PRIFYSGOL BANGOR / BANGOR UNIVERSITY

PRIFYSGOL
BANGOR
UNIVERSITY

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.

Grade, Ana; Chairi, Hicham; Lallias, Delphine; Power, Deborah; Ruano, Francisco; Leitao, Alexandra; Drago, Teresa; King, Jonathan; Boudry, Pierre; Batista, Frederico

Aquatic Living Resources

DOI:
10.1051/alr/2016035

Published: 01/12/2016

Peer reviewed version

Cyswllt i'r cyhoeddiad / Link to publication

Dyfyniad o'r fersiwn a gyhoeddwyd / Citation for published version (APA):
Grade, A., Chairi, H., Lallias, D., Power, D., Ruano, F., Leitao, A., ... Batista, F. (2016). 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. *Aquatic Living Resources*, *29*(4), [404]. https://doi.org/10.1051/alr/2016035

Hawliau Cyffredinol / General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal ?

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

15. Oct. 2019

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

Ana Grade[1,2], Hicham Chairi[3], Delphine Lallias[4,5], Deborah M. Power[6], Francisco Ruano[2], Alexandra Leitão[1,7], Teresa Drago[1,8], Jonathan W. King[4], Pierre Boudry[9], Frederico M. Batista[1, 6 a]

[1] Instituto Português do Mar e da Atmosfera, Estação Experimental de Moluscicultura de Tavira, Av. 5 de Outubro, 8700-305 Olhão, Portugal.

[2] Instituto Português do Mar e da Atmosfera, Av. de Brasília 1449-006 Lisboa, Portugal.

[3] Université Abdelmalek Essaâdi, Faculté Polydisciplinaire de Larache, Département de Biologie, B.P. 745, 92004, Larache, Morocco.

[4] Centre for Applied Marine Sciences, School of Ocean Sciences, College of Natural Sciences, Bangor University, Menai Bridge, Anglesey LL59 5AB, UK.

[5] Current address: GABI, INRA, AgroParisTech, Université Paris-Saclay, 78350 Jouy-en-Josas, France.

[6] Centro de Ciências do Mar (CCMAR), Universidade do Algarve, Campus de Gambelas, 8005-139 Faro, Portugal.

[7] Environmental Science Center (ESC), Qatar University, P. O. Box: 2713 Doha, Qatar.

[8] Instituto Dom Luiz, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, Edifício C1, Piso , 1749-016 Lisboa, Portugal.

23 [9] Ifremer, Unité de recherche Physiologie Fonctionnelle des Organismes Marins, Laboratoire
24 des Sciences de l'Environnement Marin (UMR 6539 LEMAR CNRS/UBO/IRD/Ifremer), ZI
25 de la Pointe du Diable, CS 10070, 29280 Plouzané, France.

