (size), meaning that organisms tend to be thinner as they grow (Farías-Tafolla et al. 2015). The shell morphology of *C. gigas* is highly variable and depends on the type of substrate on which it is grown and the degree of crowding. Shell shape depends on several environmental factors and habitat, including temperature, salinity, population density, with length-width ratios being higher in molluscs living in or on soft substrates than hard substrates (Harding 2007). The two valves are *solid*, but unequal in size and *shape* (CIESM: http://www.ciesm.org/atlas/Crassostreagigas.html, accessed on June 2nd 2020). Specimens are permanently attached to hard substrates (i.e. on rocks) or live on soft sediments (i.e. muddy sand) in temperate tidal and sub-tidal zones (Nehls and Büttger 2007; Hughes 2008). Due to its rapid growth and broad tolerance to varying environmental conditions, *C. gigas* has become the oyster of choice for cultivation in many regions of the world. While its native area is in Japan and South-East Asia, where it has been cultivated for centuries, it has been widely introduced elsewhere, most significantly along the USA's western coast in the 1920's and France in 1966.

These, and many other introductions aimed to reinforce existing aquaculture programs, allowed the species to further expand along coastlines since the late 1990s, and have progressively invaded all European waters (Nehring 2011). As such, *C. gigas* has also been reported from the northern Black Sea, where it has been both accidentally and deliberately introduced (Zolotarev 1996; Zaitsev and Öztürk 2001; Slynko et al. 2018), as well as from the Sea of Marmara (Yüksek 1989; Albayrak 2011; Gökçek et al. 2020), the Aegean Sea and the Levantine Sea (Öztürk et al. 2014). However, the presence of this species on the Turkish Coast of the Black Sea was hitherto unknown.

The present study reports the first record of *C. gigas* along southern Black Sea coasts by providing a DNA sequence and morphometric characterization of *C. gigas* in this newly colonized area. We investigated several morphological parameters, including shell length, shell width, meat weight, shell thickness, and total weight of this species and confirmed its identification by DNA barcoding (Hsiao et al. 2016).

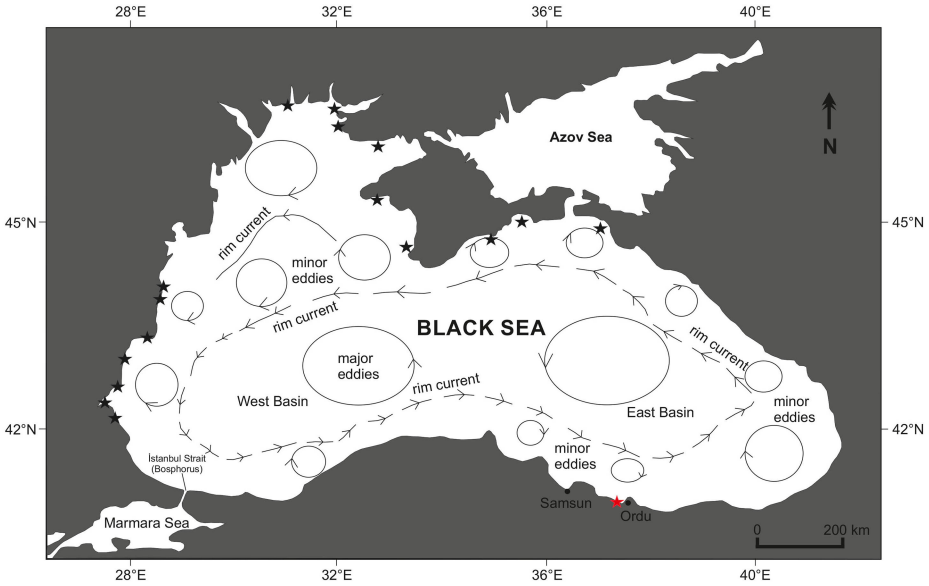

Figure 1. Map showing the study location and circulation patterns in the Black Sea (modified from Tuzhilkin 2008; Kershaw 2015). The black stars indicate the distribution of non-native *Crassostrea gigas* (Thunberg, 1793) in the Black Sea (modified from Mitov et al. 2020) and the red star displays the sampling locality reported in this study.

