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Genetic characterisation of oyster populations along the north-eastern coast of Tunisia

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Abstract:

The taxonomy of oysters has been traditionally based on characteristics of the shell. More recently, the analysis of protein and DNA polymorphism has provided a means to overcome difficulties in distinguishing the different species of oysters based solely on shell morphology. In order to identify oysters of the Tunisian north-east coast, we sequenced a 16S rRNA mitochondrial fragment from 68 oysters sampled from the Bizert Lagoon and the Gulf of Hammamet in northern Tunisia. Comparison of oyster 16S rRNA sequences available in GenBank showed the presence of both *Ostreola stentina* and *Crassostrea gigas* in our samples, which could not be detected on the basis of shell morphology only. These data confirmed that *C. gigas*, a non-native species, is now naturalised in the Bizert Lagoon. Furthermore, significant levels of genetic divergence among the 16S rRNA haplotypes from *O. stentina* populations have been observed. Specifically, the haplotypes found in the Bizert Lagoon are closer to those previously detected from Morocco and Portugal, whereas those in the Gulf of Hammamet are closer to the haplotypes from the south of Tunisia, with a divergence ranging from 2.1% to 2.7% between the northern and eastern Tunisian haplotypes. The possible impact of the Siculo-Tunisian Strait on the phylogeography of *O. stentina* is discussed.

Keywords: 16S rRNA, *Crassostrea gigas*, genetics, Mediterranean Sea, *Ostreola stentina*, polymorphism

1. Introduction

A primary aim in marine biology is the description of the diversity of marine life and the classification of marine organisms according to modern taxonomic methods in a consistent systematic framework. This is fundamental to understanding the ecology and evolution of marine organisms as well as to propose actions towards the exploitation of marine resources (Levinton 2001). Taxonomy and systematics have been carried out for many decades by comparative studies of morphology, anatomy and ecology. Bivalves have shells that are easily collected, and therefore were among the first marine organisms to be studied in detail. However, the high variability of shell morphology in response to different environments has often made it impossible to produce evolutionarily coherent systems of classification in many groups of bivalves (Saavedra and Peña 2006).

Molecular methods have been used for phylogenetic analysis of oysters, with encouraging results (Banks *et al.* 1993, Littlewood 1994, O'Foighil *et al.* 1995, 1998, O'Foighil and Taylor 2000, Lapègue *et al.* 2002). Several workers have used genetic distances in mitochondrial and nuclear DNA sequences (i.e. partial Cytochrome Oxidase subunit I [COI], 28S rRNA and 16S rRNA). These three genes have been used extensively as molecular markers in distinguishing flat oysters Ostreidae, especially species of *Crassostrea* (O'Foighil 1995, 1998, Ladron de Guevara *et al.* 1996, Boudry *et al.* 1998, Hedgecock *et al.* 1999, Leitão *et al.* 1999a, b, Lam *et al.* 2003, Lam and Morton 2004, Wang *et al.* 2004a, b). One of the major advances in phylogenetic studies in Ostreidae is based on partial mitochondrial 16S rRNA sequence data (Jozefowicz and O'Foighil 1998). In several cases of classification, or misidentification, of flat oysters, DNA molecular data have provided valuable new insights (Kenchington *et al.* 2002, Morton *et al.* 2003).

The European flat oyster *Ostrea edulis* has a high commercial value. However, there has been a marked decline in its production due to overexploitation and as a result of two severe epizootic episodes of parasitic infection during the 1960s and 1970s (Yonge 1960, Pichot *et al.* 1979). In Europe, *O. edulis* colonises the Atlantic and Mediterranean coasts. However, the province oyster, or dwarf oyster, *Ostreola stentina* (Payradeau 1826), is also present along the these coastlines, as well as along the southern Mediterranean coast and south-western coast of the Iberian Peninsula, and the African Atlantic coast as far South Africa (Lapègue *et al.* 2006). Leal (1984) noted the abundant presence of *O. stentina* in the Tejo and Sado estuaries (Portugal).

