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SHORT COMMUNICATION

Range expansion of the non-native oyster *Crassostrea gigas* in the Adriatic Sea

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The Pacific oyster, Crassostrea gigas*, was introduced into the Europe in the late 1960s for aquaculture purposes and has since spread extensively in Mediterranean waters. For the first time, screening of the four ecologically most suitable bays along the Eastern Adriatic coast was conducted to detect the possible presence of non-native oysters. Sequencing analysis of a fragment of mitochondrial 16S rRNA was examined to demonstrate the presence of the exotic species* C. gigas *in the natural environments of the northern and central coastal areas of the eastern Adriatic. The likely routes of invasion are discussed and the importance of monitoring the distribution of feral* C. gigas *populations emphasized, with a focus on the potential direct and indirect effects on the native European flat oyster,* Ostrea edulis.

Key words: Pacific oyster, invasion, Adriatic Sea, ecological impact, European flat oyster

INTRODUCTION

Aquaculture, the trade of aquatic organisms and maritime transport have facilitated the spread of non-native species across the world and the establishment of many species beyond their native ranges. More than 5% of marine species in the Mediterranean are now considered non-native (ZENETOS *et al.,* 2012). According to the latest regional reviews, 13.5% of those species are classed as being invasive in nature, with macrophytes as the dominant group in the western Mediterranean and Adriatic Seas, and polychaetes, crustaceans, molluscs and fishes in the eastern and central Mediterranean (GALIL, 2009; ZENETOS *et al.,* 2012).

The main recognized pathways of introduced species into aquatic ecosystems are aquaculture practices and transport by ships (KELLER *et al.*, 2011). For aquaculture purpose, live oysters have been moved for a long period around the world and planted in new waters to support commercial cultivation or, in some instances, to establish a capture fishery. Still, the ecological consequences of such introductions are not fully understood.

The European flat oyster, *Ostrea edulis* L., is a native European oyster species that is traditionally cultured in Croatia, with the stable production of 50 tons over the years (Source: Croatian Ministry of Agriculture, Directorate of Fisheries). In several European countries, however, flat oyster production has declined dramatically, beginning in the last century (ABOLLO *et al.*, 2008; CULLOTY & MULCAHY, 1996; VAN BANNING, 1991). Overharvesting, habitat loss and high mortalities due to the parasitic diseases, marteiliosis (caused by the protozoan *Marteilia refringens*) and the more serious bonamiosis (caused by the protozoan *Bonamia ostreae*) have been identified as the main causes of the significant decrease in *O. edulis* production (MACKENZIE *et al.*, 1997; GOSLING, 2003; BUESTEL *et al.*, 2009). To compensate for the scarcity of flat oysters, the Pacific oyster *Crassostrea gigas* has been introduced to Europe, mainly for aquaculture purposes (GRIZEL & HÉRAL, 1991). Today, *C. gigas* has become the leading species in global shellfish culture, with an estimated production of 555 thousand tons in 2013 (FAO, 2005a-2014). However, in the continental NW European estuaries, *C. gigas* has established self-sustaining wild populations with massive reef formations, likely in response to recent climate change and increasing water temperature (TROOST, 2010). Given that *C. gigas* has demonstrated high invasion success, concerns have been raised about the potential effects on other indigenous species (DIEDERICH, 2005; MARKERT *et al.,* 2009), such as the native oyster species *O. edulis,* which is traditionally exploited by the aquaculture industry in Croatian waters.

The main potential environmental impacts related to *C. gigas* introduction include competition for space and food with other intertidal species (NEHRING, 2011), habitat modification by invasive ecosystem engineer (GUTIÉRREZ *et al.*, 2003), hybridization with local oyster species (HUVET *et al.*, 2004) and transfer of parasites and diseases (FORD & SMOLOWITZ, 2007). Feral populations of *C. gigas* have been recorded in the northern Adriatic, i.e. the Gulf of Trieste (CROCETTA, 2011) and along the entire Slovenian coast, mainly in the lower mediolittoral belt (LIPEJ *et al.*, 2012). In Croatian waters, the first larvae of Pacific oyster were recorded thirty years ago in Lim Bay in the northern Adriatic (FILIĆ & KRAJNOVIĆ-OZRETIĆ, 1978; HRS-BRENKO, 1982). To date, the presence of *C. gigas* on boats and at mariculture sites has been reported only sporadically (PEĆAREVIĆ *et al.,* 2013), as Croatian shellfish mariculture supports only long-line cultures of European flat oyster *O.edulis* and Mediterranean mussel *Mytilus galloprovincialis*. To date, there has been no evidence of self-sustaining populations, although the eco-physiological requirements of *C. gigas* could be met within the local environmental conditions in bays such as Lim, Budava or Mali Ston Bay.

