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EVIDENCE FOR THE PRESENCE OF THE PORTUGUESE OYSTER, *CRASSOSTREA ANGULATA*, IN NORTHERN CHINA

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ABSTRACT The Pacific oyster, *Crassostrea gigas* (Thunberg), and the Portuguese oyster, *C. angulata* (Lamarck), are two closely related taxa. Although these two taxa were both introduced from Asia into Europe, one (*C. gigas*) was voluntarily introduced in the early 1970s, whereas the other (*C. angulata*) was presumed to be present in Europe for at least four centuries, but nearly disappeared because of disease. Few *C. angulata* populations remained in southern Portugal, Spain and Morocco and their putative origin was traced in Taiwan. The present paper reports evidence for its presence in Northern China. We reanalyzed recently published mitochondrial cytochrome oxidase C subunit I (COI) sequence data from presumed Dalianwan oysters (*C. talienwhanensis*) and compared them with those of *C. gigas* and *C. angulata*. Additionally, two new *C. angulata* haplotypes from Portugal were identified. The results clearly showed that some of the *C. talienwhanensis* sequences cluster with *C. angulata* sequences. The relative divergence between *C. gigas*, *C. angulata*, and *C. talienwhanensis* haplotypes indicated that *C. angulata*-like oysters are present in northern China. This opens new perspectives in terms of genetic resources and population genetics of *C. gigas* and *C. angulata*, two oyster species of aquacultural importance.

KEY WORDS: cupped oysters, *Crassostrea angulata*, *Crassostrea gigas*, *Crassostrea talienwhanensis*, cytochrome oxidase C subunit I gene, phylogeography

INTRODUCTION

The phenotypic plasticity of oysters and their wide range of distribution are known to have led to numerous taxonomic misclassification or redundancy in species names. However, during the last decade, molecular tools have contributed in highlighting and resolving several of these cases. For example, Anderson and Adlard (1994) proposed that *Saccostrea commercialis* and *S. glomerata* should be regarded as synonymous taxa based on rDNA internal transcribed spacer sequence data. More recently, Kenchington et al. (2002) suggested that *Ostrea edulis* and *O. angasi* are conspecific. In addition, O'Foighil et al. (1999) confirmed the transoceanic range (New Zealand and Chile) of *O. chilensis* using mitochondrial COI sequence data and proposed that dispersal by rafting was the most likely explanation for this distribution. Similarly, the mangrove oyster *Crassostrea gasar* was shown to be present not only along the coasts of Western Africa but also along the Atlantic coasts of South America where some specimens had been wrongly described as *C. rhizophorae* (Lapègue et al. 2002).

In this context, the relative taxonomic status of the Portuguese oyster, *C. angulata*, and the Pacific oyster, *C. gigas*, may be considered as a case study. *C. gigas* and *C. angulata* were classified as two different species by Thunberg in 1793 and Lamarck in 1819, respectively. This classification was chiefly due to apparently separated geographical distribution of the two species, because *C. angulata* was described in Europe and *C. gigas* in Asia. However, following morphologic comparison (Ranson 1948), experimental hybridization (reviewed by Gaffney & Allen 1993, Huvet et al. 2001, Huvet et al. 2002) and allozyme data (Mathers et al. 1974, Buroker et al. 1979, Mattiucci & Villani 1983), the authors concluded that there was only a single species, grouping Portuguese and Pacific oysters. Yet, significant phenotypic differences between the two taxa were observed, in terms of production yield (Bougrier et al. 1986, Héral 1986, Parache 1989, Soletchnick et al. 2002), and eco-physiologic characteristics (His 1972, Goulletquer et al. 1999, Haure et al. 2003). Furthermore, differences have now been observed (1) from karyotype analyses (Leitão et al. 1999b) although these two taxa exhibited a close genetic similarity in comparison with other cupped oyster species (Leitão et al. 1999a); (2) in the genetics of the two taxa based on the mitochondrial COI gene (Boudry et al. 1998, O'Foighil et al. 1998) and nuclear microsatellites (Huvet et al. 2000).