In Tunisia, oyster aquaculture began in the 20th century by cultivating the indigenous *O. edulis* (Azzouz 1966). Attempts were made to cultivate introduced species, initially (in 1931) with the Portuguese oyster *Crassostrea angulata*, and then in the early 1970s with the Pacific oyster *Crassostrea gigas*, after the disappearance of *O. edulis* as a result of infestation to an iridovirus. Dridi *et al.* (2006) studied the sexual cycles of *C. gigas* in the Bizert Lagoon and showed that the reproduction of this cupped oyster takes place in that region of Tunisia. Those authors first reported gametogenesis of *C. gigas* in the lagoon in March 2002, with spawning occurring in June and September, followed by a reproductive resting period. The sexual cycle of *C. gigas* was completed successfully in the lagoon and the newly recruited juveniles were observed fixed on the shell of adult oysters (Dridi *et al.* 2006). In Tunisia, at least three species of oysters occur: *O. edulis*, *O. stentina*, and *C. gigas*.

Allozymic markers allowed the differentiation of *O. edulis* and *O. stentina* in the Nador Lagoon in Morocco (Blanc *et al.* 1986), in the Bay of Cadiz in Spain, the Mira Estuary in Portugal (Amezuca *et al.* 2001) and the Mar Menor Lagoon in Spain (Gonzalez-Wangüemert *et al.* 2004). A molecular analysis by Lopez-Flores *et al.* (2004) based on a centrometric satellite DNA family showed a high degree of differentiation between *O. edulis* and *O. stentina*. Data, based on the sequence analysis of the mitochondrial 16S and COI fragments

of oysters from Portugal, southernTunisia and Morocco, also supported a high degree of differentiation between *O. stentina* and *O. edulis*, and a close relationship between *O. stentina* and both *Ostrea aupouria* and *Ostreola equestris* (Lapègue *et al.* 2006).

The first objective of this study was to identify the oysters from two locations in Tunisia, the Bizert Lagoon and the Gulf of Hammamet, and to confirm the presence of naturalised *C. gigas*. The second aim was to characterise the differentiation between the Tunisian populations of *O. stentina* from the northern and the eastern coasts.

2. Material and Methods

Biological sampling

A total of 120 wild (i.e. not taken from bags) oysters were sampled in August 2006 from the Bizert Lagoon (37°09'N, 9°53'E) [60] and the Gulf of Hammamet [60] (36°15'N, 10°35'E), n northern Tunisia (Figure 1).

These oysters, first characterised as C. gigas, were separated into three groups on the basis of their origin and their shell morphology, size, total weight and internal anatomy. The morphological characterisation of those from the Bizert Lagoon showed the presence of two groups of oysters. One group (referred hereafter to 'BS') comprised 30% of the sample, in which the shell was elongated dorso-ventrally with a spatulate form and with a cupped left valve. The mean length of the shell in this group was 88 mm and the mean weight was 92 g. In the other group (referred to as 'Biz') comprising 70% of the sample, the maximal size was 70 mm, the shell was elongated dorso-ventrally, the right valve was flat to gently convex and the left valve was slightly convex. Oysters sampled in the Gulf of Hammamet (refereed to as 'H') had a mean length of 42 mm (maximum 67 mm) and some individuals had a cupped left valve (Figure 2).

Extraction, amplification and sequencing of DNA

DNA extraction of ethanol-preserved gill fragments of 5 mm^2 was performed using the Wizard DNA clean-up system (Promega, Madison, WI). The 16S rRNA fragment was amplyfied with primers according to Banks *et al.* (1993) and to the protocol detailed in Boudry *et al.* (1998). Polymerase Chaine Reaction (PCR) products were purified by alcohol precipitation using ammonium acetate. They were then sequenced in both directions using the ABI prism BigDye v3 Terminator Cycle sequencing Kit (Applied Biosystems) and the sequences were analysed on an ABI 3100 Avant genetic analyser (Applied Biosystems) using the Seqscape software. The sequencing reaction consisted of a first step of denaturation (2 min, 92°C) and 30 cycles (30s, 95°C, denaturing; 30s, 50°C, annealing; 1 min, 60°C, elongation). All the novel sequences were submitted to the Genbank nucleotide sequence database.

DNA sequence analysis

The 16S rRNA sequences obtained were aligned using CLUSTALW (Thompson *et al.* 1994), with some sequences already obtained by Boudry *et al.* (2003) for *C. gigas* (haplotype D), by Jozefowicz and O'Foighil (1998) for *O. edulis* and by Lapègue *et al.* (2006) for *O. stentina* from the southern Tunisia, Portugal and Morocco respectively Pairwise sequence divergences between species were estimated with the DNADIST program in PHYLIP

(Felsenstein 1989) according to Kimura's (1980) two-parameter model. Phylogenetic analyses were conducted using the program NEIGHBOR. Bootstrap analysis with 100 replicates was performed using the SEQBOOT and CONSENSE programs. The average number of nucleotide differences, a nucleotide diversity index, was estimated with ARLEQUIN vs 3.1 (Excoffier *et al.* 2005) according to Nei (1987), as well as the differentiation parameter *Fst* according to Wright (1951). An analysis of molecular variance approach (AMOVA; Excoffier *et al.* 1992) was performed using ARLEQUIN version 3.0 (Markov chain length: 10 000; Schneider *et al.* 2000) to examine structuring among the two populations.