26

27 [a] corresponding author: fmbatista@yahoo.com

28

29 **Abstract**

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

45

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

47

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

73 Batista et al. 2009). Based on the sequence of the mitochondrial genome the two species were
74 estimated to have diverged about 2.7 million years ago (Ren et al. 2010). The close
75 relationship between *C. angulata* and *C. gigas* has led several authors to suggest that they
76 have a recent common origin (e.g. Ranson 1960). Phylogenetic analysis by O'Foighil et al.
77 (1998) suggested that *C. angulata* has been recently introduced to Europe from Asia.
78 Moreover, Boudry et al. (1998) based on PCR-RFLP data were the first to report the presence
79 of *C. angulata* in Taiwan. Since then, several studies have reported the presence of *C.*
80 *angulata* in Asia, namely in China (Làpegue et al. 2004; Xia et al. 2009; Wang et al. 2010)
81 and more recently in Okinawa Island, Japan (Sekino and Yamashita 2013).
82 The absence of *Crassostrea* sp. shells in shell middens in Europe, contrasts with the
83 abundance of shells from the European flat oyster *Ostrea edulis* (e.g. Gutiérrez-Zugasti et al.
84 2011; Drago et al. unpublished data) and is an additional argument in support of the
85 hypothesis that *C. angulata* was introduced from Asia into Europe, rather than being part of
86 its natural range. Shell middens in Portugal (southern Portugal and Tagus River valley) from
87 different periods (Mesolithic and late Islamic occupation of Iberia) contained *O. edulis* shells
88 but none which were undoubtedly from *Crassostrea* sp. (Drago et al., unpublished data).
89 The presence of *C. angulata* in Europe dates back at least to its description by
90 Lamarck in 1819. *C. angulata* is of high aquaculture importance, now recognized as one of
91 the most produced species in China (Qin et al. 2012) and sometimes known as the "Fujian
92 oyster". It was the main bivalve species cultured in France until the 1970's when massive
93 mortalities almost led to its disappearance (Comps 1988). Nowadays, the only known sites in
94 NE Atlantic where *C. angulata* is present and *C. gigas* has not been detected at least in large
95 numbers are the Mira and Sado estuaries in Portugal, the Cadiz region in Spain and Tahaddart
96 in Morocco (Fabioux et al. 2002). However, these *C. angulata* beds are threatened by the
97 invasion of *C. gigas* (Huvet et al. 2000).

98 Many questions remain unanswered about the introduction of *C. angulata* into Europe
99 and the potential consequences. The objective of the present study was to estimate the genetic
100 diversity and differentiation of *C. angulata* sampled in NE Atlantic and Asian sites. A
101 phylogeographic study was performed using samples collected in Europe, Africa and Asia,
102 and sequence data from a highly polymorphic mitochondrial DNA region.
103

104 # 2 Material and Methods

105 ## 2.1 Sampling sites

106 Oysters presumed to be *C. angulata* were sampled from the following sites: Mira estuary in
107 Portugal (37°42'N, 8° 44'W) in 2010; Sado estuary in Portugal (38° 25'N, 8°39'W) in 2010;
108 Tahaddart bay in Morocco (35°35'N, 5°59'W) in 2013; and Nanao island, Shantou,
109 Guangdong province in China (23°25'N, 117°04'E) in 2012. In addition, samples of *C.*
110 *angulata* collected in Keelung (Taiwan) previously analysed by Boudry et al. (1998) were
111 also studied. A piece of gill tissue (~2-3 mm^2) was dissected from each oyster and stored in
112 ethanol (70-95%). The number of oysters analyzed from each site is given in Table 1.
113

114 ## 2.2 DNA extraction, amplification and sequencing

115 DNA was extracted from gill tissue samples using a DNeasy®blood & tissue kit (QIAGEN)
116 following the manufacturer's instructions. Extracted DNA was then quantified using a
117 spectrophotometer (Nano-Drop Technologies). A 739-bp fragment of the major noncoding
118 region (MNR) of mitochondrial DNA was amplified by PCR using the forward (5'-
119 TCACAAGTACATTTGTCTTCCA-3') and reverse (5'-
120 AACGTTGTAAGCGTCATGTAAT-3') primers designed by Aranishi and Okimoto (2005).
121 PCR reactions were performed in a final reaction volume of 25 µl and contained 2.5 µl of
122 10X reaction buffer, 16 µl of distilled water, 1.5 µl of dNTP (2.5mM), 1.5 µl of MgCl2 (25