The introduction of the Pacific oyster was relatively well documented because it was a recent voluntary introduction. Hence, the introduction of *C. gigas* from Japan into Europe was made in the early 1970s to replace the Portuguese oyster in the shellfish industry (Grizel & Héral 1991) that nearly disappeared probably due to an iridoviral disease (Comps 1969). As indicated by its common name, the Portuguese oyster, was believed to originate from Portugal or at least southern Europe. However, results from nuclear and mitochondrial DNA studies (Boudry et al. 1998, O'Foighil et al. 1998, Huvet et al. 2000) suggested an explanation for the separated geographical distribution of these genetically closely-related taxa by supporting the hypothesis of the introduction of *C. angulata* from Asia (and more precisely Taiwan) to the Portuguese coast by merchant ships during the 16th century. Until now, no *C. angulata* specimens were observed in any other Asian location (see Lam et al. 2003, Boudry et al. 2003).

China is the country with the largest *C. gigas* production (in 1997: Mainland China: 2.3 10^6 metric tonnes, Taiwan: 24 10^3 metric tonnes, Hong Kong: 66 metric tonnes, according to FAO, 1999). However, Guo et al. reported in 1999 that it is not *C. gigas*, but another species, *C. plicatula*, that accounts for the main 50% to 60% of the production, *C. ariakensis* for 20% to 30% and *C. gigas* for only 10% to 20%. Qi (1989) also cited the Dalianwan oyster (*C. talienwhanensis*) as of commercial importance after the Zhe oyster (*C. plicatula*) and the Suminoe oyster (*C. ariakensis*) (Qi 1989). Many other different taxa have been reported along Chinese coasts and species identification is often uncertain. In Northern China, *C. talienwhanensis*, *C. plicatula* and *C. gigas*

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were considered as sibling species (Liu & Dai 1998). In the East and South China, at least nine species have been described (Bernard et al. 1993). Recently, a new species was described in Hong Kong (Lam & Morton 2003, Boudry et al. 2003). Finally, a recent study based on mitochondrial DNA sequences showed that *C. talienwhanensis* specimens from Northern China were genetically close to *C. gigas* specimens which suggested that the Dalianwan and Pacific oysters belong to the same species (Yu et al. 2003). Similarly, their results suggested that *C. plicatula* and *C. ariakensis* are closely related.

In the present paper, we compared DNA sequence data of *C. gigas*, *C. talienwhanensis*, and *C. angulata* to establish their genetic relationships. To do so, we reanalyzed the mitochondrial COI sequence data of *C. talienwhanensis* specimens from Yu et al. (2003) and compared with those of known haplotypes from *C. gigas*, *C. angulata* (Boudry et al. 1998) and two newly identified *C. angulata* haplotypes.

MATERIALS AND METHODS

New Crassostrea angulata *Sequence Data*

A total of 218 cupped oysters from Portugal were sampled in October of 2002 from 2 locations, 109 in the Sado estuary (Monte da Pedra site: 38°25′N, 8°39′W) and 109 in the Mira estuary (Roncão Velho site: 37°42′N, 8°44′W). DNA extraction of ethanol-preserved gill fragments was performed by a phenol/chloroform method, as described by Moore (1993). A partial COI fragment was amplified using the primers and conditions detailed in Folmer et al. (1994). Polymorphism was first studied using restriction enzymes as described by Boudry et al. (1998). Some of these PCR products were sequenced as described by Boudry et al. (2003). All the novel sequences have been submitted to the EMBL nucleotide sequence database.

DNA Sequence Analysis

The COI sequences of the new haplotypes, together with some sequences already obtained for *Crassostrea gigas* and *C. angulata* (Accessions AJ553901, AJ553902, AJ553903, AJ553904, AJ553905; Boudry et al. 2003), *C. virginica*, *C. ariakensis*, and *C. sikamea* (Accessions AF152566, AF152569, AF152568; O'Foighil et al. 1998) and *C. talienwhanensis* (Yu et al. 2003) were aligned with CLUSTALW (Thompson et al. 1994). Pairwise sequence divergences between species were estimated with the DNADIST program in PHYLIP (Felsenstein 1989) according to Kimura's two-parameter model (Kimura 1980). Phylogenetic analyses were conducted using the program FITCH. Bootstrap analysis with 100 replicates was performed with the SEQBOOT and CONSENSE programs.