3. Results

In all, 68 Sixty-eight 16S rRNA sequences were obtained from samples from the Biz (20),, BS (5) and H (43) populations.. The polymorphic sites, described in Table 1, led to the detection of four new haplotypes detected; two in samples from the H population, labelled H16 and H43, and two in samples from the Biz populations, Biz1 and Biz7. They were given Accession numbers EU409056, EU409057, EU409060 and EU409061 respectively.
The alignment of the 346 bp fragment with published sequences for *O. stentina* from southern Tunisia (DQ313180), Portugal (DQ313179), and Morocco (DQ313180), and *O. edulis* (AF052068) and *C. gigas* (AJI553904), allowed genetical characterisation of those oysters. All the BS samples were *C. gigas* (see null divergence between BS8 haplotype and *C. gigas* in Table 2), although those in Biz and H were *O. stentina*. This confirmed our second grouping of oysters on the basis of their shell length and internal anatomy. No individual among the 68 sequenced were *O. edulis*.

The genetic distances confirmed that oysters from the Biz and H populations were genetically very close to the *O. stentina* samples that were studied by Lapègue *et al.* (2006). The nucleotide divergence (Table 2 and Figure 3) between the southern Tunisian haplotype and the two new sequences from the H population was 0.3%, and 0.3% between the Portugal-Morocco haplotype and the two sequences from the Biz population. However, the nucleotide divergence between the new H and Biz haplotypes ranged between 2.1% and 2.7%. The divergence between all the *O. stentina* sequences (from this study) and *O. edulis* (from previous studies) ranged between 8.1% and 8.5% (Table 2) and from 20.8% to 23.4% between *O. stentina* sequences and the BS and *C. gigas* sequences.

It should be noted that (1) a smaller sequence of 346 bp was taken into account in our analysis, 393 bp in Lapègue *et al.* (2006), which provided an identical sequence between *O. stentina* from Morocco and Portugal; (2) the estimation of the nucleotide divergence does not take into account the indels, but the only substitutions. Consequently, haplotype Biz7 and *O. stentina* from Morocco or Portugal might appear to be the same according to their nucleotide divergence (Table 2) but they are in fact different due to indels (Table 1, Fig. 3).
In the Biz and H populations, 10% (2 among 20) and 95% (41 among 43) respectively of the individuals had the same partial 16S rRNA sequence as the one described from southern Tunisia, and 65% (13 among 20) in Biz and 0% in H had the same sequence as the one described from Portugal or Morocco. The four new haplotypes were rare (Table 1). The mean number of nucleotide pairwise differences was 0.5 for Biz and 0.1 for H. An analysis of the distribution of all these haplotypes showed that they were almost all population-specific haplotypes, except for two individuals from the Biz population which exhibited the southern Tunisian *O. stentina* haplotype (Table 1). This translates to a very high differentiation between both populations, with a *Fst* value of 0.71. Structuring of the populations using the AMOVA analysis gave a similar result of high differentiation between the two populations: the among-populations component variance was estimated at 0.294 (variance of 71.4%) and the

within-population component variance 0.117 (variance of 28.6%), giving an estimated *Fst* value of 0.71 ($p < 0.001$).