Materials and methods

Regional setting

The study area (41°04′44″N; 37°49′00″E) is located 23 km west of Ordu, situated in the Black Sea region, northern Turkey (Figure 1). August is the month with the warmest seawater in Ordu, with an average sea temperature of 26.2 °C (https://www.weather-tr.com, accessed on June 2nd 2020). According to the Navy Coastal Ocean Model (NCOM), seawater salinity in Ordu is around 18‰ (https://www.ncdc.noaa.gov/data-access/model-data/model-datasets/navoceano-ncom-glb, accessed on June 2nd 2020). The Black Sea is the largest anoxic basin globally (Jones and Gagnon 1994), and İstanbul Strait (Bosphorus) is the only connection of the Black Sea to the world ocean via the Sea of Marmara and the Dardanelles Strait. Warm and saline Mediterranean water enters the Black Sea basin as an undercurrent, creating a delta fan structure with its levée-channel system and mixing with deep Black Sea waters (Özsoy and Ünlüata 1997). The shallow sill depth of the İstanbul Strait and the oxygen consumption caused by organic matter mineralisation are responsible for establishing a permanent oxic-anoxic boundary (chemocline). Today, this oxic-anoxic boundary is situated at a depth of 100–150 m.

Sampling

Sampling was performed by handpicking in the southern Black Sea region in 2019 at depths of 0–5 m on rocky shore substrates near the harbour of Ordu (northern Turkey). A total of 235 adult specimens was collected in an area of about 200 m².

Morphometric measurements

Shell length (SL), shell width (SWi) and shell thickness (ST) of the 235 specimens were measured with digital calipers to the closest 0.1 mm. Subsequently, specimens were dried on drying paper and the total weight (W), meat weight (MW) and shell weight (SW) of individuals measured with a Precisa balance (up to 0.01 g).

Length-weight relationship (LWR) was estimated using the equation $W = a.L^b$ (W: Weight (g); L: Length (mm); where "a" is a coefficient and "b" is a slope and an exponent indicating isometric growth when b = 3. In contrast, b > 3 indicates positive allometric growth and b < 3 indicates negative allometric growth (Froese 2006; Shingleton et al. 2009). Student's t-test was used to evaluate the type of growth. Student's t-tests were also used to evaluate growth-in-length conversion to growth-in-weight equations (Ricker 1975).

The SL and W of individuals were selected for determination of growth performance following Ricker (1975):

$$\text{SL increase (\%)} = [(SL_n - SL_{n-1}) / SL_{n-1}] \times 100, \text{ n is a length class.}$$

$$\text{Weight gain (\%)} = [(W_n - W_{n-1}) / W_{n-1}] \times 100, \text{ n is a weight class.}$$

All relationships between SL and W, SWi, ST, SW, MW, between SWi and W, ST, SW, MW, between ST and W, MW, SW, between SW and W, MW and between MW and W were analyzed using regression equations and are presented in Figures 2–3.

DNA identification

Individual genomic DNA was extracted from adductor muscle tissue from five specimens using the Nucleospin® Tissue Kit (Macherey-Nagel) according to the manufacturer's instructions.

Two mitochondrial gene fragments (COI and 16S rRNA) were PCR-amplified. COI was amplified with the primer pair LCO1490 and HCO2198 (Folmer et al. 1994), and 16S rRNA with 16Sar and 16Sbr (Simon et al. 1994). PCR reactions were performed with 1.5 µl DNA extract in a total volume of 20 µl. The Qiagen® Multiplex PCR Kit was used to amplify the COI fragment according to the manufacturer's instructions, at an annealing temperature of 40 °C. For the 16S rRNA, the PCR reaction contained final concentrations of 0.2 mM dNTP's, 0.2 µM of each primer, 0.5 U of Qiagen® Taq DNA polymerase and 1.5 mM $MgCl_2$ in the 1x PCR buffer. The thermal cycler program for the 16S rRNA amplification consisted of an initial denaturation step of 5 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 45 °C and 60 s at 72 °C, with a final extension of 5 min at 72 °C. PCR products were checked with agarose gel electrophoresis and subsequently purified with Exonuclease I and FastAP™ (Thermo Scientific). Both DNA strands were sequenced (same primers as for PCR), using the Big Dye Terminator v3.1 chemistry (Applied Biosystems, USA).