This paper presents the results of screening of the four ecologically most suitable bays along the Croatian coast for a natural population of non-native oysters. Since oysters (*Ostreidae*) manifest a high degree of phenotypic plasticity, the objective of the study was to confirm species identification using a molecular approach and to characterize genetically the species of *Crassostrea* occurring along the Croatian coast.

MATERIAL AND METHODS

Four bays along the Croatian coast were surveyed for the presence of oysters during 2015 (Fig. 1). A total of 20 individuals with shell characteristics Ω to *C. gigas* were collected at six sampling points from different localities in Lim Bay, Budava Bay, Marina Bay and Mali Ston Bay (Table 1, Fig. 1). The oysters were collected from natural recruitment and were attached to the rocky surface of the littoral zone. Additionally, six specimens morphologically identified as *Ostrea edulis* were sampled in Lim Bay, and served as the control group (Table 1). All surveyed localities are under the influence of underground freshwater springs or rivers, and, as such, encompass shellfish farming sites with the exclusive cultivation of European flat oyster *O. edulis* and Mediterranean mussel *M. galloprovincialis*.

Muscle sections of each sampled oyster were stored separately in 96% ethanol and later used in genetic analyses to confirm species identification. DNA was extracted using the DNeasy 96 Tissue Kit (Qiagen) according to the manufac-