123 mM), 1 µl of each primer (10 µM), 0.5 µl of Taq polymerase (Supreme NZYTaq DNA
124 polymerase, NZYTECH) and 1.0 µl of template DNA (ca. 50 ng). The amplification cycle
125 consisted of an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of 20 s at
126 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
127 at 72 °C was performed. PCR products were purified using NZYgel pure (NZYTECH)
128 following the manufacturer's instructions. Amplicons were separated by electrophoresis on
129 1.5% agarose gels, stained with GreenSafe (NZTECH) and visualized under UV light to
130 confirm the expected size and absence of non specific amplification. The purified amplicons
131 were sequenced using the kit ABI PRISM BigDye Terminator v3.1 and an automatic
132 sequencer (Genetic Analyzer 3130xl Applied Biosystems). All singletons were confirmed by
133 repeating the PCR and sequencing analyses.
134
135 **2.3 Data analysis**
136 Chromatograms of the nucleotide sequences were analyzed using Bioedit version 7.0.9.0
137 (Hall 1999). After editing, multiple sequence alignments were performed using ClustalW
138 (Thompson et al. 1994). Dnasp (version 5.00.03; Rozas et al. 2003) was used to calculate the
139 number of variable sites, number of haplotypes, mean number of nucleotide differences,
140 haplotype diversity and nucleotide diversity. In order to analyze the demographic history of
141 *C. angulata*, Tajima's D (Tajima 1989), Fu's F (Fu 1997) and R_2 (Ramos-Onsins and Rozas
142 2002) were also calculated using Dnasp to test constant population size versus population
143 growth. Negative Tajima's D and Fu's F values are expected under a scenario of recent
144 population expansion but can also be a signature of selection (non-neutral evolution). Low R_2
145 values are expected after a recent severe population growth event (Ramos-Onsins and Rozas
146 2002). The statistical significance of Tajima's D, Fu's F and R_2 values was calculated with
147 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.

173 Haplotype H6 was common to all sites and had the highest overall frequency (16.9%). It was
174 the most frequent haplotype observed in the Shantou site (33%, Figure 1). The analysis of a
175 Median-joining network constructed using all the haplotypes identified in the study showed
176 that haplotype H6 had a central position and hence is likely to be an ancestral haplotype
177 (Figure 2). Ten out of 11 haplotypes observed in the Shantou site were only observed in this
178 site (i.e. private haplotypes). Among the 60 haplotypes detected, only four were shared
179 between the NE Altantic sites (Mira, Sado and Tahaddart) and Asian sites (Shantou and
180 Keelung).
181 The frequency of unique haplotypes observed in each site ranged from 19.2 to 64.3%.
182 Haplotype diversity ranged from 0.832 to 0.960 and nucleotide diversity from 0.00594 to
183 0.00791 (Table 1). Significant pairwise genetic differentiation (Fst estimates between 0.102
184 and 0.228) after Bonferroni correction was observed between all NE Atlantic sites and the
185 two Asian sites (Table 2). However, no significant Fst estimates (0.003 - 0.072) were
186 observed among the NE Atlantic sites. A significant Fst value of 0.151 was estimated
187 between Shantou and Keelung (Table 2). Significant negative Tajima's D values were
188 observed in Shantou and Keelung sites (Table 3) and significant Fu's F negative values were
189 observed in Mira and Keelung sites. Moreover, significant R_2 values were observed in all
190 sites (Table 3).
191

192 4 Discussion

193 The analysis of the nucleotide sequence of a highly polymorphic non-coding mitochondrial
194 region of *C. angulata* samples collected in Europe, Africa and Asia provided new insight into
195 the introduction of this taxon from Asia into the NE Atlantic region. We observed high
196 genetic diversity in *C. angulata* from the NE Atlantic region and genetic differentiation
197 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

248 (Shafee 1985 cited in Fabioux et al. 2002). In contrast to *C. gigas* in Europe, which showed a
249 typical star-shaped haplotype phylodiversity characteristic of expanding populations
250 (Moehler et al. 2011; Lallias et al. 2015), the haplotype network of *C. angulata* did not show
251 such a pronounced star-shape. Significant negative Tajima's D values were only found for *C.*
252 *angulata* samples from Asia, although Fu's F tests were significant for Mira and Keelung
253 samples. Significant R_2 values were observed for all sites. For small samples the R_2 test is
254 more powerful than Tajima's D and Fu's F tests for detecting population growth (Ramos-
255 Onsins and Rozas 2002). Thus, our results suggested that a demographic expansion event
256 occurred recently in *C. angulata*. This is in agreement with the results of Hsiao et al. (2016)
257 who suggested that *C. angulata* in Asia experienced a sudden population expansion after the
258 last glacial maxima. However, the results from Tajima's D, Fu's F and R_2 tests obtained in
259 the present study and in other studies have to be considered cautiously since the coalescent
260 model used may be inappropriate for highly fecund species such as *C. angulata* (Steinrucken
261 et al. 2013).
262

