Past and present geographical distribution of populations of Portuguese (*Crassostrea angulata*) and Pacific (*C. gigas*) oysters along the European and north African Atlantic coasts

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ABSTRACT

Today, it is commonly accepted that *C. angulata* and *C. gigas* are a single species according to morphological, genetic and experimental hybridization data. Following the viral disease that affected *C. angulata* and the subsequent expansion of *C. gigas* in Europe, it was of interest to examine the geographical distribution of both taxa in Europe. We studied the genetic composition of seventeen populations of cupped oysters, sampled in France, Spain, Portugal, Morocco and Italy, using a diagnostic marker, the mitochondrial Cytochrome Oxydase I gene. Results showed two distinct geographical zones. All the populations located in the northern part of the European sampled zone were mainly composed of *C. gigas* haplotypes whereas populations from southern Europe and Morocco were mainly composed of *C. angulata* haplotypes. No natural populations were found between Coruña (northern Spain) and Lisbon (western Portugal). Very limited mixtures of the two taxa were observed in France and northern Spain, which might correspond to ancestral polymorphism and suggest the past presence of *C. angulata* in these regions. However, some notable mixtures were observed in the south of Portugal. This could be the result of importation of *C. gigas* spat to this region. These results indicate a recent change in genetic composition of populations in southern Portugal and show that human activities have created contact zones between the two taxa although no natural sympatric zones exist in Europe.

RÉSUMÉ

Distribution géographique passée et présente de populations de l'huître portugaise (*Crassostrea angulata*) et de l'huître japonaise (*C. gigas*) le long des côtes européennes et nord-africaines.

Les deux huîtres creuses *Crassostrea gigas* et *Crassostrea angulata* sont aujourd'hui communément classées dans la même espèce du fait de leur similarité morphologique et génétique et de leur hybridation en conditions expérimentales. La forte expansion de *C. gigas* en Europe ayant succédée à la disparition des

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populations de *C. angulata*, affectées par une infection virale, il était intéressant d'étudier la distribution actuelle des deux taxons d'huîtres en Europe. Dans la présente étude, la composition génétique de dix-sept populations d'huîtres creuses échantillonnées en France, en Espagne, au Portugal, au Maroc et en Italie, a été analysée avec un marqueur diagnostique, le gène mitochondrial de la Cytochrome Oxydase I. Deux zones géographiques distinctes ont ainsi été mises en évidence. Toutes les populations, situées dans le nord de la zone échantillonnée en Europe, sont principalement constituées par des haplotypes *C. gigas*. Inversement, les populations sud européennes et Marocaines sont principalement composées d'haplotypes *C. angulata.* Aucune population sauvage d'huître creuse n'a été trouvée entre La Corogne (Nord Espagne) et Lisbonne (Ouest Portugal). Les résultats ont montré de très faibles mélanges des deux taxons dans quelques populations en France et dans le nord de l'Espagne qui pourraient être imputés à du polymorphisme ancestral suggérant l'ancienne présence de *C. angulata* sur ces côtes. Néanmoins, des mélanges notables des deux taxons ont été observés dans le sud du Portugal où la présence de *C. gigas* résulterait d'importations de naissain dans cette région. Cette étude suggère une récente modification de la composition génétique des populations dans le sud du Portugal et montre que les activités humaines ont créées des zones de contact entre les deux taxons alors qu'aucune zone naturelle de sympatrie n'a été mise en évidence en Europe.

