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New insights about the introduction of the Portuguese oyster, *Crassostrea angulata*, into the North East Atlantic from Asia based on a highly polymorphic mitochondrial region

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Abstract

It is commonly presumed that the Portuguese oyster *Crassostrea angulata* was introduced into the North East (NE) Atlantic from Asia. The analysis of the nucleotide sequence of a highly polymorphic non-coding mitochondrial region (major noncoding region - MNR) of *C. angulata* samples collected in Europe (Portugal), Africa (Morocco) and Asia (Shantou and Taiwan) provided new insight into the introduction of this species into the NE Atlantic. Sixty haplotypes and a nucleotide diversity of 0.0077 were observed in 130 analyzed sequences. Higher nucleotide diversity levels were observed in NE Atlantic sites than in Asian sites and significant genetic differentiation was found between the two. Our results suggest that *C. angulata* might have been introduced to the NE Atlantic by multiple introductory events, though the exact origins remain unknown since none of the analyzed Asian sites seemed to have been a source of introduction. The nucleotide diversity of *C. angulata* was higher than that previously reported for Pacific oyster *C. gigas* in Europe and Asia for the same mitochondrial region. The results obtained in the present study suggest that NE Atlantic *C. angulata* stocks are a unique genetic resource, which highlights the importance of their conservation.

Keywords: *Crassostrea angulata* / phylogeography / genetic signature / biological invasions

1 Introduction

The Portuguese explorer Vasco da Gama (ca. 1460-1524) was the first European to reach India by sea in 1498 by sailing around the Cape of Good Hope and across the Indian Ocean. In 1511, the contest of Malaca led by the Portuguese general Afonso de Albuquerque was of crucial importance for the establishment of a commercial maritime route between Europe and Asia (India and China) (Boxer 1969). As many marine organisms can survive attached to the hull of vessels it is possible that Asian species may have been introduced via this route as early as the XVI century, but this has remained poorly documented until recently (Seebens et al. 2013).

Biological invasions can have dramatic ecological and economic impacts (Simberloff et al. 2013). However, some species introduced involuntary or voluntary have a high social and/or economic importance, which is notably the case in Europe for several marine bivalve species such as the Pacific oyster *Crassostrea gigas* (Thunberg, 1793) and the Manila clam *Ruditapes philippinarum* (Adams and Reeve, 1850).

C. gigas was originally described in Asia whereas the Portuguese oyster *C. angulata* (Lamarck, 1819) was first described in the NE Atlantic. Several comparative studies based on larval shell morphology (Ranson 1960) and allozyme markers (Mathers et al. 1974; Buroker et al. 1979) suggested that the two taxa were the same species. Studies using other types of genetic markers also reached a similar conclusion (López-Flores et al. 2004; Cross et al. 2006). Several studies have reported the production of F1 hybrids between *C. angulata* and *C. gigas* under experimental conditions (Imai and Sakai 1961; Menzel 1974; Huvet et al. 2002; Batista et al. 2007). Hybrids between *C. angulata* and *C. gigas* have also been reported in the wild (Huvet et al. 2004) and first generation hybrids were shown to be fully viable and fertile under experimental conditions (Huvet et al. 2002). However, several phenotypic and genetic differences were also reported between *C. angulata* and *C. gigas* (for review see

Batista et al. 2009). Based on the sequence of the mitochondrial genome the two species were estimated to have diverged about 2.7 million years ago (Ren et al. 2010). The close relationship between *C. angulata* and *C. gigas* has led several authors to suggest that they have a recent common origin (e.g. Ranson 1960). Phylogenetic analysis by O'Foighil et al. (1998) suggested that *C. angulata* has been recently introduced to Europe from Asia. Moreover, Boudry et al. (1998) based on PCR-RFLP data were the first to report the presence of *C. angulata* in Taiwan. Since then, several studies have reported the presence of *C. angulata* in Asia, namely in China (Làpegue et al. 2004; Xia et al. 2009; Wang et al. 2010) and more recently in Okinawa Island, Japan (Sekino and Yamashita 2013).