263 ## 5 Conclusions

264 We observed higher nucleotide diversity for *C. angulata* in NE Atlantic sites than in the
265 studied Asian sites. Moreover, significant genetic differentiation was detected between *C.*
266 *angulata* from three NE Atlantic and two Asian sites. Our results suggested that *C. angulata*
267 might have been introduced into the NE Atlantic through multiple introductory events.
268 However, further analyses in Asia are needed to identify the source(s) of the introduction(s)
269 since none of the Asian sites studied seemed to have been the origin. The NE Atlantic *C.*
270 *angulata* stocks appear to be unique genetic resources, which highlights the importance of
271 their conservation.
272

273 *Acknowledgments.* This work was funded by the Marinaqua project (03-01-FPE-0013), the
274 European Union Atlantic Area Transnational Programme (2007–2013) project SEAFARE
275 (Grant agreement No. 2009–1/123) and the ShareBiotech consortium within the ShareBiotech
276 project co-financed with the support of the ERDF-Atlantic Area programme (priority 1
277 INTERREG IVB). However, it does not reflect the European Union's, INTERREG IV B
278 Atlantic Area programmes or local Governments views and in no way anticipates their future
279 policy in this area. F.M. Batista was funded by FCT (SFRH/BPD/108591/2015). We would
280 like to thanks Professor Ziniu Yu for providing samples from Shantou (China). We also
281 would like to thank two anonymous reviewers for their constructive and helpful comments.

282

283 ## References

284 Aranishi F., Okimoto T., 2005, Sequence polymorphism in a novel noncoding region of
285 Pacific oyster mitochondrial DNA. J. Appl. Genet. 46, 201–206.
286 Bandelt H., Forster P., Röhl A., 1999, Median-joining networks for inferring intraspecific
287 phylogenies. Mol. Biol. Evol. 16, 37–48.
288 Batista F.M., Leitão A., Fonseca V. G., Ben-Hamadou R., Ruano F., Henriques M.A.,
289 Guedes-Pinto H., Boudry P., 2007, Individual relationship between aneuploidy of gill
290 cells and growth rate in the cupped oysters *Crassostrea angulata*, *C. gigas* and their
291 reciprocal hybrids. J. Exp. Mar. Biol. Ecol. 352, 226-233.
292 Batista F.M., Leitão A., Huvet A., Lapègue S., Heurtebise S., Boudry P., 2009, The
293 taxonomic status and origin of the Portuguese oyster *Crassostrea angulata* (Lamark,
294 1819). Special Edition of the Proceedings of the 1st & 2nd International Oyster
295 Symposiums 24, pp. 5–13.
296 Boudry P., Heurtebise S., Collet B., Cornette F., Gérard. A., 1998, Differentiation between
297 populations of the Portuguese oyster, *Crassostrea angulata* (Lamark) and the Pacific