4. Discussion

Oyster classification remains problematic and the taxonomic status for many species has not been satisfactorily resolved, particularly along the coast of North-East Tunisia. Our study contributes to characterising three wild oysters species along that coast using the large mitochondrial 16S rRNA. The phylogenetic analysis suggests that both Biz and H populations are of the genus *Ostreola*, because they cluster with known members of that genus and not with *Crassostrea*. More precisely, our The molecular DNA analysis of the 16S rRNA sequences presented here helps to distinguish Biz and H populations, identified as *O. stentina* from *C. gigas* (BS) (20.8 – 23.4%) and from *O. edulis* (7.4 – 8.4%). This finding confirms our second grouping of oysters on the basis of their shell length and internal anatomy. Consequently, *C. gigas* and *O. stentina* both inhabit the Bizert Lagoon and are sympatric in that area. Furthermore, the presence of 'wild' *C. gigas*, confirms that this species is reproducing successfully in the lagoon (Dridi *et al*. 2006). Although the level of success of this reproduction is unknown, the number of these naturalised oysters may have increased, given the greater production of Tunisian oysters in the recent years. The production rose from 1 000 t in 2000 to 16 000 t in 2002 and 2003, and represented 0.14% of the total production of molluscs in Tunisia (FAO 2006). This is of particular interest given the invasive character of *C. gigas* in Europe (Ruesink et al., 2005). The Pacific oyster, which originated from north-eastern Asia, has been introduced and translocated, mainly for aquaculture purpose, into a number of countries worldwide (NIMPIS 2002). In North America, the species is found from South-East Alaska to Baja California, whereas in European waters the species is cultured along the coast from Norway to Portugal, as well as in the Mediterranean Sea (McKenzie *et al.* 1997). *C. gigas* is found over wide a range of environments, although it is usually found in coastal and estuarine areas within its natural range. The degree of invasion by *C. gigas* is highly variable (Ruesink *et al.* 2005) and it is sometimes considered as a pest or a noxious species areas along the south-western Atlantic coast (Orensanz *et al.* 2002). In Australia, transfer restrictions and eradication programmes have been implemented (Ayres 1991). However it has adapted well , and is the second highest (by volume) and the first (in value) of shellfish production along the European coast (FAO 2006). Although it has been naturalising since its introduction along the southern French Atlantic, this species is now naturalising over a wide range, especially in northern Europe (e.g. Wadden Sea; Nehls *et al*. 2006). Massive recruitment of the species has been observed in the Thau Lagoon (Mediterranean). Our study substantiates the increasing range in the successful reproduction and naturalisation of *C. gigas.*

O. stentina is easily confused with *O. edulis* because of their similar morphological characteristics, except in their maximum size. Several studies have provided useful discrimination techniques (Leal 1984, Blanc *et al.* 1986, Gonzalez-Wangüemert *et al.* 2004, Lapègue *et al.* 2006). In our study, samples from Biz and H populations were first morphologically identified as *C. gigas* based on their shell morphology (Figures 2a, b). In a second step, molecular tools allowed to avoid the confusion between O. stentina and C. gigas (Fig. 2c). According to Rosique et al. (1995), *O. stentina* does not exceed 20 g in weight and 45 mm in size, but the *O. stentina* identified for the first time in the Bizert Lagoon attained a weight and size of 21 g and 70 mm respectively. Also, individuals of that species from the Gulf of Hammamet attained 34.8 g and 67 mm. Thus, the shell characteristics of *O. stentina* are very plastic, marking classification difficult, particularly in small individuals. Also,, the effect of the environment upon valve morphology in oysters can be pronounced (Seilacher *et al.* 1985). There is clearly a need for molecular techniques in the identification of this species, as demonstrated in the current study.

Our study describes significant levels of genetic divergence among the 16S rRNA haplotypes from *O. stentina* from the Biz and H populations. The haplotypes found in the Biz population are closer to those from Morocco and Portugal, although those found in the H population are closer to those from the south of Tunisia with a divergence ranging between 2.1% and 2.7% for the northern and eastern Tunisian haplotypes respectively. Molecular techniques have been applied to discriminate between oysters and to quantify genetic divergence within and among species. For example, Boudry *et al.* (2003) collected what they presumed to be *C. gigas* and *C. angulata* from France, Spain, Portugal, the United Kingdom and China. Within *C. gigas*, divergence was <0.5% for COI and <0.2% for 16S rRNA,and within *C. angulata*, divergence was respectively <1.1% and 0.2%. Our results for the 16S rRNA sequences are in the same range of level of divergence within the Biz and H haplotypes (0.3%), but are much higher when considering the between Biz and H haplotypes divergence: 2.1–2.7% compared with 0.5–1% between *C. gigas* and *C. angulata*. This finding also contrasts with those obtained from 16S rRNA sequencing in the quagga mussel *Dreissena bugensis*, for which no intraspecific variation was found between Eurasian and North American populations (Stepien *et al.* 1999). However, for the scallop *Chlamys farreri*, Kong *et al.* (2003) found that the average number of nucleotide differences in Asian populations ranged from 1.03% to 3.5%, which is high and in the same range as the Bizert Lagoon value of 2.6% However, in the Asian scallop study, there was little geographic structure among samples. On the contrary, in our study the high level of differentiation between both species could indicate that there is very restricted gene exchange between both sides of the Siculo-Tunisian strait. On the basis of the high level of differentiation observed, the two populations could be considered as encompassing two different subspecies or taxa, as has been proposed for *C. gigas* and *C. angulata* (Batista *et al.* 2006). It should be noted that, because the current analysis is based on a single mtDNA gene, our findings should be regarded as preliminary. More rigorous analyses, using several loci, are necessary to substantiate the conclusions drawn from this study