The absence of *Crassostrea* sp. shells in shell middens in Europe, contrasts with the abundance of shells from the European flat oyster *Ostrea edulis* (e.g. Gutiérrez-Zugasti et al. 2011; Drago et al. unpublished data) and is an additional argument in support of the hypothesis that *C. angulata* was introduced from Asia into Europe, rather than being part of its natural range. Shell middens in Portugal (southern Portugal and Tagus River valley) from different periods (Mesolithic and late Islamic occupation of Iberia) contained *O. edulis* shells but none which were undoubtedly from *Crassostrea* sp. (Drago et al., unpublished data).

The presence of *C. angulata* in Europe dates back at least to its description by Lamarck in 1819. *C. angulata* is of high aquaculture importance, now recognized as one of the most produced species in China (Qin et al. 2012) and sometimes known as the "Fujian oyster". It was the main bivalve species cultured in France until the 1970's when massive mortalities almost led to its disappearance (Comps 1988). Nowadays, the only known sites in NE Atlantic where *C. angulata* is present and *C. gigas* has not been detected at least in large numbers are the Mira and Sado estuaries in Portugal, the Cadiz region in Spain and Tahaddart in Morocco (Fabioux et al. 2002). However, these *C. angulata* beds are threatened by the invasion of *C. gigas* (Huvet et al. 2000).

Many questions remain unanswered about the introduction of *C. angulata* into Europe and the potential consequences. The objective of the present study was to estimate the genetic diversity and differentiation of *C. angulata* sampled in NE Atlantic and Asian sites. A phylogeographic study was performed using samples collected in Europe, Africa and Asia, and sequence data from a highly polymorphic mitochondrial DNA region.

2 Material and Methods

2.1 Sampling sites

Oysters presumed to be *C. angulata* were sampled from the following sites: Mira estuary in Portugal (37°42'N, 8° 44'W) in 2010; Sado estuary in Portugal (38° 25'N, 8°39'W) in 2010; Tahaddart bay in Morocco (35°35'N, 5°59'W) in 2013; and Nanao island, Shantou, Guangdong province in China (23°25'N, 117°04'E) in 2012. In addition, samples of *C. angulata* collected in Keelung (Taiwan) previously analysed by Boudry et al. (1998) were also studied. A piece of gill tissue (~2-3 mm^2) was dissected from each oyster and stored in ethanol (70-95%). The number of oysters analyzed from each site is given in Table 1.

2.2 DNA extraction, amplification and sequencing

DNA was extracted from gill tissue samples using a DNeasy®blood & tissue kit (QIAGEN) following the manufacturer's instructions. Extracted DNA was then quantified using a spectrophotometer (Nano-Drop Technologies). A 739-bp fragment of the major noncoding region (MNR) of mitochondrial DNA was amplified by PCR using the forward (5'-TCACAAGTACATTTGTCTTCCA-3') and reverse (5'-AACGTTGTAAGCGTCATGTAAT-3') primers designed by Aranishi and Okimoto (2005). PCR reactions were performed in a final reaction volume of 25 µl and contained 2.5 µl of 10X reaction buffer, 16 µl of distilled water, 1.5 µl of dNTP (2.5mM), 1.5 µl of MgCl2 (25

mM), 1 µl of each primer (10 µM), 0.5 µl of Taq polymerase (Supreme NZYTaq DNA polymerase, NZYTECH) and 1.0 µl of template DNA (ca. 50 ng). The amplification cycle consisted of an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of 20 s at 95 °C, 20 s at 58 °C, and 1 min at 72 °C. At the end of these cycles a final extension of 7 min at 72 °C was performed. PCR products were purified using NZYgel pure (NZYTECH) following the manufacturer's instructions. Amplicons were separated by electrophoresis on 1.5% agarose gels, stained with GreenSafe (NZTECH) and visualized under UV light to confirm the expected size and absence of non specific amplification. The purified amplicons were sequenced using the kit ABI PRISM BigDye Terminator v3.1 and an automatic sequencer (Genetic Analyzer 3130xl Applied Biosystems). All singletons were confirmed by repeating the PCR and sequencing analyses.