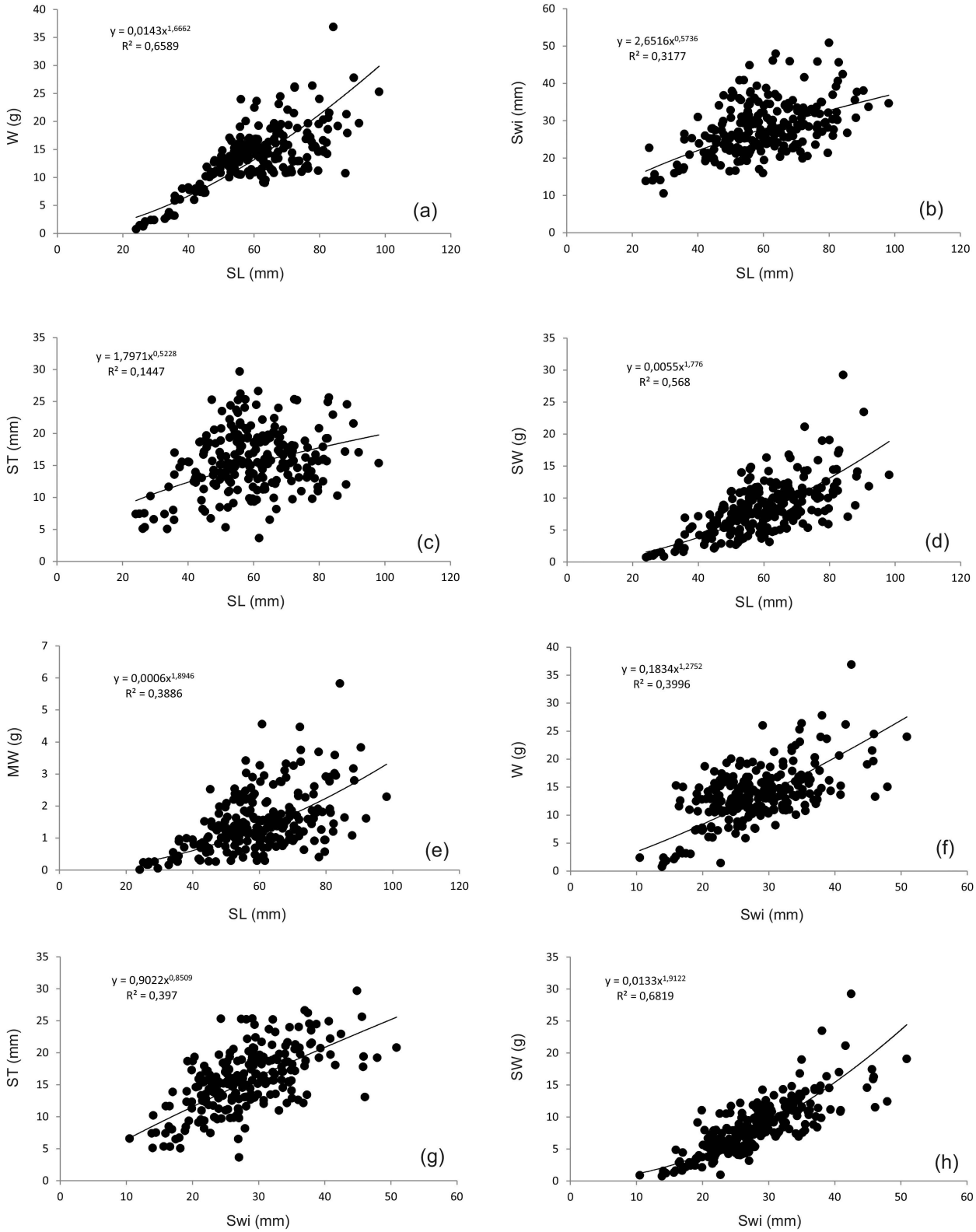

Figure 2. Morphometrics of *Crassostrea gigas* from Ordu, Turkey. Relationships between shell length (SL) and a) total weight (W), b) shell width (SWi), c) shell thickness (ST), d) shell weight (SW), e) meat weight (MW), and relationships between shell width (SWi) and (f) total weight (W), (g) shell thickness (ST), h) shell weight (SW).

DNA sequences were assembled and corrected using CodonCode Aligner version 8.0.2 (https://www.codoncode.com/). Each gene fragment was aligned separately, including the new sequences from Ordu and