INTRODUCTION

The two cupped oysters *Crassostrea gigas* and *Crassostrea angulata* were first described respectively by Thunberg in Japan in 1793 and by Lamarck in Portugal in 1819. Discovered on the opposite sides of the world, they were initially described as two different species. However, their taxonomic status had been debated for a long time and they are now commonly classified as single species (Menzel, 1974; Huvet *et al.*, 2002). The definitions of a species in the biological species concept are multiple but are mainly based on three criteria: similarity, descent and interfertility (Mayr, 1963; Lherminer and Solignac, 2000). The two taxa have indistinguishable prodissoconch and adult shells (Ranson 1948; Ranson 1960). Experimental hybridizations and fertility of hybrids showed the absence of reproductive barriers (Gaffney and Allen, 1993; Huvet *et al.*, 2001; Huvet *et al.*, 2002). All these data support the grouping of *C. gigas* and *C. angulata* within a single species, as proposed by Menzel (1974). Several studies based on allozyme markers, showing no genetic differentiation between populations of the two taxa are also concordant with a single species (Mathers *et al.*, 1974; Buroker *et al.*, 1979; Mattiucci and Villani, 1983). However, differences between the karyotypes of the two taxa were observed, notably for chromosome pair 7, but these data do not disagree with the close genetic identity of both taxa (Leitão *et al.*, 1999 *a,b*). Two studies on the Cytochrome Oxydase I (COI) mitochondrial gene (O'Foighil *et al.*, 1998; Boudry *et al.*, 1998) also showed significant genetic differences between *C gigas* and *C. angulata* populations. This locus was considered as a diagnostic marker of the two taxa (Boudry *et al.*, 1998 and Huvet *et al.*, 2000) because the PCR-RFLP (Polymerase chain Reaction - Restriction Fragment Length Polymorphism) technique revealed haplotypes specific for each taxon. Other small but significant nuclear genome differences between *C. gigas* and *C. angulata* populations were revealed using microsatellite markers (Huvet *et al.,* 2000). Lastly, *Crassostrea gigas* was shown to have a superior production yield in the natural environment in France (His, 1972; Héral *et al.,* 1986), and a growth rate twice as high as the one observed for the Portuguese oyster (Bougrier *et al.*, 1986). Differences were also revealed in terms of their ecophysiological characteristics (filtration: His, 1972; oxygen consumption: Goulletquer *et al.*, 1999).

Mitochondrial gene analyses revealed the Asian origin of *C. angulata* (O'Foighil *et al.*, 1998; Boudry *et al.*, 1998) and, more precisely, its probable introduction from Taiwan to Portugal by commercial ships in the 16th or 17th century. Introduced from Portugal to French Atlantic coasts in 1868 (Lambert *et al.*, 1929; Cochard and Dardignac, 1977), *C. angulata* spread rapidly and became the principal farmed oyster species in France, with a yearly production of up to 100 000 tons. However, in the early 1970s, an iridovirus infection resulted in major mortality (Comps, 1970) that decimated French cultivated stocks of *C. angulata*. The same symptoms also affected populations in Portugal (Ferreira and Dias, 1973). To sustain the oyster industry, *C. gigas* was imported from Japan and Canada into France in the early 1970's (Grizel and Héral, 1991). *Crassostrea gigas* then settled widely along the Atlantic coast and became the main cultivated oyster in Europe with a yearly production of over 150 000 tons. Following the viral disease that affected *C. angulata* and the subsequent expansion of *C. gigas*, the present distribution of remaining populations of *C. angulata* in Europe was poorly known and only a few populations were known to exist in southern Portugal (Boudry *et al.*, 1998; Huvet *et al.*, 2000). The aim of the present study was therefore to further document the present genetic composition of cupped oyster populations in Europe and, more precisely, to find out to what extent mixed populations (composed by *C. gigas* and *C. angulata*) exist along the Atlantic coasts.

MATERIAL AND METHODS

Biological material

Wild oyster samples were collected from 16 locations along a geographical north-south gradient from French to Moroccan coasts plus from one Mediterranean location (Table 1, Figure 2). Oysters of the sixteen sampled populations come from natural spat collection. Each sample was represented by an abbreviation of three capital letters. The northern part of the studied area was represented by 4 French populations from Brest (ROZ) to Vieux-Boucau (BOU). Seudre (SEU) and Arcachon (ARC) populations are located in the two main oyster farming regions in France. Five populations were sampled in Spain. Four of these were taken along the northern coast at Orio (ORI), Islares (ISL), Ribadesella (RIB) and Castropol (CAS), and one in the south near Cadiz (CAD). As far as we know, Castropol is the only Spanish location where oyster farming activities are developed. The sampling transect was interrupted from Corona (northern point of Spain) to Porto (western Portugal) because no cupped oysters could be found in this zone. In southern Portugal, 5 sites were sampled from Setubal (SET1) to Rio Formosa (RFA). Two other populations were sampled along the Atlantic coast in Morocco: Oualidia lagoon (OUA) and Tahaddart (TAH). Finally, one Mediterranean population was sampled in Italy, near Venice (VEN). Six out of these 17 populations (see Table 1: SEU, ARC and BOU in France, RFA and MIR in Portugal and CAD in Spain) were previously studied, using COI and microsatellite markers (Huvet *et al.*, 2000). In total, 732 individuals (16 to 50 per population) were sampled and analysed.