298 oyster, *Crassostrea gigas* (Thunberg) revealed by mtDNA RFLP analysis. J. Exp. Mar.
299 Biol. Ecol. 226, 279-291.
300 Boxer C.R., 1969, The Portuguese seaborne empire, 1415-1825, [1st American ed.], A. A.
301 Knopf, New York.pp.426
302 Buroker N.E., Hershberger W.K., Chew K. K., 1979, Population genetics of the family
303 Ostreidae. I. Intraspecific studies of *Crassostrea gigas* and *Saccostrea commercialis*.
304 Mar. Biol. 54, 157-169.
305 Comps M., 1988, Epizootic diseases of oysters associated with viral infections. In: Fisher,
306 W.S. (Ed.), Disease processes in marine bivalve molluscs. American Fisheries Society
307 Special Publication Bethesda, MD 8, pp. 23–37.
308 Cross I., Rebordinos L., Diaz E., 2006, Species identification of *Crassostrea* and *Ostrea*
309 oysters by polymerase chain reaction amplification of the 5S rRNA gene. J. AOAC Int.
310 89, 144-148.
311 EFSA, 2010, EFSA Panel on Animal Health and Welfare (AHAW); scientific opinion on the
312 increased mortality events in Pacific oyster (*Crassostrea gigas*). EFSA J. 8, 1894.
313 Excoffier L., Laval G., Schneider S., 2005, Arlequin (version 3.0): an integrated software
314 package for population genetics data analysis. Evol. Bioinform. 1, 47–50.
315 Hsiao S.-T., Chuang S.-C., Chen K.-S., Ho P.-H., Wu C.-L., Chen C. A., 2016, DNA
316 barcoding reveals that the common cupped oyster in Taiwan is the Portuguese oyster
317 *Crassostrea angulata* (Ostreoida; Ostreidae), not *C. gigas*. Scientific Reports, 6: 34057.
318 Fabioux C., Huvet A., Lapègue S., Heurtebise S., Boudry P., 2002, Past and present
319 geographical distribution of populations of Portuguese (*Crassostrea angulata*) and
320 Pacific (*C. gigas*) oysters along the European and north African Atlantic coasts. Haliotis
321 31, 33-44.

322 Fu Y. X., 1997, Statistical tests of neutrality of mutations against population growth,
323 hitchhiking and background selection. Genetics 147, 915–925.
324 Gutiérrez-Zugasti I., Andersen S. H., Araújo A.C., Dupont C., Milner N., Monge-Soares
325 A.M., 2011, Shell midden research in Atlantic Europe: State of the art, research problems
326 and perspectives for the future. Quaternary International 239, 70e85.
327 Hall T.A., 1999, BioEdit: a user-friendly biological sequence alignment editor and analysis
328 program for Windows 95/98/NT. Nucleic Acids Symp. Ser. 41, 95–98.
329 Huvet A., Lapègue S., Magoulas A., Boudry P., 2000, Mitochondrial and nuclear DNA
330 phylogeography of *Crassostrea angulata*, the Portuguese oyster endangered in Europe.
331 Conserv. Gen. 1, 251-262.
332 Huvet A., Gérard A., Ledu C., Phélipot P., Heurtebise S., Boudry P., 2002, Is fertility of
333 hybrids enough to conclude that the oysters *Crassostrea gigas* and *Crassostrea angulata*
334 are the same species? Aquat. Living Resour. 15, 45-52.
335 Huvet A., Fabioux C., McCombie H., Lapègue S., Boudry P., 2004, Natural hybridization
336 between genetically differential populations of *Crassostrea gigas* and *C. angulata*
337 highlighted by sequence variation in flanking regions of a microsatellite locus. Mar.
338 Ecol. Prog. Ser. 272:141-152.
339 Imai T., Sakai S., 1961, Study of breeding of Japanese oyster, *Crassostrea gigas*. Tohoku J.
340 Agric. Res. 12, 125-171.
341 Lallias D., Boudry P., Batista F.M., Beaumont A., King J.W., Turner J.R., Lapègue S., 2015,
342 Invasion genetics of the Pacific oyster *Crassostrea gigas* in the British Isles inferred
343 from microsatellite and mitochondrial markers. Biol. Invasions 17(9), 2581-2595.
344 Lapègue S., Batista F.M., Heurtebise S., Yu Z., Boudry P., 2004, Evidence for the presence
345 of the Portuguese oyster, *Crassostrea angulata*, in Northern China. J. Shellfish Res. 23,
346 759-763.