RESULTS

PCR-amplified fragments from the COI gene were obtained for 218 individuals from the newly sampled Portuguese populations. The PCR-RFLP analysis detected 2 new haplotypes, which were called F and G here. Haplotype G lacked a *Sau*3A restriction site when compared with the others haplotypes of *Crassostrea gigas* and *C. angulata* described by Boudry et al. (1998). For haplotype F a new restriction site was observed using *Mse*I when compared with the same haplotypes. These new haplotypes are rare, each one being present in one of the two populations: the frequency of haplotype F is 5% in Mira and the frequency of haplotype G is 2% in Sado. The COI sequences of haplotypes F and G, together with haplotype J—that had been described by Boudry et al. (2003), but not sequenced—were respectively registered as Accessions AY397685, AY397686, and AY455664. We compared the sequences of these haplotypes, F, G, and J, with those of haplotypes from *C. gigas* (haplotype C, D, and E; Boudry et al. 2003) *C. angulata* (haplotype A and B; Boudry et al. 2003), and *C. talienwhanensis* (haplotype talienw1, talienw2, talienw3, talienw4, and talienw5; Yu et al. 2003). The multiple alignment showed that haplotypes C and talien1 had exactly the same sequence. The distances computed after this alignment is presented in Table 1. The phylogenetic tree obtained from sequence divergence of the COI fragment according to Kimura's model (Kimura

TABLE 1.

Pairwise sequence divergences, for the mt COI DNA fragments (*C. angulata* haplotypes are in bold).

	Haplotype E	Haplotype C	Haplotype D	talienw2	talienw3	**Haplotype A**	**Haplotype B**	**Haplotype G**	**Haplotype J**	**Haplotype F**	talienw4	talienw5	*C. sikamea*	*C. ariakensis*	*C. virginica*
Haplotype E	0														
Haplotype C	0,0036	0													
Haplotype D	0,0055	0,0018	0												
talienw2	0,0055	0,0018	0,0036	0											
talienw3	0,0055	0,0018	0,0036	0,0036	0										
Haplotype A	0,0279	0,0241	0,0222	0,0260	0,0260	0									
Haplotype B	0,0337	0,0299	0,0279	0,0318	0,0318	0,0055	0								
Haplotype G	0,0317	0,0279	0,0260	0,0299	0,0299	0,0036	0,0055	0							
Haplotype J	0,0375	0,0337	0,0317	0,0356	0,0356	0,0091	0,0110	0,0091	0						
Haplotype F	0,0317	0,0279	0,0260	0,0298	0,0298	0,0110	0,0129	0,0110	0,0166	0					
talienw4	0,0298	0,0260	0,0279	0,0279	0,0279	0,0055	0,0073	0,0055	0,0073	0,0129	0				
talienw5	0,0279	0,0241	0,0260	0,0260	0,0260	0,0036	0,0055	0,0036	0,0091	0,0110	0,0018	0			
C. sikamea	0,1051	0,1053	0,1031	0,1075	0,1075	0,1007	0,0986	0,1007	0,0984	0,1050	0,1029	0,1007	0		
C. ariakensis	0,1623	0,1625	0,1650	0,1650	0,1601	0,1652	0,1725	0,1701	0,1723	0,1723	0,1676	0,1652	0,1679	0	
C. virginica	0,2590	0,2538	0,2512	0,2565	0,2512	0,2513	0,2567	0,2567	0,2569	0,2592	0,2594	0,2567	0,2627	0,2789	0

talien1 and haplotype C have the same COI sequence.

1980) is given in Figure 1. The 12 haplotypes clustered into 2 groups (100% bootstrap *P* value) with a divergence varying from 2.2% to 3.7% depending the pairs of haplotypes being compared. One group encompassed the *C. gigas* haplotypes (C, D, and E) and the *C. talienwhanensis* haplotypes (talienw1, talienw2, and talienw3). The second group included the *C. angulata* haplotypes (A, B, J, F, and G) and the *C. talienwhanensis* haplotypes (talienw4 and talienw5). The nucleotide divergence within the first group varies from 0.2% to 0.5% and in the second group from 0.2% to 1.7%. *C. sikamea* and *C. arienkensis*, two other Asian cupped oysters species, respectively exhibited about 10% and 16% divergence with the *C. gigas* and *C. angulata* haplotypes. The American oyster, *C. virginica*, showed about 26% divergence with the *C. gigas* and *C. angulata* haplotypes and was considered as an outgroup.