Mantle or gills of each sample were dissected and preserved in 95% ethanol before DNA extraction.

Table 1. Location, sample size (N) and genetic composition (*Crassostrea gigas* and/or *C. angulata*) of the 17 populations sampled in Europe and North Africa.

Country	Location	Latitude and longitude	Abbreviation	Taxa	N
France	Roz estuary, Brest	48°N 3°W	ROZ	*C. gigas*	50
	Seudre Estuary *	45 °N 1°W	SEU	*C. gigas*	49
	Arcachon Bay *	44°N1°W	ARC	*C. gigas*	50
	Vieux-Boucau Bay *	43°N 1°W	BOU	*C. gigas / C. angulata*	49
Spain	Orio	43°N 2°W	ORI	*C. gigas*	50
	Islares	43°N 3°W	ISL	*C. gigas*	48
	Ribadesella, Gijon	43°N 5°W	RIB	*C. gigas / C. angulata*	50
	Castropol	43°N 7°W	CAS	*C. gigas*	45
	Cadiz*	36°N 6°W	CAD	*C. angulata*	44
Italy	Venice	45°N 12°W	VEN	*C. gigas / C. angulata*	39
Portugal	Setubal	38°N 8°W	SET1	*C. angulata*	50
	Rio Mira Estuary *	37°N 8°W	MIR	*C. angulata*	30
	Barrinha-Faro	37°N 7°W	BAR	*C. angulata / C. gigas*	42
	Tavira	37°N 7°W	TAR	*C. angulata / C. gigas*	40
	Ria Formosa *	37°N 7°W	RFA	*C. angulata / C. gigas*	35
Morocco	Tahaddart	35°N 6°W	TAH	*C. angulata / C. gigas*	16
	Oualidia lagoon	32°N 9°W	OUA	*C. gigas / C. angulata*	45

(*) Previously determined in Huvet *et al*. (2000).

Mitochondrial DNA analysis

DNA was extracted by the Phenol/Chloroform method (Moore, 1993). A DNA fragment was amplified by PCR using primers described by Banks *et al.* (1993) and Folmer *et al.* (1994) and under conditions used by Boudry *et al.* (1998). The mitochondrial amplified fragment was 710 bp in length and corresponded to the Cytochrome Oxydase C subunit I (COI) gene. Restriction reactions were performed on DNA fragments with 4 polymorphic endonucleases (*Taq*I, *Sau3*A, *Hha*I and *Mse*I) following conditions in the protocol of Boudry *et al.* (1998).

RESULTS

Six different haplotypes, previously named A, B, C, D, E and J (Boudry *et al.*, 1998), were observed in the studied populations. Two haplotype clusters (Figure 1) can be distinguished, haplotypes C and E were associated with *C. gigas* and haplotypes A, B, D and J with *C. angulata* (Boudry *et al.*, 1998; Huvet *et al.*, 2000). The frequencies of these 6 haplotypes in the 17 populations studied are presented in Table 2. The geographical distribution of the *C. gigas* specific haplotypes (C and E) and of the *C. angulata* specific haplotypes (A, B, D and J) is also presented in Table 1 and graphically in Figure 2.