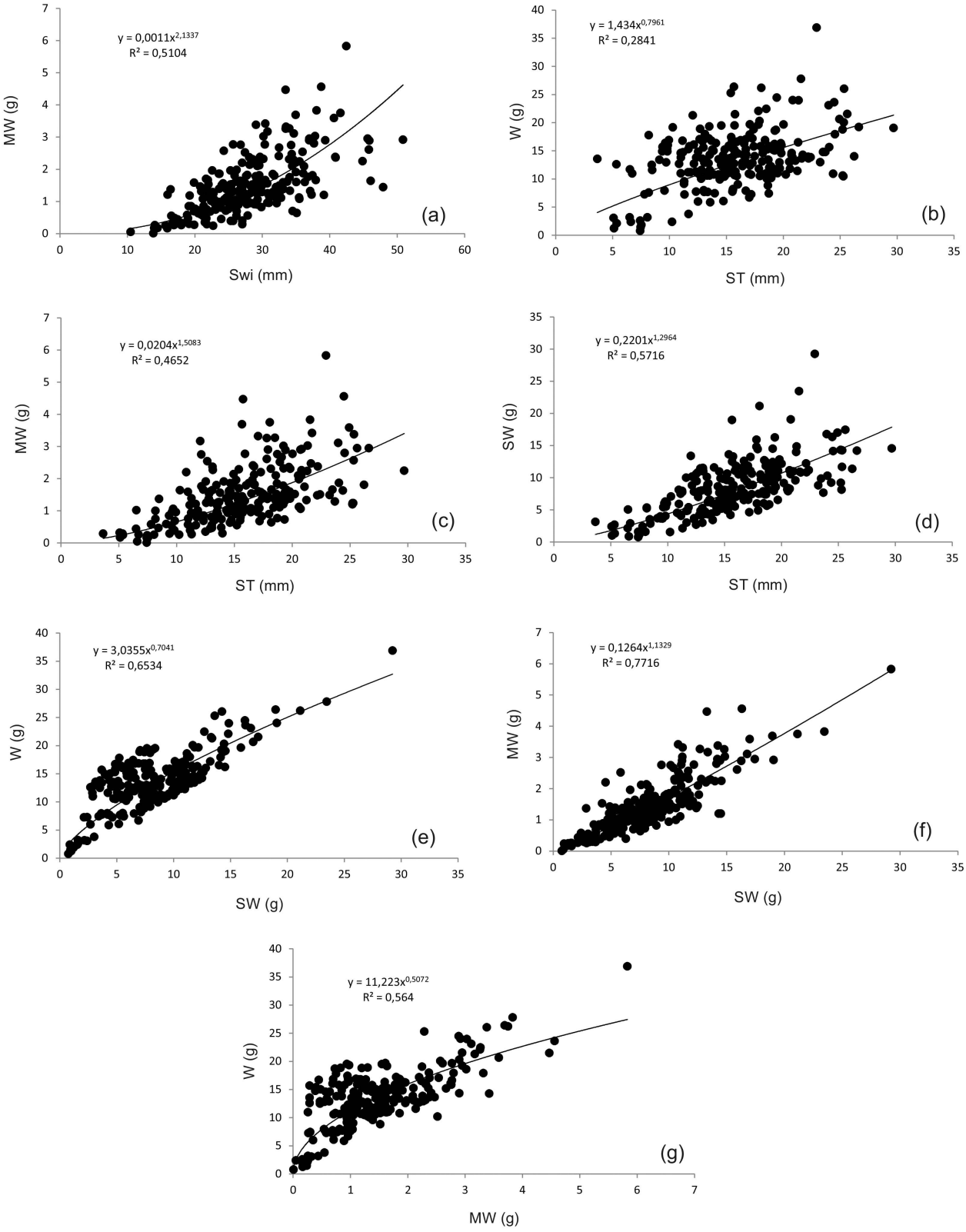

Figure 3. Morphometrics of *Crassostrea gigas* from Ordu, Turkey. Relationships between shell width (SWi) and a) meat weight (MW) and between shell thickness (ST) and b) total weight (W), c) meat weight (MW), and between shell weight (SW) and d) shell thickness (ST), e) total weight (W), f) meat weight (MW), and g) meat weight (MW) and W.

Crassostrea sequences retrieved from Genbank and BOLD. For the COI alignment a search in BOLD was performed on "*Crassostrea*" and "*Magallana gigas*" (19/04/2021); as such, 1678 sequences were extracted. A search in

Table 1. MEGA 6: Mean p-distance for COI and 16S rRNA within and between *Crassostrea gigas* and *C. angulata* based on the present data and unique sequences available in GenBank.

	# unique sequences	Mean p-distance
COI *C. gigas*	15	0.0053
COI *C. angulata*	13	0.0053
COI *gigas* vs. *angulata*	28	0.0279
16S rRNA *C. gigas*	7	0.0035
16S rRNA *C. angulata*	5	0.0030
16S rRNA *gigas* vs. *angulata*	12	0.0098

Genbank on "*Crassostrea* and 16S ribosomal" (20/04/2021) gave 351 sequences. After cleaning the sequence data files, aligning with CLUSTAL W (Thompson et al. 1994), restricting the length of the alignments, and generating the haplotypes in DnaSP (Rozas et al. 2017), the final COI dataset has a length of 506 bp and consists of 616 unique sequences. The 16S rRNA alignment includes 81 unique sequences and has a length of 438 bp. The COI and 16S alignments are available upon request. Sequences from *Saccostrea cucullata* (Born, 1778) were added as an outgroup. Neighbor-Joining (NJ) trees were inferred with MEGA 6 using p-distances and with pairwise deletion of indels. Branch support (BS) was assessed by bootstrapping with 1000 replicates.