347 López-Flores I., Hérran R., Garrido-Ramos M.A., Boudry P., Ruiz-Rejón C., Ruiz-Rejón M.,
348 2004, The molecular phylogeny of oysters based on a satellite DNA related to
349 transposons. Gene 339, 181-188.
350 Mathers, N.F., Wilkins N.P., Walne P.R., 1974, Phosphoglucose isomerase and esterase
351 phenotypes in *Crassostrea angulata* and *C. gigas*. Biochem. System. Ecol. 2:93-96.
352 Menzel R.W., 1974, Portuguese and Japanese oysters are the same species. J. Fisheries Res.
353 Board Canada 31: 453-456.
354 Moehler J., Wegner K.M., Reise K., Jacobsen S., 2011, Invasion genetics of Pacific oyster
355 *Crassostrea gigas* shaped by aquaculture stocking practices. J. Sea Res. 66, 256–262.
356 O'Foighil D., Gaffney P.M., Wilbur A.E., Hilbish T.J., 1998, Mitochondrial cytochrome
357 oxidase I gene sequences support an Asian origin for the Portuguese oyster *Crassostrea*
358 *angulata*. Mar. Biol. 131, 497-503.
359 Qin J., Huang Z., Chen J., Zou Q., You W., Ke C., 2012, Sequencing and de novo analysis of
360 *Crassostrea angulata* (Fujian Oyster) from 8 different developing phases using 454
361 GSFlx. PLoS One 7 (8), e43653.
362 Ramos-Onsins S.E., Rozas J., 2002, Statistical properties of new neutrality tests against
363 population growth. Mol. Biol. Evol. 19, 2092–2100.
364 Ranson G., 1960, Les prodissoconques (coquilles larvaires) des ostréides vivants. Bull. Inst.
365 Océanogr. Monaco 1, 1-41.
366 Ren J., Liu X., Jiang F., Guo X., Liu B., 2010, Unusual conservation of mitochondrial gene
367 order in *Crassostrea* oysters: evidence for recent speciation in Asia. BMC Evol. Biol. 10,
368 394.
369 Ren J., Hou Z., Wang H., Sun M.A., Liu X., Liu B., Guo, X., 2016, Intraspecific variation in
370 mitogenomes of five *Crassostrea* species provides insight into oyster diversification and
371 speciation. Mar. Biotechnol. 18, 242-254.

372 Rozas J., Sanchez-DelBarrio J.C., Messeguer X., Rozas R., 2003, DnaSP, DNA
373 polymorphism analyses by the coalescent and other methods. Bioinformatics 19, 2496–
374 2497.
375 Seebens H., Gastner M., Blasius B., 2013, The risk of marine bioinvasion caused by global
376 shipping. Ecology Letters 16(6), 782-790.
377 Sekino M., Yamashita H., 2013, Mitochondrial DNA barcoding for Okinawan oysters: a
378 cryptic population of the Portuguese oyster *Crassostrea angulata* in Japanese waters.
379 Fish. Sci. 79, 61–76.
380 Simberloff D., Martin J.-L., Genovesi P., Maris V., Wardle D.A., Aronson J., Courchamp F.,
381 Galil B., García-Berthou E., Pascal M., Pyšek P., Sousa R., Tabacchi E., Vila M., 2013,
382 Impacts of biological invasions: what's what and the way forward. Trends Ecol. Evol.
383 28, 58–66.
384 Steinrücken M., Birkner M., Blath J., 2013, Analysis of DNA sequence variation within
385 marine species using Beta-coalescents. Theor. Popul. Biol. 87, 15-24
386 Tajima F., 1989, Statistical method for testing the neutral mutation hypothesis by DNA
387 polymorphism. Genetics 123, 585–595.
388 Thompson J.D., Higgins D.G., Gibson T.J., 1994, CLUSTALW: improving the sensitivity of
389 progressive multiple sequence alignment through sequence weighting, position specific
390 gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.
391 Wang H., Qian L., Liu X., Zhang G., Guo X., 2010, Classification of a common cupped
392 oyster from southern China. J. Shellfish Res. 29, 857–866.
393 Xia J., Yu Z., Kong X., 2009, Identification of seven *Crassostrea* oysters from the South
394 China Sea using PCR-RFLP analysis. J. Mollusc. Stud. 75, 139-146.
395
396