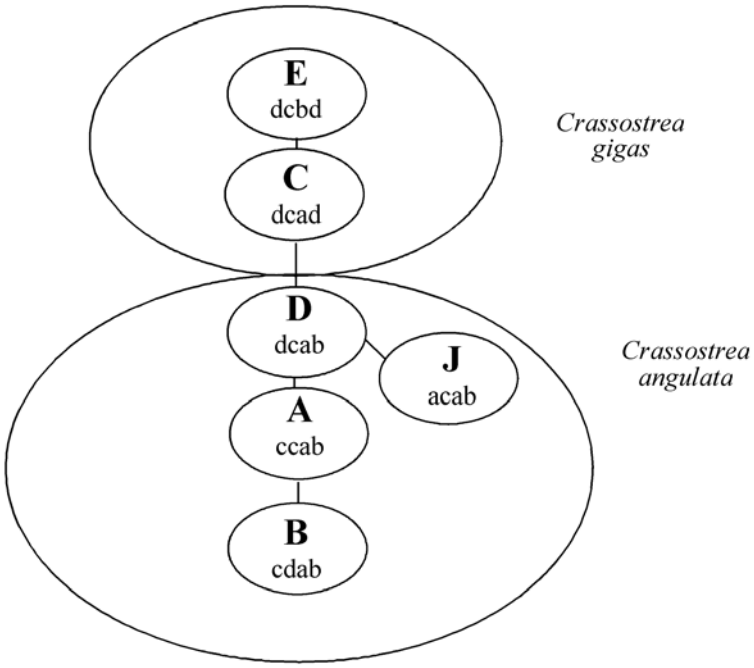

Figure 1. Minimum spanning tree of RFLP haplotypes at the COI marker (Boudry *et al.*, 1998) and their taxonomic specificity among *C. gigas* (C and E) and *C. angulata* (A, B, D and J). Small letters correspond to profiles obtained with the four restriction endonucleases, *Taq*I, *Sau3*A, *Hha*I and *Mse*I respectively. Each circle is separated from adjacent circles by a single restriction site.

Table 2. Haplotype frequencies in each population.

			Haplotypes					
Country	Population	N	A	B	C	D	E	J
France	ROZ	50			0.96		0.04	
	SEU	49			0.98		0.02	
	ARC	50			0.98		0.02	
	BOU	49	0.08		0.90		0.02	
Spain	ORI	50			0.98		0.02	
	ISL	48			1			
	RIB	50	0.04		0.93		0.03	
	CAS	45			1			
	CAD	50	0.96	0.02		0.02		
Italy	VEN	39			0.92			0.08
Portugal	SET1	50	0.94					0.06
	MIR	30	1					
	BAR	42	0.83	0.1	0.05			0.02
	TAR	40	0.72		0.23			0.05
	RFA	35	0.86	0.03	0.11			
Morocco	TAH	16	0.87	0.13				
	OUA	45	0.1		0.85		0.05	