All new *Crassostrea* sequences from Ordu (northern Turkey) have been deposited in GenBank under the accession numbers: MT350568–MT350572 for 16S rNA and MT350630–MT350634 for COI. The five sequenced individuals and additional voucher material have been deposited in the collections of FDBF-PIS/2018-2 in the Fatsa Faculty of Marine Sciences, Ordu University (Turkey).

Results

DNA identification

The lengths of *Crassostrea gigas* gene fragments from Ordu were 649 bp for COI and 488 bp for 16S rRNA. Blast searches for the five individuals from Ordu yielded the following best species match scores: for COI *C. gigas* with 99.85% identity and next best *C. angulata* with 97.69%; for 16S rRNA *C. gigas* with 100% and next best *C. angulata* with 99.39%. The mean p-distances within and between *C. gigas* and *C. angulata* are provided in Table 1. They show that for both COI and 16S rRNA the mean p-distances within each taxon are comparably small, whereas the mean p-distances between the two taxa are much higher, as would be expected when comparing different species.

The NJ tree for COI clustered the *Crassostrea* sequences from Ordu with *C. gigas* with very high support values, viz. BS = 98 (Figure 4). The 16S rRNA showed the same clustering pattern, though with a support of 76% (Figure 5). Yet, in no instances did *Crassostrea* from Ordu cluster with *C. angulata* or any other *Crassostrea* species.