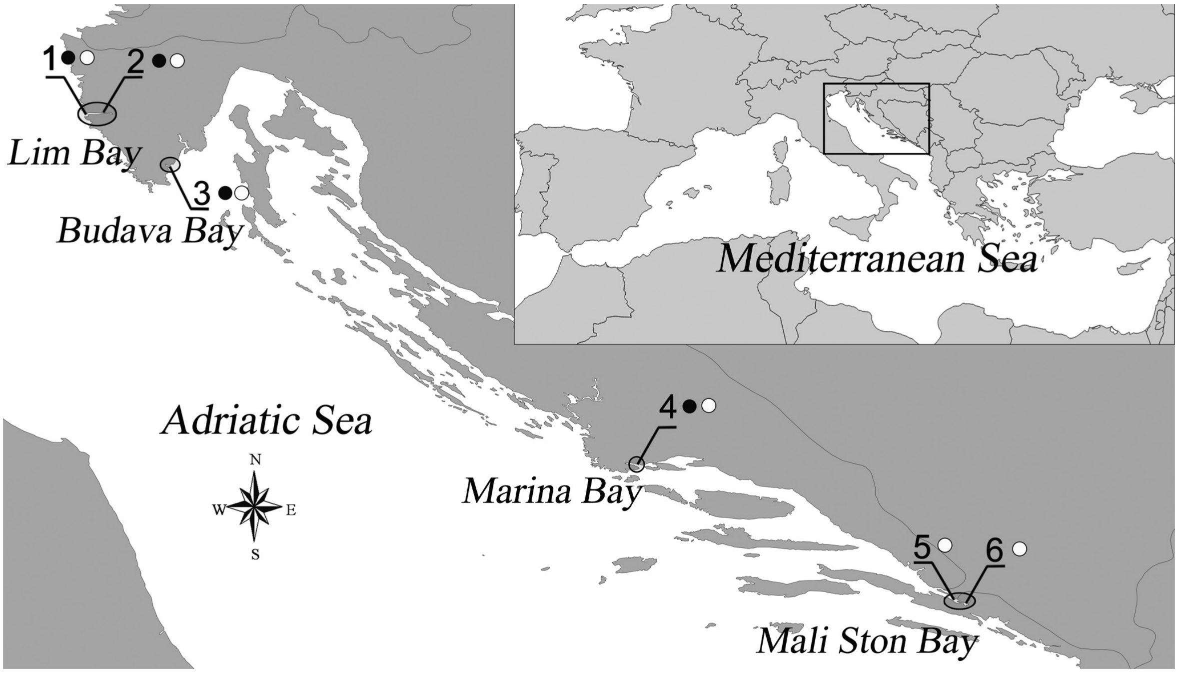

Fig. 1. Sampling locations in the eastern Adriatic Sea with distributions of C. gigas *(●) and* O. edulis *(○) at collection sites (Lim Bay, sites 1–2; Budava Bay, site 3; Marina Bay, site 4; Mali Ston Bay, sites 5–6).*

turer's instructions. A partial mitochondrial large ribosomal subunit (16S) segment was amplified using the primers 16SAR (5'-CGCCTGTTTATCAAAAACAT-3') and 16SBR (5'-CCGGTCTGAACTCAGATCACGT-3') (KESSING *et al.*, 1989), a marker previously shown to be diagnostic for the Ostreidae (JOZEFOWICZ & O'FOIGHIL, 1998). Each PCR reaction contained 1 µl DNA extraction solution, 1 × PCR buffer (Invitrogen), 2.0 mM $MgCl_2$ (Invitrogen), 0.1 µM of each primer, 200 µM of each dNTP, and 0.625 U of Taq Platinum polymerase (Invitrogen), finalizing the volume to 25 µL with ultrapure water. PCR amplification protocol consisted of 3-min denaturation at 94°C and 35 cycles at 94, 52 and 72°C for 1 min each, followed by a 7-min extension at 72°C. PCR products were electrophoresed on 1% agarose gels stained with SYBR Safe (1%) and visualized under UV transillumination. Amplicons were submitted for purification and direct sequencing to Macrogen (Macrogen Inc., Seoul, Korea). Each sample was sequenced from both directions. The quality of the chromatogram was evaluated using the Chromas Pro 1.5 software (http://www.technelysium.com.au). For sequence identification, the program BLASTn (NCBI, available online) was used. Sequence alignment was carried out using the ClustalW tool, Mega v6 software (TAMURA *et al.*, 2013). DnaSP 5.19 software (LIBRADO & ROZAS, 2009) was applied to calculate haplotype diversity. Additionally, the 16S sequences of *C. gigas* and *C. angulata* corresponding to COI haplotypes A, B, C, D and

Surveyed bays	Site	Latitude	Longitude	Sample size	Shell characteristics	Molecular *C. gigas* identification
Lim Bay	1	45.131819	13.615125	7	*C. gigas*	4
	2	45.12972	13.66722	5	*C. gigas*	4
Budava Bay	3	44.89458	13.98544	4	*C. gigas*	4
Marina Bay	4	43.51558	16.15475	2	*C. gigas*	2
Mali Ston Bay	5	42.875329	17.645552	1	*C. gigas*	-
	6	42.86917	17.69956	1	*C. gigas*	-
Lim Bay Control	7	45.122968	13.622264	6	*O. edulis*	-

Table 1. Details regarding Adriatic oyster collections analyzed in present study

E (Accessions AJ553901, AJ553902, AJ553903, AJ553904 and AJ553905; BOUDRY *et al.*, 2003), together with sequences for *C. angulata* (Accession KC847117), *Crassostrea virginica* (Accession KC429253; SHARMA *et al.,* 2013) and *Ostrea edulis* (Accessions DQ093488, JF808187; SÁNCHEZ *et al.,* 2014) were included in the phylogenetic analysis.