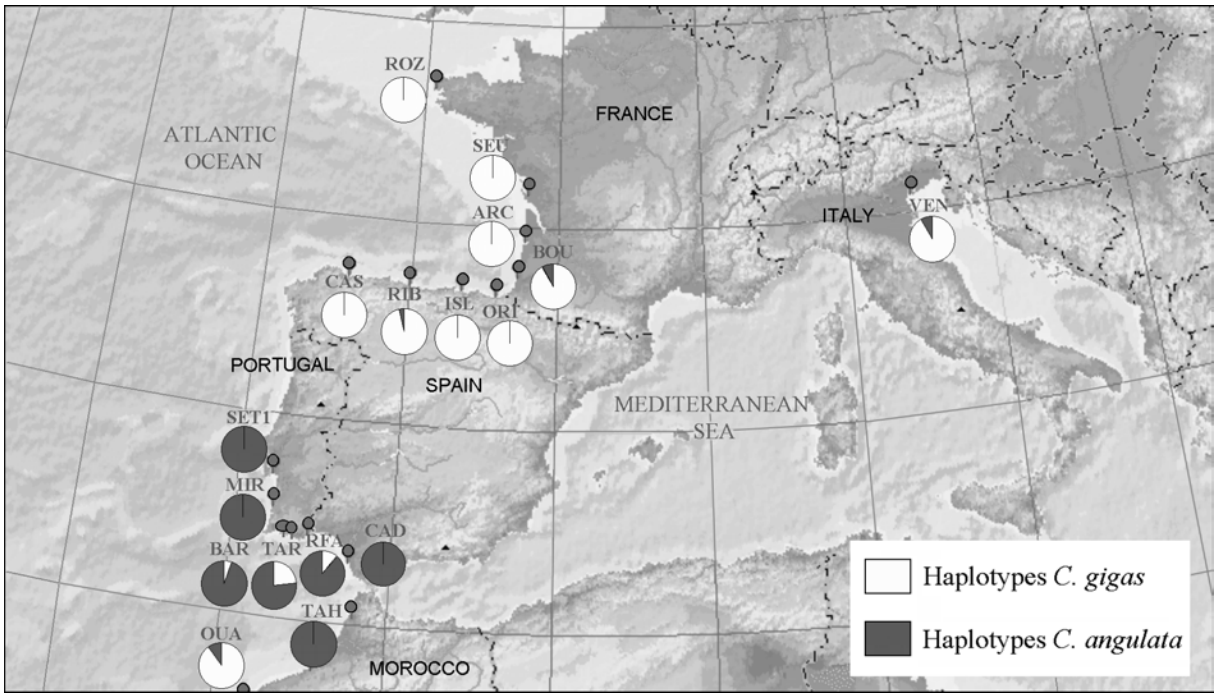

Figure 2. Specific haplotype frequencies of the 17 populations of *Crassostrea gigas* and *C. angulata* sampled in Europe and north Africa. For each population, a pie chart presents the frequencies of haplotypes associated with *C. gigas* (light grey) and *C. angulata* (dark grey).

Populations located in the northern part of the sampled zone (in France: ROZ, SEU and ARC; in northern Spain: ORI, ISL and CAS) showed high frequencies of haplotype C (ranging from 0.90 to 1), associated with low frequencies of haplotype E (ranging from 0.02 in 0.04). Two populations appeared to be monomorphic for haplotype C (ISL and CAS). Two populations, BOU in France and RIB in northern Spain, also showed high frequencies of haplotype C and low frequencies of haplotype E, but haplotype A, known to be specific to *C. angulata* (Boudry *et al.*, 1998) was also present at low frequencies (0.08 and 00.4 respectively). Similarly, the Mediterranean population VEN was mainly composed by haplotype C (0.98), but haplotype J was also observed at a low frequency (0.08).

Conversely, in southern Portugal and southern Spain, all the sampled populations showed high frequencies of haplotype A (ranging from 0.72 to 1), and low frequencies of haplotypes B, D or J. MIR was the only sample to be monomorphic for haplotype A. Three populations (RFA, TAR and BAR) also displayed low to medium frequencies of haplotypes C (0.05 to 0.23) or E (0.02 to 0.05), known to be specific to *C. gigas*.

Further south, the two Moroccan samples (OUA and TAH) were found to be very different from each other. The most southern sample (OUA), showed a genetic composition similar to populations RIB and BOU, *i.e.* a high frequency of haplotype C (0.85) with low frequencies of haplotypes E (0.05) and A (0.1). Conversely, TAH, showed a genetic composition similar to southern Portuguese and southern Spanish samples, with a high frequency of haplotype A (0.87) and a low frequency of haplotype B (0.13).