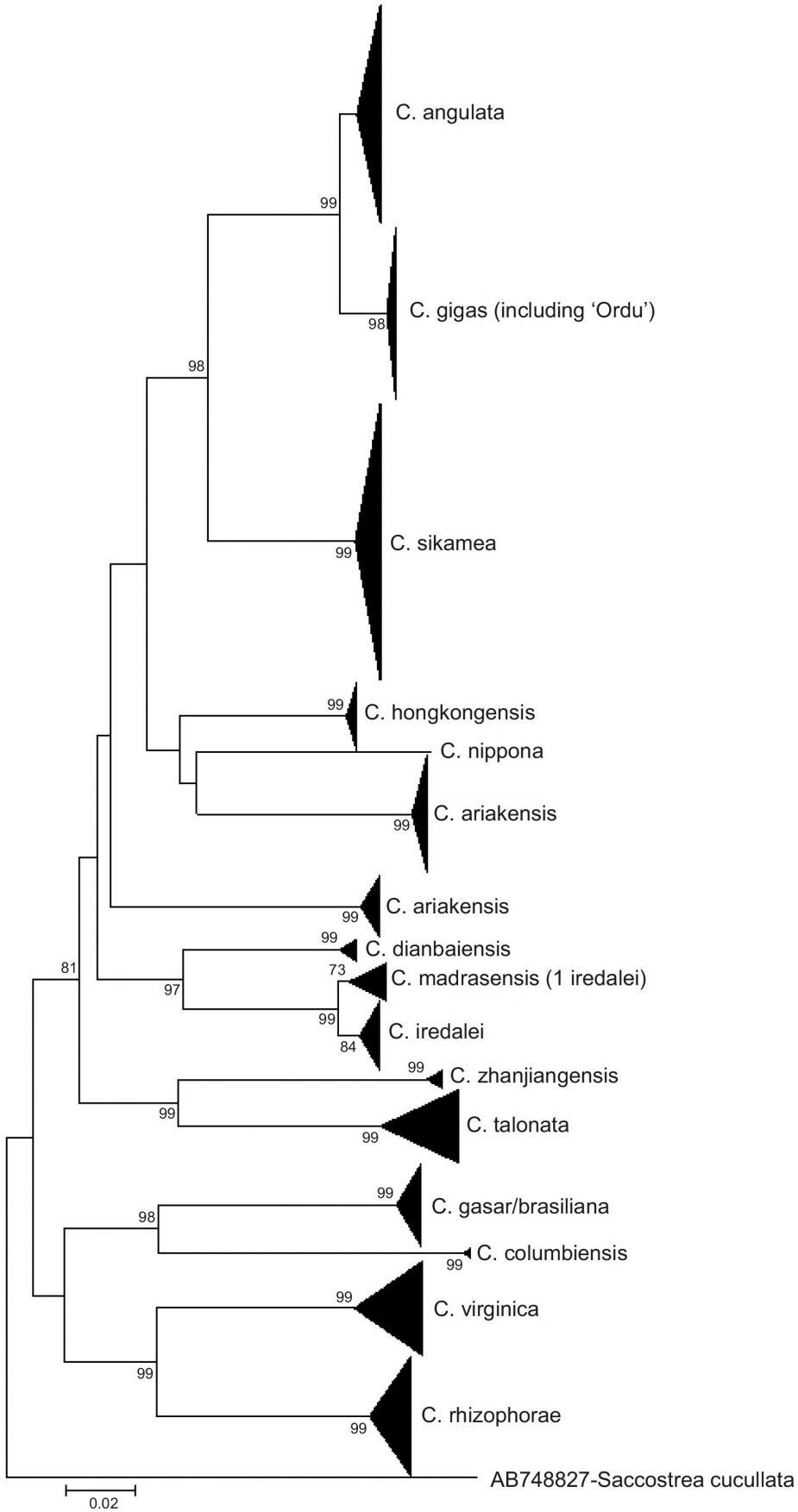

Figure 4. Neighbor-Joining tree of *Crassostrea* species based on COI sequences downloaded from BOLD, including *C. gigas* recorded from the southern Black Sea (Ordu); numbers at the branches correspond to bootstrap values higher than 70%.

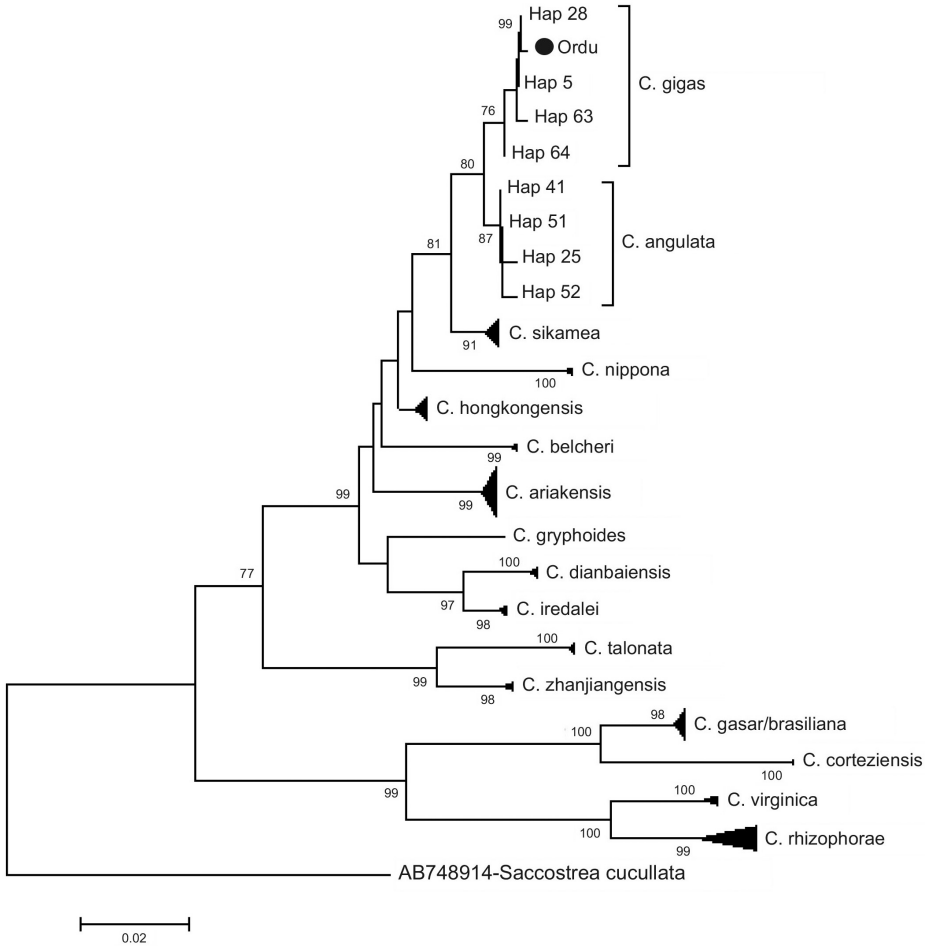

Figure 5. Neighbor-Joining tree of *Crassostrea* species based on 16S rRNA; numbers on branches correspond to bootstrap values higher than 70%.