Besides the Strait of Gibraltar may act as a phylogeographic transition between Atlantic and Mediterranean Sea (see Patarneoo et al 2007 for recent discussion), our study provides some evidence that an intra-Mediterranean barrier could play a role in the distribution of marine species. This barrier is possibly the Siculo-Tunisian Strait, which extends from the Tunisian Cap Bon to Marsala (Sicilia), and is also considered to be a biogeographical boundary between the eastern and western Mediterranean basins (Quignard 1978). During the last glaciation, the lower sea level modified the coastline of the Mediterranean basin, splitting the eastern and western basins (Bonatti 1966, Thiede 1978). Since then, the two basins have had different hydrographic regimes. The water circulation in the Siculo-Tunisian Strait is characterised by a unidirectional east-south-east flow of currents (from the Atlantic via Gibraltar), which rounds Tunisian Cap Bon and leave the coast of Tunisia at Kelibia (36°50'N). On the other hand, eastern Mediterranean waters remain in the Lybico-Tunisian Gulf. The circulation is not very dynamic in the rest of the eastern Mediterranean, with the Adriatic and Aegean seas, both under the influence of cool and low salinity waters, showing cyclonic circulation which causes isolation of their northern regions (Pinardi *et al.* 1997). These hydrographic patterns may have allowed progressive genetic differentiation. Supportive evidence of this was found by Borsa *et al*. (1997) in their study of the genetic differences between populations from 16 species of tropical and subtropical boreal fish, and coastal invertebrates in the north-eastern Atlantic and the Mediterranean Sea. For most species, there was a fairly strong genetic cline between each side of the Gibralatar strait area, and for some between each side of the Siculo-Tunisian strait. It is premature to suggest that the Siculo-Tunisian Strait has a similar impact on *O. stentina* populations until a comprehensive study has been made on the genetic structure of more populations of the species along the Mediterranean coast.

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Tables

Table 1: Polymorphic sites corresponding to the four new *O. stentina* haplotypes detected from a 346-bp fragment of the mitochondrial 16S rRNA gene and compared with the sequence from the south of Tunisia, and Portugal, and the frequency of haplotypes in the Biz and H populations. Columns 3-13 indicate the position of the polymorphism along the sequence. Dashes indicate a deletion. Columns 14 and 15 indicate the number of individuals exhibiting each haplotype in the two populations

Haplotype	Accession number	556	565	1000	1007	1448	1554	1555	1887	2112	2433	2533	Biz	H
O. stentina Tunisia	DQ313178	A	T	T	T	A	T	_	A	T	C	_	2	41
H16	EU409056	A	C	T	T	A	T	_	A	T	C	_	0	1
H43	EU409057	A	T	T	T	A	T	_	G	T	C	_	0	1
Biz7	EU409060	G	T	C	C	G	C	C	A	G	A	_	4	0
Biz1	EU409061	G	C	C	C	G	C	C	A	G	A	_	1	0
O. stentina Portugal/Morocco	DQ313179	G	T	C	C	G	C	C	A	G	A	T	13	0

Table 2: Pairwise sequence divergences for the four Biz and H new 16S rRNA haplotypes, the BS haplotype, three *O. stentina* known haplotypes, *C. gigas* and *O. edulis*

Hoplotype	*5. Biz 7*	*5.1. Biz 1*	*5.2. O. stentina (Portugal)*	*O. stentina (Morocco)*	*6. H 43*	*7. H16*	*7.1. O. stentina (Tunisia)*	*7.2. BS8*
Biz 1	0.0029							
O. stentina (Portugal)	0.0000	0.0029						
O. stentina (Morocco)	0.0000	0.0029	0.0000					
H 43	0.0267	0.0237	0.0267	0.0267				
H 16	0.0237	0.0207	0.0237	0.0237	0.0029			
O. stentina (Tunisia)	0.0207	0.0237	0.0207	0.0207	0.0029	0.0029		
Ostrea edulis	0.0807	0.0839	0.0807	0.0807	0.0846	0.0839	0.0806	
BS8	0.2288	0.2326	0.2288	0.2288	0.2159	0.2121	0.2083	
Crassotrea gigas	0.2281	0.2319	0.2281	0.2281	0.2153	0.2115	0.2077	0.0000