Phylogenetic analysis based on 16S sequence alignment was carried out using Maximum likelihood (ML) and Bayesian (BA) approaches, with *Crassostrea virginica* as the outgroup. JModeltest 2.1.4 (DARRIBA *et al.*, 2012) was used to determine the best substitution model based on Akaike information criterion (AIC) corrected values and TVM+G model was identified as the best available one. A Bayesian tree was constructed using MrBayes 2.0.6 (RONQUIST & HUELSENBECK, 2003) as implemented in Geneious (v. 2.0.3) running at least two independent Monte Carlo Markov Chain (MCMC) analyses with 2,200,000 generations sampled every 400 generations, with a 200,000 tree burn-in. Maximum likelihood trees were constructed using the PhyML (GUINDON *et al.,* 2010) plug-in of Geneious with the BEST topology search option and 1000 bootstrap replicates. Distance analysis was conducted using MEGA v6.

RESULTS

The sequences of 456 bp fragments of the 16S gene were obtained for 6 control individuals and 20 individuals that had shell characteristics common to *C. gigas*. Bayesian and maximum likelihood analyses produced highly similar

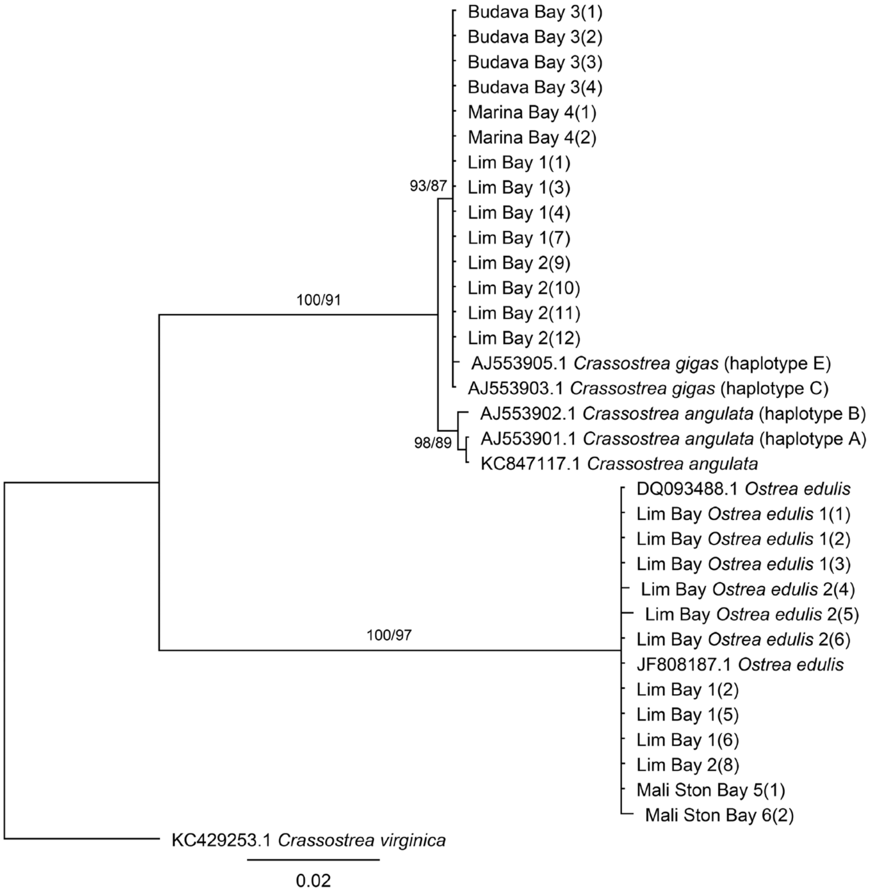

Fig. 2. Phylogenetic tree inferred by the Bayesian and maximum likelihood analyses of partial mitochondrial 16S sequences where identical topologies were produced. Sequences obtained in this study are labelled according to the bay of origin and specific site location (individual no. in parenthesis). Sequences from GenBank are included for O. edulis, C. gigas, C. angulata *and* C. virginica *(outgroup), where* C. gigas *and* C. angulata *are represented by different haplotypes. Posterior probabilities followed by bootstrap values are included at the nodes.*