DISCUSSION

The geographic distribution of the closely related taxa *Crassostrea gigas* and *C. angulata* in southwestern Europe is now well documented, so is the genetic variability within the populations and the genetic differentiation between the populations. *C. gigas* was observed in northern Europe bordered by the headland of northern Spain (La Corogne) in the south. *C. angulata* was observed in southern Spain, Portugal, and Morocco (Boudry et al. 1998, Fabioux et al. 2002, Huvet et al. 2004). According to the grouping of the haplotypes observed here, haplotype F and G, detected in the new samples from the Mira and Sado Portuguese populations, can be considered as two new *C. angulata* haplotypes. This is confirmed by the divergence values that are of the order of those reported by Boudry et al. (2003): less than 0.5% for the *C. gigas* group compared with 0.2% to 0.5% in this study, and less than 1.1% for the *C. angulata* group compared with 0.2% to 1.7% in this study. When observing the results for the *C. talienwhanensis* haplotypes from Yu et al. (2003), talienw1 (identical to haplotype C), talienw2 and talienw3 are grouped with the *C. gigas* haplotypes, and talienw4 and talienw5 with the *C. angulata* haplotypes. Consequently, oysters of talienw1, talienw2 and talienw3 can be considered as *C. gigas*, as proposed by Yu et al. (2003), and the others (oysters of talienw4 and talienw5) can be considered as *C. angulata*. This partly confirms that the Dalianwan oyster, described by Zhang and Lou (1956), is another name for the Pacific oyster in China (Li & Qi 1994), but also supports that *C. talienwhanensis* being considered as *C. angulata*. After the evidence of the presence of *C. angulata* in Taiwan (Boudry et al. 1998), this species is now found existing in the northern China (Dalian, Liaoning province and Rongcheng, Shandong province) suggesting a broader Asian geographical distribution. Additionally, it should be noted that the cupped oysters specimens found in Hong-Kong (Lam et al. 2003, Boudry et al. 2003) and considered as belonging to a putative new taxa, cannot be considered as *C. talienwhanensis*.