DISCUSSION

Past and present geographic distribution of C. angulata ***and*** C. gigas

Based on the observed haplotype frequencies, two distinct geographical zones can be outlined. From the French to the northern Spanish coasts (ROZ to CAS in Figure 2), *C. gigas* appears to be the dominant taxon. In this area, only 6 out of 391 individuals were found with *C. angulata* specific haplotypes. In France, the viral disease that affected *C. angulata* in the early 1970's, followed by the successful introduction of *C. gigas* and the subsequent development of the farming of this taxon is a likely explanation of this current situation. Indeed, the large-scale importation of *C. gigas* from Japan and Canada led to a fast extension of the *C. gigas* cultivated stocks along the French coasts (Barré, 1981). Furthermore, Marennes-Oléron and Arcachon bays are the two main areas for spat collection and this seed supply is the basis of oyster production in France. Up to now, no data were available about the genetic composition of cupped oyster populations along Spanish coasts. Our results showed that *C. gigas* is the dominant taxon in the populations sampled in the northern Spain as observed in France. Unlike the French situation, the significant presence of *C. gigas* in northern Spain cannot be explained by human activities, because no oyster farming has been developed in this region, except in Castropol (CAS). In northern Spain, oyster populations could have resulted from settlement of larvae originating from southern coasts of France. Larvae could follow marine currents and spread along the northern Spanish coast from one favourable site to another. Only two populations, Vieux-Boucau (BOU) in the south of France and Ribadesella (RIB) in the north of Spain, showed a low frequency of the *C. angulata* haplotype A, with a large majority of *C. gigas* haplotypes. The absence of *C. gigas* oyster farming in these two sites might explain the persistence of rare *C. angulata* haplotypes and indicate the past presence of *C. angulata* in these regions.

Concerning the Italian population (VEN), it is known that cupped oysters were first imported from France into Italy in 1966. At this time, the first spat from Japan had probably already been introduced to the Bay of Marennes-Oléron (Grizel and Héral, 1991) so the taxonomic nature of these introduced oysters was unsure (Biocca and Matta, 1982). Our sample is mainly composed of the *C. gigas* haplotype C (0.92) but haplotype J is also present at low frequency (0.08). Haplotype J is also present, at low frequencies, in three of our Portuguese samples (SET1, BAR and TAR) and was previously reported in Taiwanese *C. angulata* populations (Huvet *et al.*, 2000). It was also observed on the French Atlantic coast, but only for a single individual (Boudry *et al.*, 1998). Haplotype J can therefore be considered to be specific to *C. angulata*. As a consequence, our Italian sample could have a genetic composition similar to the BOU and RIB samples; *i.e.* a majority of *C. gigas* with a low frequency of *C. angulata*. The fact that haplotype J is present but not haplotypes A or B could be due to genetic drift.

The second geographical area identified in this study is composed of southern Portugal and southern Spain (SET1 to CAD in Figure 2). In this region, samples show high frequencies of *C. angulata* haplotypes. This area represents the remaining populations of *C. angulata* in Europe and is most probably much more limited than it was before the beginning of the 1970s. To date, according to the statistics of the Food and Agriculture Organization (FAO, 1999) only Portugal produces *C. angulata* oysters (618 tons in 1997). Nevertheless, the presence of *C. gigas* was detected in three sites along the southern European coasts in Tavira, Rio Formosa and Barrinha-

Faro. The level of *C. gigas* in the mixture is limited (less than 25%), but significantly higher than the *C. angulata* observed in the *C. gigas* populations in northern Europe.

An interesting situation is observed in the two Moroccan populations. The northern population, Tahaddart (TAH), showed only *C. angulata* haplotypes whereas the southern population, Oualidia (OUA), was dominated by *C. gigas* ones. Moroccan lagoons have been reported to host natural oyster beds of *Ostrea edulis*, *Ostrea stentina* and *Crassostrea angulata* (Dollfus, 1934; Collignon, 1960 and Beaubrun, 1976). Moreover, *C. angulata* spat had been imported into Morocco from Spain and Portugal from 1952 (Shafee, 1985). They were farmed in coastal waters up to marketable size. But oyster farmers preferred to cultivate the Pacific oyster because it gave a higher yield than other species (Shafee and Sabatie, 1986). In Oualidia, the culture of *C. gigas* has been developed since 1972 and is based on spat importation from France (Shafee and Sabatie, 1986), and more precisely from Arcachon bay (A. Gérard, pers. com.). The genetic composition of the Oualidia sample could therefore be considered as a result of the recent development in oyster farming. Conversely, the Tahaddart population has remained in its "original" genetic composition, *i.e. C. angulata*. These data confirm the past presence of *C. angulata* along the coasts of northern Africa. The highly contrasted genetic composition between populations separated by less than 200 kilometres implies a very limited gene flow between Oualidia and Tahaddart since *C. gigas* was introduced into Morocco. A fine-scale study of *Crassostrea* populations in Morocco, for example in the estuary of the Sabou Wadi where *Crassostrea* populations were previously described (Pasteur-Humbert, 1962) would document the genetic differentiation of the populations of the two taxa in northern Africa.