2.3 Data analysis

Chromatograms of the nucleotide sequences were analyzed using Bioedit version 7.0.9.0 (Hall 1999). After editing, multiple sequence alignments were performed using ClustalW (Thompson et al. 1994). Dnasp (version 5.00.03; Rozas et al. 2003) was used to calculate the number of variable sites, number of haplotypes, mean number of nucleotide differences, haplotype diversity and nucleotide diversity. In order to analyze the demographic history of *C. angulata*, Tajima's D (Tajima 1989), Fu's F (Fu 1997) and R_2 (Ramos-Onsins and Rozas 2002) were also calculated using Dnasp to test constant population size versus population growth. Negative Tajima's D and Fu's F values are expected under a scenario of recent population expansion but can also be a signature of selection (non-neutral evolution). Low R_2 values are expected after a recent severe population growth event (Ramos-Onsins and Rozas 2002). The statistical significance of Tajima's D, Fu's F and R_2 values was calculated with coalescence simulations implemented in Dnasp. ARLEQUIN (version 3.11) was used to

148 calculate pairwise genetic differentiation (Fst) using 1000 permutations (Excoffier et al.
149 2005). A median joining network (Bandelt et al. 1999) was constructed using PopArt (version
150 1.7; http://popart.otago.ac.nz).
151

152 ## 3 Results

153 A total of 60 haplotypes were identified comparing 130 individual nucleotide sequences
154 (length 656 – 658 bp) of the major noncoding region (MNR) fragment of the mitochondrial
155 genome of *C. angulata* (Genbank accession numbers KY217737-KY217796). The number of
156 total variable sites was 64 and the mean number of nucleotide differences was 5.07. Five
157 indels were observed and the other mutations detected were 59 transitions and 5
158 transversions.
159 All haplotypes (with the exception of two sequences from Tahaddart) shared high nucleotide
160 sequence similarity (>98%) for the corresponding region of *C. angulata* mitochondrial
161 genomes (Ren et al. 2010; 2016). Some haplotypes observed in the present study (H5, H6,
162 H11, H32 and H33) were identical to the respective region of the *C. angulata* mitochondrial
163 genomes (Genbank accession numbers KJ855246, KJ855247, KJ855248, KJ855249 and
164 EU672832) (Ren et al. 2016). Only two haplotypes, detected in Tahaddart, showed a high
165 nucleotide identity (99.8 and 100%) with haplotypes previously described for *C. gigas*
166 (KJ855243; Ren et al. 2006); they were excluded from the analysis. The nucleotide
167 divergence among *C. angulata* MNR haplotypes ranged from 0.2 to 1.9 %. The nucleotide
168 divergence between *C. angulata* and *C. gigas* MNR haplotypes (haplotypes identified by
169 Moehler et al. 2011 with the accession numbers JF505202-JF505277 and position 10615 to
170 11253 bp of EU672831) ranged from 8.2 to 10.2 %.
171 Table 1 shows the number of *C. angulata* haplotypes detected at each site. The Sado and
172 Mira sites shared seven haplotypes, which was the highest number of shared haplotypes.

Haplotype H6 was common to all sites and had the highest overall frequency (16.9%). It was the most frequent haplotype observed in the Shantou site (33%, Figure 1). The analysis of a Median-joining network constructed using all the haplotypes identified in the study showed that haplotype H6 had a central position and hence is likely to be an ancestral haplotype (Figure 2). Ten out of 11 haplotypes observed in the Shantou site were only observed in this site (i.e. private haplotypes). Among the 60 haplotypes detected, only four were shared between the NE Altantic sites (Mira, Sado and Tahaddart) and Asian sites (Shantou and Keelung).

The frequency of unique haplotypes observed in each site ranged from 19.2 to 64.3%. Haplotype diversity ranged from 0.832 to 0.960 and nucleotide diversity from 0.00594 to 0.00791 (Table 1). Significant pairwise genetic differentiation (Fst estimates between 0.102 and 0.228) after Bonferroni correction was observed between all NE Atlantic sites and the two Asian sites (Table 2). However, no significant Fst estimates (0.003 - 0.072) were observed among the NE Atlantic sites. A significant Fst value of 0.151 was estimated between Shantou and Keelung (Table 2). Significant negative Tajima's D values were observed in Shantou and Keelung sites (Table 3) and significant Fu's F negative values were observed in Mira and Keelung sites. Moreover, significant R_2 values were observed in all sites (Table 3).