Table 2. Shell length (SL), shell width (SWi), shell thickness (ST), shell weight (SW), total weight (W), and meat weight (MW) of *Crassostrea gigas* (n = 235) from Ordu, Turkey.

	SL (mm)	SWi (mm)	ST (mm)	W (g)	MW (g)	SW (g)
Mean	59.57	28.05	15.80	13.62	1.50	8.47
Minimum	24.09	10.50	3.65	0.78	0.01	0.75
Maximum	98.17	50.87	29.69	36.89	5.83	29.24
± SE	13.65	6.91	4.74	5.03	0.90	4.10

Shell morphometric properties of Crassostrea gigas

Bivalves are usually defined in terms of shell dimensions, e.g, shell length (SL), shell thickness (ST) and shell width (SWi) (Lucas et al. 2019). SL of *Crassostrea gigas* from Ordu, ranged from 24.09 to 98.17 mm, with a mean value of 59.57 mm and with 54% of the shells ranging between 55 and 65 mm. W ranged from 0.78 to 36.89 g with a mean value of 13.62 g and SW ranged from 0.75 to 29.24 g with a mean value of 8.47 g. An overview of all measured shell morphometric properties is provided in Table 2. Regressions between morphometric characters are given in Table 3.

The strongest regression was found between SW and SWi (r^2 = 0.6819) and the weakest regression between ST and SL (r^2 = 0.1447). Negative allometric growth implies the *C. gigas* becomes more slender as it becomes

Table 3. Regression relationships and formulas of morphometric characters of *Crassostrea gigas* from Ordu, Turkey.

Regression Formula	r^2	*t*	*P*
$W = 0.0143\ SL^{0.16662}$	0.6589	15.841	***
$W = 0.1834\ SWi^{1.2752}$	0.3996	2.753	**
$W = 1.434\ ST^{0.7961}$	0.2841	4.417	***
$SWi = 2.6516\ SL^{0.5736}$	0.3177	7.701	***
$SWi = 0.9022\ ST^{0.8509}$	0.3970	9.878	***
$MW = 0.0204\ ST^{1.5083}$	0.4652	14.238	***
$ST = 1.7971\ SL^{0.5228}$	0.1447	6.278	***
$SW = 0.0133\ SWi^{1.9122}$	0.6819	15.299	***
$SW = 0.2201\ ST^{1.2964}$	0.5716	11.315	***
$SW = 0.0055\ SL^{1.7760}$	0.5680	13.165	***
$MW = 0.0011\ SWi^{2.1337}$	0.5104	10.352	***
$MW = 0.0006\ SL^{1.8646}$	0.3886	6.292	***

W: Total weight; SL: Shell length; SWi: Shell width; ST: Shell thickness; SW: Shell weight; MW: Meat weight; Explanations: **, regression is significant at the 0.01 level (2-tailed); ***, regression is significant at the 0.001 level (2-tailed).

Table 4. Growth performance values of *Crassostrea gigas* from Ordu, Turkey. Bold values indicate an increase in length for 45 mm (SL) and an increase in weight for 35 mm (SL).

SL (mm)	N	%	Mean SL (mm)	Mean W (g)	Increase in length (%)	Increase in weight (%)
25	2	0.9	24.62	1.12	–	–
35	12	5.1	31.78	3.2	29.1	**185.7**
45	21	8.9	43.03	8.09	**35.4**	152.8
55	64	27.2	52.29	12.94	21.5	60.0
65	63	26.8	60.80	14.55	16.3	12.4
75	41	17.4	69.88	16.07	14.9	10.4
85	26	11.1	79.82	17.85	14.2	11.1
95	6	2.6	90.86	20.46	13.8	14.6
Total	235		59.57 ± 13.65	13.62 ± 5.03		

longer, as indicated by $b < 3$. Positive allometric growth implies the *C. gigas* becomes relatively more stout or deeper-bodied as it increases in length, as indicated by $b > 3$. Growth showed negative allometry with $b < 3$ for all relationships.

This is the first information of LWR parameters for *C. gigas* in the southern Black Sea. Maximum growth in length was found within the 45 mm-length groups showing a length increase of 35.4%. Maximum growth in weight was observed within the 35 mm-length groups showing a weight increase of 185.7% (Table 4).

Discussion

DNA based species identification

Results from the DNA analysis leave no doubt that the oyster specimens from Ordu are *Crassostrea gigas*. The DNA differentiation with respect to *C. angulata* was clear, well-supported and consistent, supporting the practice of treating these two taxa as separate species.