topologies, where all nodes were strongly supported by posterior probabilities or bootstrap values. The obtained results genetically confirmed the presence of the exotic species *C. gigas* in natural environments at three of the four sampling localities (Table 1), i.e. in the bays Budava, Lim and Marina. Namely, phylogenetic reconstruction showed the presence of two distinct clades (Fig. 2) corresponding to the presence of two different oyster genera, i.e. *Crassostrea* and *Ostrea*. The *Crassostrea* clade clearly separated the *C. gigas* group from the *C. angulata* group where 14 *Crassostrea*-like individuals clustered with *C. gigas* GenBank sequences (AJ553903.1, AJ553905.1). All six control samples and *O. edulis* GenBank sequences (DQ093488.1, JF808187.1) were assigned to the *Ostrea* clade. Due to high phenotypic plasticity (Fig. 3), disagreement with morphological identification was observed in *Crassostrea*-like individuals where 6 of 20 individuals were assigned to the *O. edulis* clade. The sequences of 14 individuals genetically identified as *C. gigas* from three different localities were monomorphic (Accession number KX394620) and corresponded to the COI haplotype C (BOUDRY *et al.*, 2003), which is the most common haplotype in *C. gigas* (HUVET *et al.*, 2000). *O. edulis* sampled from three different bays presented four haplotypes (Accession numbers KX394616 - KX394619) with a haplotype diversity (*Hd*) of 0.455. Interspecific sequence divergence (p - distance) for *C. gigas* vs. *O. edulis* was 0.158.

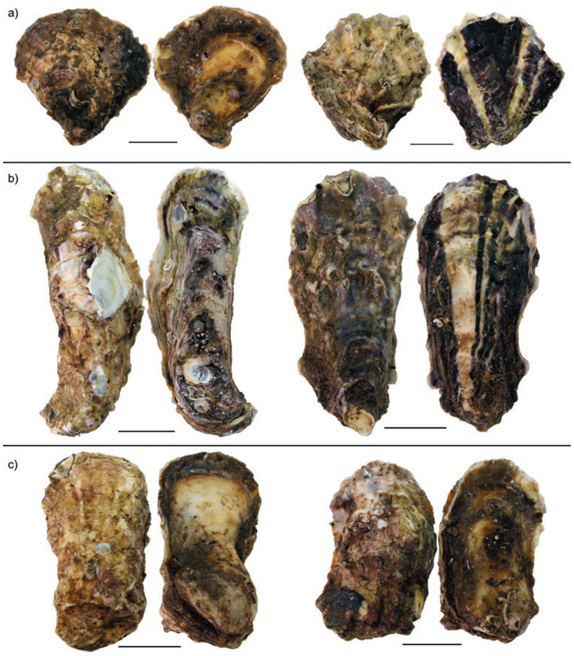

Fig. 3. Oysters specimens collected in Lim Bay, eastern Adriatic Sea: a) O. edulis*; b)* C. gigas*; and c)* O. edulis *misidentified as a* Crassostrea sp. *based on morphological criteria. See text for additional details*

DISCUSSION

Mitochondrial marker sequencing data provide evidence that self-sustaining populations of *C. gigas* are present in the northern (Lim and Budava Bay) and central (Marina Bay) parts of the eastern Adriatic, while *C. gigas* populations have not been identified in the southern part (Mali Ston Bay). Self-sustaining populations of *C. gigas* are primarily limited by temperature for successful gametogenesis (9–16°C), gamete release (16–20°C), and normal larval development (24–28°C) (RUIZ *et al.*, 1992). Interestingly, in all studied areas, the environmental conditions are suitable for *C. gigas* establishment, i.e., depending on the intensity of freshwater inflow, the temperature ranges from 9°C to 26°C in February and August and salinity varies from 31 to 38.5 over the year (NINČEVIĆ-GLADAN *et al.*, 2008).

In the eastern Adriatic areas where the Pacific oyster was not intentionally introduced in aquaculture, the question remains as to the species dispersal capacity and alternative pathways of introduction. Namely, *C. gigas* was introduced to the Mediterranean coast of France and to the Adriatic lagoons of Italy by the late 1960s from the Atlantic French stock originating from broodstock imported from Canada and Japan, and has since been introduced to many Mediterranean localities from Cyprus to Tunisia (RILOV & CROOKS, 2009). It is possible that, after oysters were established in aquaculture facilities in the northern Italian lagoons, they likely experienced secondary spread via both natural (larvae were passively transferred by water currents from farming locations) and human-mediated vectors (ships hulls and ballast water).