4 Discussion

The analysis of the nucleotide sequence of a highly polymorphic non-coding mitochondrial region of *C. angulata* samples collected in Europe, Africa and Asia provided new insight into the introduction of this taxon from Asia into the NE Atlantic region. We observed high genetic diversity in *C. angulata* from the NE Atlantic region and genetic differentiation between three NE Atlantic and two Asian sites. Recently, Ren et al. (2016) analyzing

198 intraspecific variation in mitogenomes of *C. gigas*, *C. angulata*, *C. sikamea*, *C. ariakensis*
199 and *C. hongkongensis* observed an overall between species nucleotide diversity ranging from
200 0.00106 to 0.00683. Using this data, we calculated a nucleotide diversity of 0.0085 from five
201 *C. angulata* mitogenomes (one of the individuals was collected in Portugal and the other four
202 in China; KJ855246–KJ855249 and EU672832) published by Ren et al. (2016) for the
203 mitochondrial region that we studied (i.e. MNR). We obtained a similar nucleotide diversity
204 of 0.0077 for *C. angulata* from the present dataset. Repeating the analysis for the MNR
205 fragments from five *C. gigas* mitogenomes (KJ855241–KJ855245, Ren et al. 2016) resulted
206 in a nucleotide diversity of 0.0049, which was higher than the overall nucleotide diversity of
207 0.0019 obtained for *C. gigas* by Ren et al. (2016). This difference between the overall
208 mitochondrial genome and MNR region confirms that the mitochondrial region analyzed in
209 the present study is indeed a highly polymorphic region, as was previously suggested by
210 Aranishi and Okimoto (2005) for *C. gigas*.
211 The nucleotide diversity of *C. angulata* observed in the present study was higher than that
212 previously reported for *C. gigas* for the same mitochondrial region. Moehler et al. (2011) and
213 Lallias et al. (2015) reported nucleotide diversity values of 0.0044 and 0.0046 for *C. gigas*,
214 respectively, with samples mainly collected in Europe but also in Canada and Japan. Hsiao et
215 al. (2016) found haplotype and nucleotide diversities (using mitochondrial cytochrome
216 oxidase I sequences) in Asia being considerably higher in *C. angulata* than in *C. gigas*. This
217 suggests that the number of *C. angulata* individuals introduced into the NE Atlantic must
218 have been sufficiently high to reflect the genetic diversity of *C. angulata* in its native range.
219 In addition to the number of individuals introduced, other factors may influence the genetic
220 diversity of introduced species such as the number of introduction events. It is possible that
221 *C. angulata* was introduced via multiple introduction events and that this may explain the
222 high nucleotide diversity observed in the NE Atlantic relative to the Asian sites. Other

223 possible explanations are that the origin of *C. angulata* was not the two Asian sites analyzed
224 in the present study (i.e. Keelung and Shantou) or that haplotype diversity strongly changed
225 in these sites since the introduction of *C. angulata* into Europe. Indeed, only four out of 60
226 haplotypes were shared between the NE Atlantic and the two Asian sites. Moreover, the
227 significant Fst values observed between the NE Atlantic sites and the Keelung and Shantou
228 sites support the hypothesis that these two sites were not the source of the introduction. Huvet
229 et al. (2000) using microsatellite loci also observed a significant Fst value of 0.0173 between
230 Mira and Keelung sites. Our results therefore do not support the hypothesis that *C. angulata*
231 might have been introduced from Taiwan (for which *C. angulata* DNA-based identification
232 was first reported) to Portugal. The hypothesis is based on previous studies using PCR-RFLP
233 haplotypes of a fragment of the mitochondrial gene cytochrome oxidase subunit I (COI)
234 (Boudry et al. 1998; Huvet et al. 2000). An explanation of this result comes from the much
235 higher polymorphism of MNR sequence data in comparison with RFLP-based COI. Since *C.*
236 *angulata* is known to be widespread along the southern coast of China (Wang et al. 2010) it is
237 likely that there are other areas that could have been the origin of the NE Atlantic *C. angulata*
238 populations. Consequently, further analyses of *C. angulata* populations in Asia are needed to
239 trace the putative source(s) of this introduction. However, it cannot be ruled out that the
240 source of *C. angulata* in Asia may have been lost due to anthropogenic activities (seed
241 exchange in relation with aquaculture production) or natural causes (e.g. disease outbreaks),
242 which would then make the NE Atlantic *C. angulata* resources unique.
243 The lack of genetic differentiation of *C. angulata* from the Sado, Mira and Tahaddart is in
244 agreement with the hypothesis that *C. angulata* was mainly introduced to a single location in
245 Europe (probably the Tagus River, which was one of the main ports for anchoring ships from
246 Asia), from where it was introduced to other locations in the NE Atlantic. For example, it is
247 documented that *C. angulata* was introduced into Morocco from Spain and Portugal in 1952