No natural zone of sympatry of the two taxa in Europe

The geographical distribution of the two taxa (i.e. *C. gigas* in northern Europe and *C. angulata* in southern Europe) (Figure 2) would allow us to suppose the existence of a zone of sympatry on the northern coast of Portugal and Galicia. However, despite our efforts, no cupped oysters could be found from Corona (northern Spain) to Lisbon (Portugal). The apparent absence of cupped oysters in this area implied that there would be no natural sympatric zone for *C. gigas* and *C. angulata* in Europe. Different hypotheses, based on ecological and/or hydrodynamic factors, could explain the absence of oysters in northern Portugal and Galicia. Tidal or surface currents could prevent larvae from migrating and/or settling. It is known that larval dispersion of such species can exceed 100 kilometres (Cameron, 1986; Butman, 1987) but more than 800 kilometres separate the northern *C. gigas* populations from the southern *C. angulata* populations. Another hypothesis is that the habitat in this area (*i.e.* environmental factors such as temperature, salinity, trophic level, pollution) could be unsuitable for larval fixation. However, this hypothesis is improbable because both mussels (*Mytilus galloprovincialis*) and flat oysters (*Ostrea edulis*) are present in this region. Our study shows that, unlike mussel populations of *Mytilus edulis* and *M. galloprovincialis* that are naturally mixed in Europe (Cousteau *et al.,* 1991; Daguin *et al.*, 2001), no natural zone of sympatry is observed in Europe for *C. gigas* and *C. angulata.*

Impact of oyster farming on wild C. angulata *populations*

In our study, a few populations showed both taxa together (up to 23% of haplotype C in the *C. angulata* population of Tavira, southern Portugal). This situation could be the result of the development of oyster farming in this area. Indeed, transportation of oysters and, more precisely, importation of *C. gigas* spat into the south of Portugal supports the increasing oyster production in Portugal. This has created an "artificial" contact zone between *C. gigas* and *C. angulata* in southern Portugal. A similar situation, even more pronounced than in Tavira, can be found in Morocco where oyster culture might be at the origin of the high frequency (0.9) of *C. gigas* haplotypes in the Oualidia population. In Tahaddart (TAH) where no oyster farming was reported, only *C. angulata* haplotypes were observed. These results show that increasing frequencies of *C. gigas* in populations seem to be correlated with oyster culture. It can be concluded that a natural sympatric zone does not seem to exist in Europe for the two taxa but some contact between *C. gigas* and *C. angulata* is created in southern Europe by human activities (i.e. transportation of *C. gigas* spat and oysters for shellfish industries in development in these regions). These mixed populations would be of great interest for the study of hybridization processes between *C. gigas* and *C. angulata*. Indeed, as *C. gigas* and *C. angulata* were totally inter-fertile under controlled conditions (Huvet *et al.*, 2002), we could except some natural hybridization in populations where they are in contact. Because the mitochondrial genome is haploid and uniparentaly inherited, it cannot be used to analyse such phenomena. Therefore, a nuclear diagnostic marker is needed and a combined analysis of mitochondrial and nuclear markers should be performed in order to study hybridization between *C. gigas* and *C. angulata* in the populations where the two taxa are now in contact.

CONCLUSION

In this paper, we report the present distribution of the European populations of *Crassostrea gigas* and *Crassostrea angulata*. We show that, in thirty years, *C. gigas* has settled extensively along the European and northern Atlantic coasts. Oyster farming activities created contact zones and consequently hybridization between both taxa might have occurred. The few remaining populations of *C. angulata* are likely to be threatened by the expansion of *C. gigas* in Europe and Northern Africa. Consequently our results highlight the question of whether *C. angulata* should be preserved in Europe. Future studies should be performed to follow the temporal evolution of the two taxa in Europe.

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