Figures

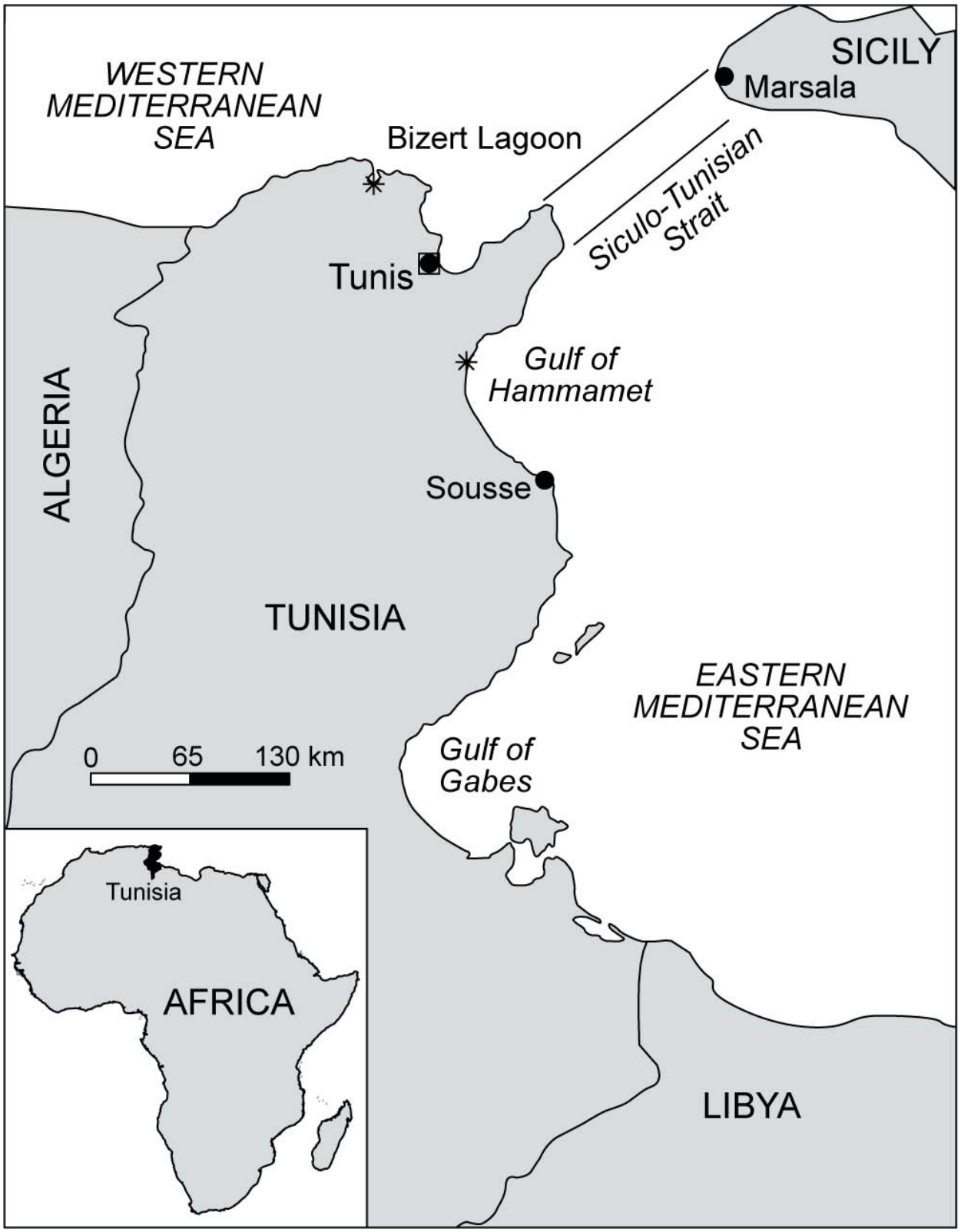

Figure 1: Map of Tunisia showing the locations of the sampling sites

Figure 2: External and internal view of the left and right valve of the haplotypes of (a) Biz, (b) H and (c) BS populations