(Shafee 1985 cited in Fabioux et al. 2002). In contrast to *C. gigas* in Europe, which showed a typical star-shaped haplotype phylodiversity characteristic of expanding populations (Moehler et al. 2011; Lallias et al. 2015), the haplotype network of *C. angulata* did not show such a pronounced star-shape. Significant negative Tajima's D values were only found for *C. angulata* samples from Asia, although Fu's F tests were significant for Mira and Keelung samples. Significant R_2 values were observed for all sites. For small samples the R_2 test is more powerful than Tajima's D and Fu's F tests for detecting population growth (Ramos-Onsins and Rozas 2002). Thus, our results suggested that a demographic expansion event occurred recently in *C. angulata*. This is in agreement with the results of Hsiao et al. (2016) who suggested that *C. angulata* in Asia experienced a sudden population expansion after the last glacial maxima. However, the results from Tajima's D, Fu's F and R_2 tests obtained in the present study and in other studies have to be considered cautiously since the coalescent model used may be inappropriate for highly fecund species such as *C. angulata* (Steinrucken et al. 2013).

5 Conclusions

We observed higher nucleotide diversity for *C. angulata* in NE Atlantic sites than in the studied Asian sites. Moreover, significant genetic differentiation was detected between *C. angulata* from three NE Atlantic and two Asian sites. Our results suggested that *C. angulata* might have been introduced into the NE Atlantic through multiple introductory events. However, further analyses in Asia are needed to identify the source(s) of the introduction(s) since none of the Asian sites studied seemed to have been the origin. The NE Atlantic *C. angulata* stocks appear to be unique genetic resources, which highlights the importance of their conservation.

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Table 1. Genetic diversity of *C. angulata* samples estimated from comparisons of sequences of the major noncoding region (position 10617-11217 bp of EU672832) of mitochondrial DNA.

Site	**n**	**S**	**h**	**k**	**Hd (SD)**	**π (SD)**
Sado (Portugal)	26	22	14	5.09	0.929 (0.028)	0.00760 (0.00068)
Mira (Portugal)	28	22	17	5.20	0.960 (0.018)	0.00791 (0.00064)
Tahaddart (Morocco)	21	24	13	4.63	0.862 (0.073)	0.00705 (0.00103)
Shantou (China)	27	27	11	3.90	0.832 (0.052)	0.00594 (0.00091)
Keelung (Taiwan)	28	36	22	3.83	0.958 (0.030)	0.00583 (0.00058)
Total	130	64	60	5.07	0.968 (0.007)	0.00770 (0.00036)

n, number of individuals analyzed; S, number of variable sites; h, number of haplotypes; k, mean number of nucleotide differences; Hd, haplotype diversity (standard deviation); π, nucleotide diversity (standard deviation).

Table 2. Pairwise genetic differentiation (Fst) for *C. angulata* samples collected in North East Atlantic and Asia.