Recently, Salvi et al. (2014) and Salvi and Mariottini (2017, 2021) suggested that the genus *Crassostrea* should be split into three genera, with

Magallana Salvi & Mariottini, 2017 and *Talonostrea* Li & Qi, 1994 for the Asian Pacific species, and *Crassostrea* Sacco, 1897 for the Atlantic species. Under this scheme, *Crassostrea gigas* should be assigned to the genus *Magallana* and hence be renamed as such. However, this nomenclatural suggestion has been strongly challenged (Bayne et al. 2017, 2019), not because the phylogenetic conclusions of Salvi et al. (2014) and Salvi and Mariottini (2017, 2021) would be wrong, but mainly because it disturbs nomenclatural stability for such a well-established name as *C. gigas*, without providing any added value (Backeljau 2018). Given that the clade formerly referred to as the genus *Crassostrea* is well supported, including in the analyses of Salvi et al. (2014) and Salvi and Mariottini (2017, 2021), dividing it into three genera only complicates the nomenclatural situation for the users of taxonomy (Backeljau 2018). Therefore, we endorse this latter position and apply the genus name *Crassostrea* in its traditional and equally correct, but more inclusive sense.

Since recent checklists did not mention the occurrence of *Crassostrea gigas* along the Turkish Black Sea coasts (e.g. Öztürk et al. 2014, 2017), the present report is the first record of this species in the southern Black Sea. It shows that the species appears to form well-established "natural" populations in this area, i.e. outside of aquaculture stations. In the western Black Sea, *C. gigas* was previously reported from rare specimens in the "wild" in Romania, where it was first recorded in 1995 (Micu 2004). Subsequently, the species was deliberately introduced in the Romanian Black Sea area for aquaculture (Zaharia and Crivăț 2017), which probably formed the source of the recently discovered population at Agigea Harbor, Constanta (Romania) (Krapal et al. 2019).

In the northern Black Sea area, *C. gigas* is known since the beginning of the 20th century (Zolotarev 1996; Alexandrov et al. 2007) from occasional, unintentionally introduced, single specimens and, since the 1980s, from specimens likely derived from experimental aquaculture stations (e.g. Zolotarev 1996; Zaitsev and Öztürk 2001; Pirkova and Demenko 2008; Slynko et al. 2018; Vyalova 2019). Given the continuous attempts to develop aquaculture of *C. gigas* in the western and northern Black Sea regions, as well as the recent establishment of free-living populations in the Sea of Marmara [doubted by Zaitsev and Öztürk (2001) and Albayrak et al. (2004), but nevertheless reported by Yüksek et al. (1989) as *C. angulata*, and later firmly confirmed by Acarli et al. (2017) and Gökçek et al. (2017, 2020)], it was to be expected that the species might spread further into the Black Sea.

Morphometrics

Negative allometric growth in *C. gigas* has also been observed in Israel (b = 2.34), Great Britain (b = 2.08), and Australia (b = 2.15) (Hughe-Games 1977; King 1977). However, this differs from other species in the area. For

example, Aydın et al. (2020) observed positive allometry (b > 3) between SWi and TW for the wedge clam *Donax trunculus* in Ordu but negative allometry (b < 3) between ST and TW and SL and TW.

Based on data of five studies conducted on *C. gigas* in different regions of northern Europe, the average length of individuals at the age of one year is 46 mm, 72.1 mm at the age of two years and 91.6 mm at the age of three years (Diederich et al. 2006; Cardoso et al. 2007; Christensen and Elmedal 2007; Wang et al. 2007; Walles et al. 2015). The regression between SL and W in *C. gigas* was $r^2 = 0.6589$. This suggests a strong relationship between SL and W, so that increasing shell length tends to correlate with increasing body weight.

Considering the shell lengths of the specimens sampled at Ordu, it seems probable that the species has spread in the region for at least three years. Moreover, *Crassostrea gigas* is increasingly observed when diving in the Black Sea since 2019, especially in ports with ship traffic and adjacent habitats (M. Aydin *unpublished observations*). *Crassostrea gigas* and other oyster species (for instance *C. virginica*) can live in extreme water conditions which is why these species have been cultivated extensively in several parts of the world (De Silva 1998; Keiner 2010; Herbert et al. 2016; Markert 2020). Based on these facts and our observations, it appears that *C. gigas* has adapted to the Turkish coasts of the Black Sea, creating breeding populations. A management plan should therefore be developed and applied to reduce the potential influence of this species on local native species in the near future.

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