Long distance dispersal capacity driven by the 20–30 day planktonic phase (SCHMIDT *et al.,* 2008) and clockwise wind-generated surface currents (ORLIĆ *et al.,* 1992) likely facilitated the expansion of the *C. gigas* population further north towards the northern tip of the Adriatic Sea. The characteristic north-easterly wind Bora causes cyclonic circulation that creates high density and nutrient enriched waters in the northern Adriatic. This has been demonstrated by the recent record of feral populations of *C. gigas* in the northern Adriatic, i.e. the Gulf of Trieste (CROCETTA, 2011) and along the Slovenian coast (LIPEJ *et al.,* 2012). *C. gigas* has colonized the entire mediolittoral and infralittoral fringes of the Gulf of Trieste, showing an invasive and mainly competing pattern with *M. galloprovincialis* (CROCETTA, 2011). Due to the relative proximity of the northern Croatian coast with the coasts of Italy and Slovenia, and the rapid population growth rate of *C. gigas*, these oysters were likely dispersed into the Lim and Budava Bays by natural means. Namely, *C. gigas* abundances can increase by five to nine times over a three-year period, as in the case of the 120 km long coastline of Germany (SCHMIDT *et al.,* 2008).

The *C. gigas* populations observed in the central Adriatic were likely unintentionally introduced via human-mediated vectors. In the absence of *O. edulis* hatchery-produced larvae and the great variability in larval dispersal and settlement, natural collection of the spat is conducted along the western coast of Istria and individuals are translocated into other geographically distinct areas for farming purpose (farmers, pers. comm.). The translocation impact of individuals from wild populations into other genetically distinct populations is an important issue for the management of exploited or endangered species (JOHNSON, 2000).

No wild populations of *C. gigas* have been recorded up to date in the southern Adriatic, though the unaffected coastlines should be viewed as potential locations for future invasions.

Despite the recent detection of the presence of feral *C. gigas* populations in the eastern Adriatic, the dispersal rate and ecological implications remain unknown. Concerns have been raised regarding the potential effects on other local species, especially on the commercially important native oyster *O. edulis*. If the non-native species displays similar settlement patterns to the native oyster and occupies the same habitats, competition between new recruits of the two species can be expected (RILOV & CROOKS, 2009). In Australian waters, reduced larval settlement of native oyster assemblages dominated by *C. gigas* has been noted (WILKIE *et al.,* 2012), with the impact strongly influenced by invader density and spatial arrangement (WILKIE *et al.,* 2013). In the Wadden Sea, *C. gigas* invaded mussel beds resulting in a decline in mussel abundance (NEHLS *et al.,* 2006; MARKERT *et al.,* 2009). Thus, it is important to develop management strategies aimed at controlling the future spread of the Pacific oyster along the eastern Adriatic coastline, with a focus on the potential interactions of *C. gigas* and *O. edulis* and how these effects may vary as invaders increase in abundance.

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Prisutnost alohtone kamenice *Crassostrea gigas* u Jadranskom moru

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SAŽETAK

Japanska ili pacifička kamenica, *Crassostrea gigas*, uvedena je u Europu kasnih 1960-ih za potrebe akvakulture i od tada se intenzivno proširila po Mediteranu. Po prvi put je izvršen pregled četiriju ekološki najprikladnijih zaljeva istočne obale Jadrana kako bi se utvrdila prisutnost alohtone kamenice. Analiza djelomičnog fragmenta mitohondrijske 16S rRNA je korištena kako bi se molekularno identificirala egzotična vrsta *C. gigas* u prirodnim staništima sjevernih i središnjih obalnih područja istočnog Jadrana. Mogući invazijski putevi su raspravljani kao i važnost praćenja distribucije populacija *C. gigas*, s naglaskom na potencijalne izravne i neizravne učinke na autohtonu europsku plosnatu kamenicu *Ostrea edulis* koja obitava u Jadranskom moru.

Ključne riječi: Japanska ili pacifička kamenica, invazija, Jadransko more, ekološki utjecaj, Europska plosnata kamenica