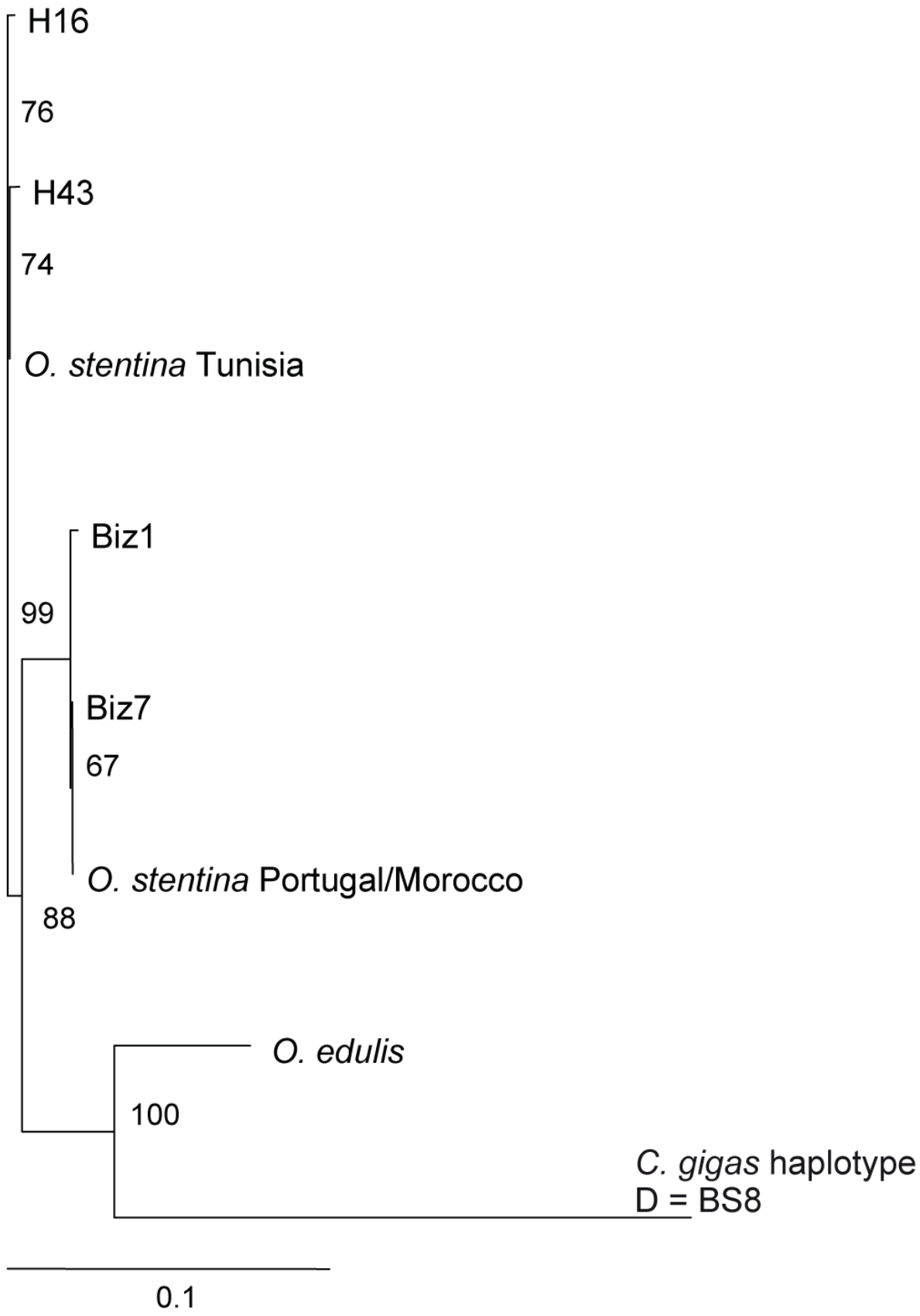

Figure 3: Phylogenetic tree obtained from sequence divergence of a 346-base nucleotide mitochondrial 16S rRNA fragment according to Kimura's (1980) model for the four Biz and H new 16S rRNA haplotypes, the BS haplotype, three *O. stentina* known haplotypes, *C. gigas* and *O. edulis*. Numbers on the branches indicate bootstrap values >50%