	1	2	3	4	5
1. Sado (Portugal)	-				
2. Mira (Portugal)	0.0641	-			
3. Tahaddart (Morocco)	0.0029	0.0717	-		
4. Shantou (China)	**0.1244**	**0.1600**	**0.1018**	-	
5. Keelung (Taiwan)	**0.2250**	**0.1418**	**0.2282**	**0.1508**	-

Values in bold are significant after Bonferroni correction ($p<0.05$)

Table 3. Neutrality and demographic tests for *C. angulata* samples estimated from sequences of the major noncoding region (position 10617-11217 bp of EU672832) of mitochondrial DNA.

Site	n	Tajima's D	Fu's F	R_2
Sado (Portugal)	26	-0.4204	-3.104	**0.1055**
Mira (Portugal)	28	-0.2869	**-5.767**	**0.1143**
Tahaddart (Morocco)	21	-1.1681	-3.833	**0.0848**
Shantou (China)	27	**-1.6214**	-1.541	**0.0703**
Keelung (Taiwan)	28	**-2.1708**	**-18.053**	**0.0448**

R_2, Ramos-Onsins and Rozas statistic. Values in bold are significantly different ($p<0.05$)

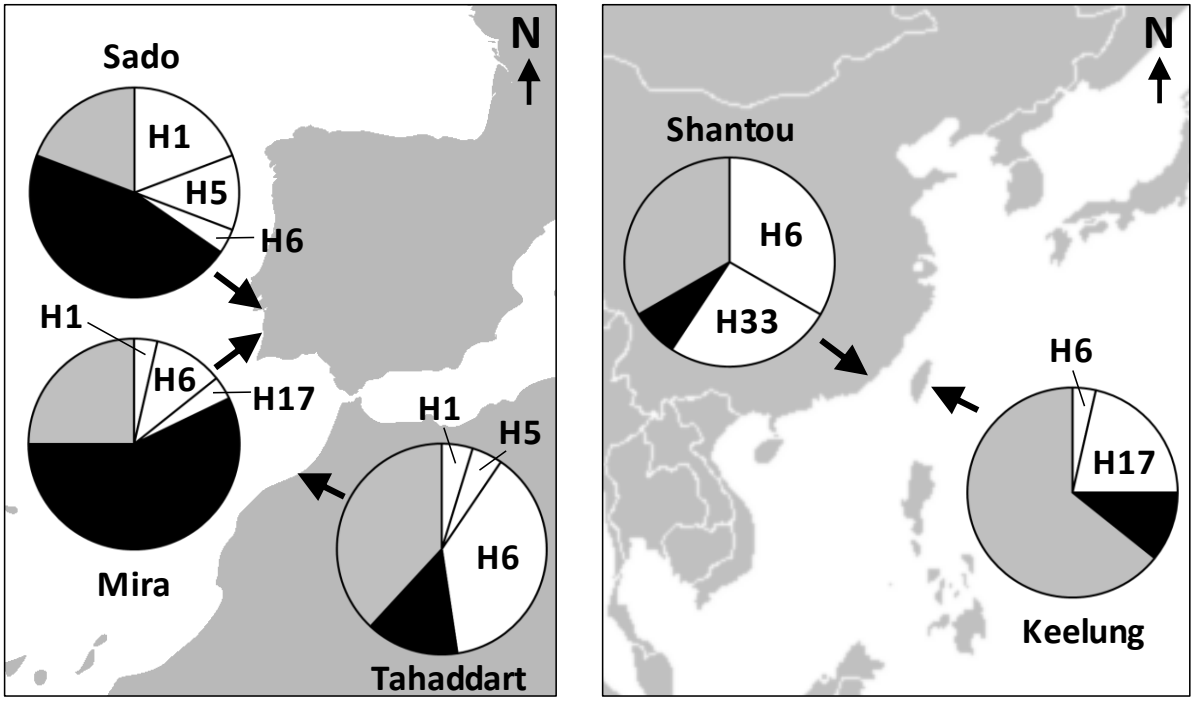

Fig. 1. Haplotype frequencies of *C. angulata* collected in Portugal (Mira and Sado estuaries), Morocco (Tahaddart), Taiwan (Keelung) and China (Shantou). The pie charts show the frequency of the main haplotypes (H1, H5, H6, H17 and H33), unique haplotypes (in gray) and haplotypes shared with other locations (in black).