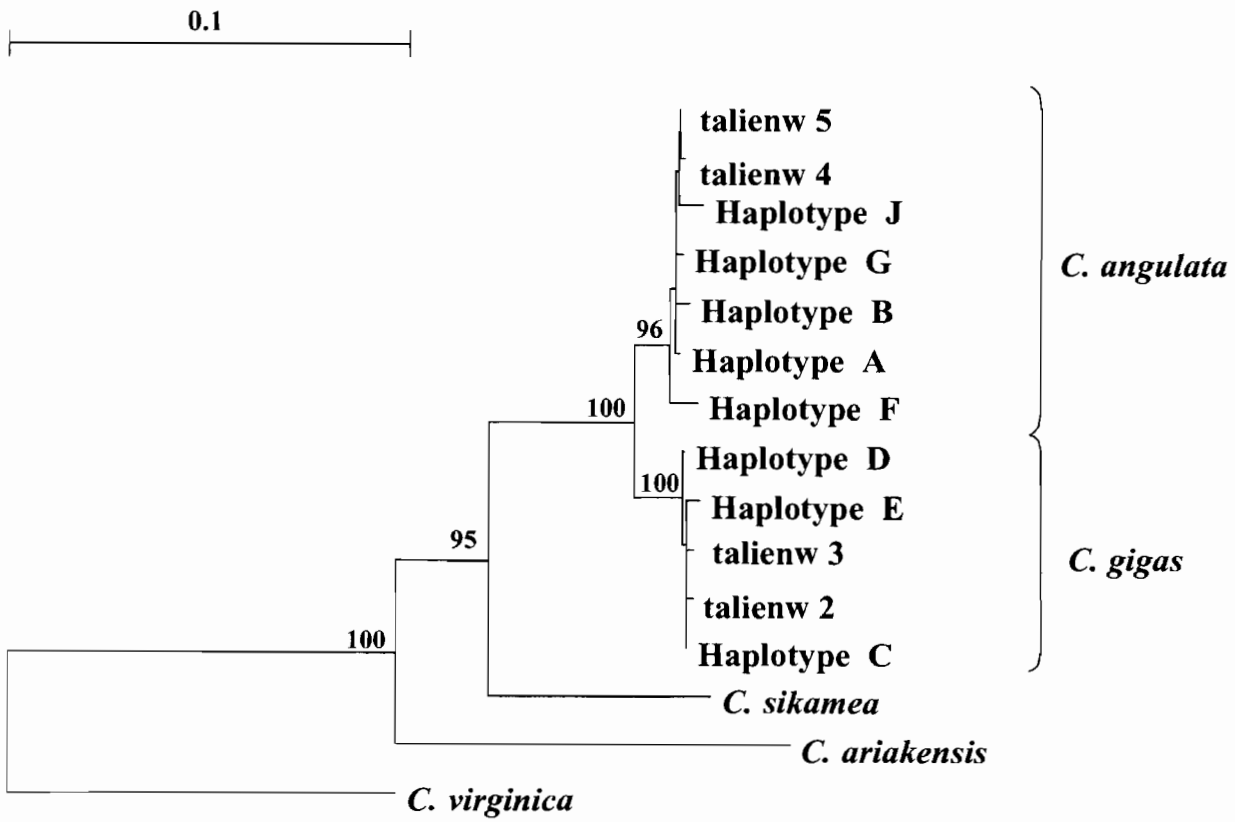

Figure 1. Phylogenetic trees obtained from sequence divergence of a 551 nucleotide mitochondrial COI DNA fragment according to Kimura's model (Kimura 1980) for *C. gigas*, *C. angulata*, and *C. talienwhanensis* haplotypes. talienw1 to 5 correspond to the haplotypes described by Yu et al. (2003). The sequences of haplotypes A, B, C, D, and were described in Boudry et al. (2003). The sequences of *C. ariakensis*, *C. sikamea* and *C. virginica* were obtained from O'Foighil et al. (1998). The sequences of haplotypes F, G, and J were described in this study. *C. virginica* was used as an outgroup. Numbers on the branches indicate bootstrap *P* values.

In total, 11 *C. angulata* COI haplotypes (A, B, and J from Boudry et al. 1998; angul1, angul2, angul3, and angul4 from O'Foighil et al. 1998; talienw4 and talienw5 reanalysed from Yu et al. 2003; F and G in this study) and 5 *C. gigas* COI haplotypes (C, D, and E from Boudry et al. 1998; talienw1 (identical to haplotype C), talienw2, and talienw3 reanalysed from Yu et al. 2003) have now been described. Interestingly, using relatively equivalent amount of research on both taxa, the level of variability of *C. gigas* appears to be lower than that of *C. angulata* for the COI sequence. Furthermore, studies based on allozyme data by Buroker et al. (1979) also showed a high genetic variability in *C. angulata*, from Portuguese populations, relative to other *Crassostrea* species. More data are needed from other mitochondrial and nuclear markers, but this high genetic variability observed in *C. angulata* opens interesting perspectives for the development of conservation programs for this taxa in Europe. Consequently this also underlines the importance of *C. angulata* as a potentially useful genetic resource for *C. gigas* aquaculture.

Although the two 16S haplotypes described for the 10 *C. talienwhanensis* individuals from Yu et al. (2003) study were each found in 2 different sampling locations (Dalian & Rongcheng), the 5 COI haplotypes (talienw1 to talienw5) distribution indicated that *C. gigas* and *C. angulata* co-occur in the two sampled locations. Hence, one individual has the talienw1, one the talienw2, one the talienw3, four the talienw4, and three the talienw5 haplotypes. This result needs to be confirmed, because the number of samples (5 in each location) is low. It would be of particular interest to focus on the sympatric status of these two taxa in this region as it was done in Southern Europe. In Portugal, there was evidence for hybridization between *C. angulata* and *C. gigas* in the wild where the two taxa are in contact due to recent transportation of *C. gigas* stocks for aquacultural production (Huvet et al. 2004). An extensive sampling protocol is clearly needed in Northern China to investigate this hybridization in the native region of these two taxa, and, more generally, their relative evolutionary history.

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