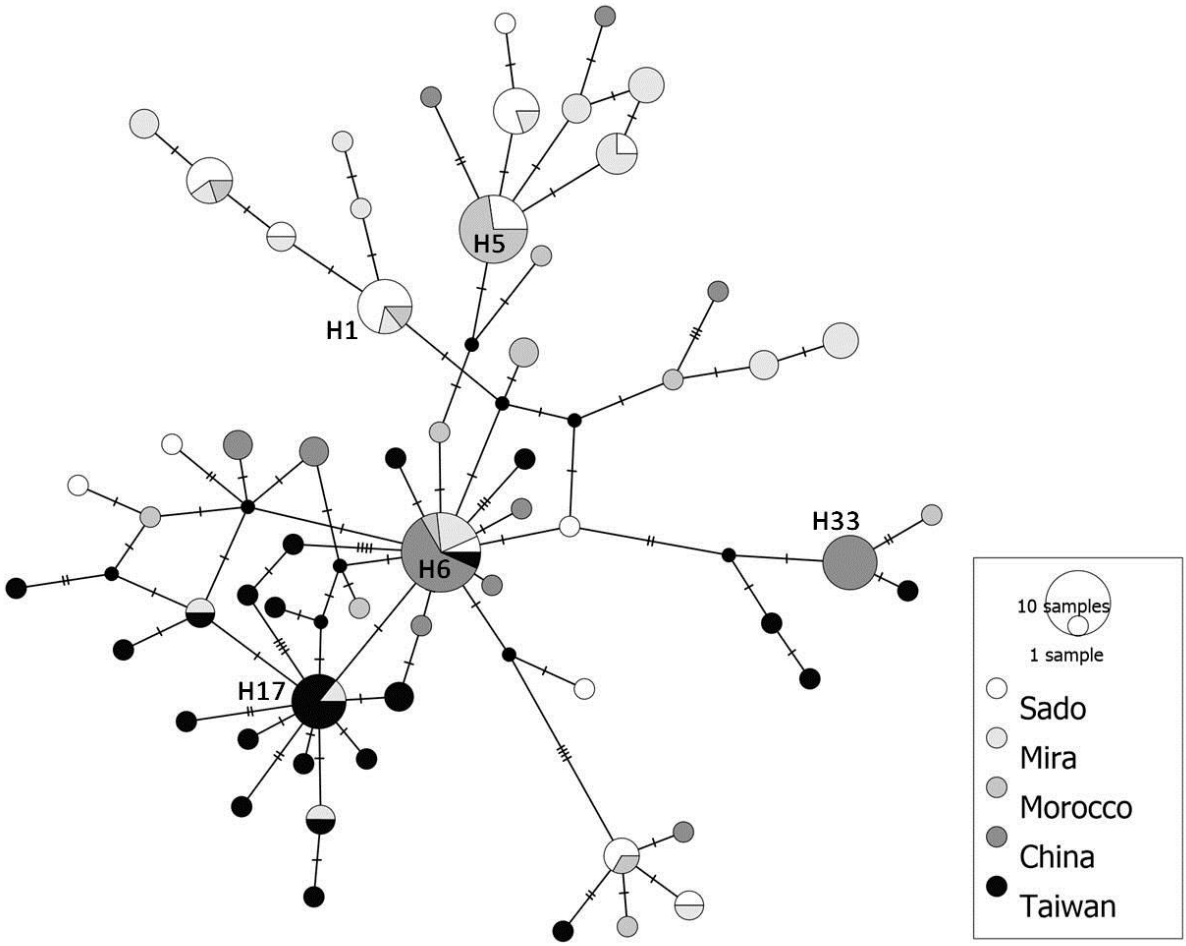

Fig. 2. Median joining network of *C. angulata* DNA haplotypes of a fragment (position 10617-11217 bp of EU672832) of the major noncoding region of the mitochondrial genome. The size of circles corresponds to haplotype frequency and the different colours represent the geographical sites where the haplotypes were observed. The number of perpendicular bars on branches represents the number of mutational steps (indels and/or substitutions) between haplotypes.