397 **Table 1.** Genetic diversity of *C. angulata* samples estimated from comparisons of sequences
398 of the major noncoding region (position 10617-11217 bp of EU672832) of mitochondrial
399 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)

400 n, number of individuals analyzed; S, number of variable sites; h, number of haplotypes; k,
401 mean number of nucleotide differences; Hd, haplotype diversity (standard deviation); π,
402 nucleotide diversity (standard deviation).
403
404 **Table 2.** Pairwise genetic differentiation (Fst) for *C. angulata* samples collected in North
405 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**	-

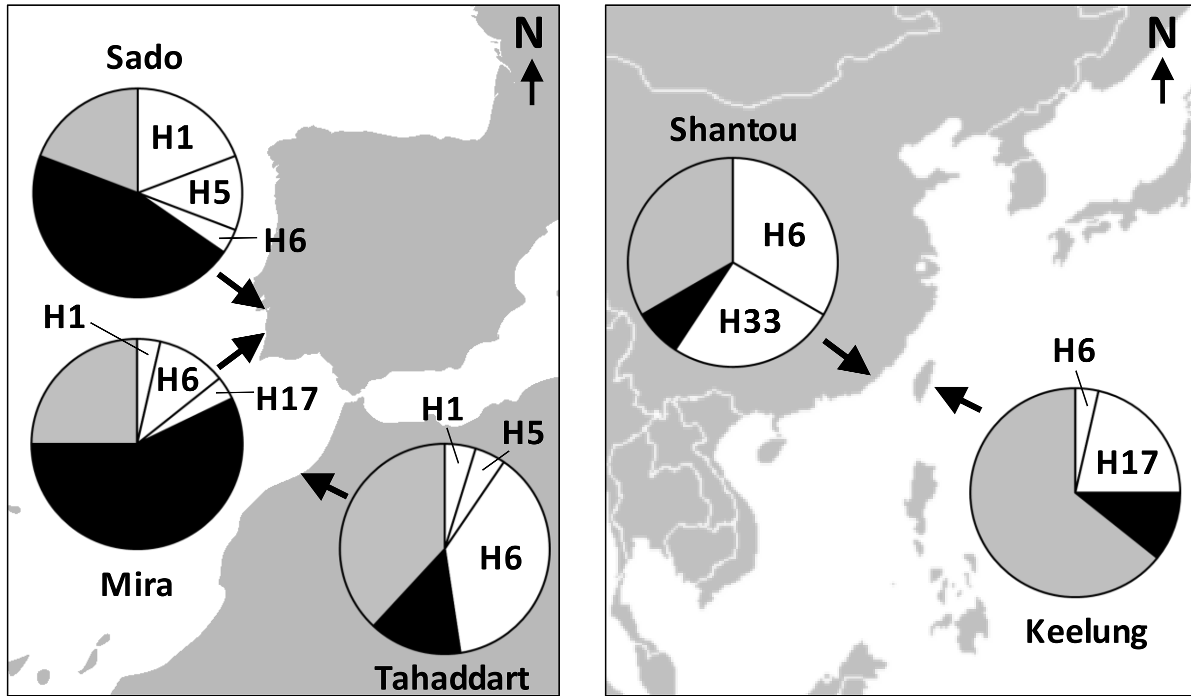
406 Values in bold are significant after Bonferroni correction ($p<0.05$)
407
408

409 **Table 3.** Neutrality and demographic tests for *C. angulata* samples estimated from sequences
410 of the major noncoding region (position 10617-11217 bp of EU672832) of mitochondrial
411 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**

412 R_2, Ramos-Onsins and Rozas statistic. Values in bold are significantly different ($p<0.05$)
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428

429



430

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

435

436

437

438

439

440

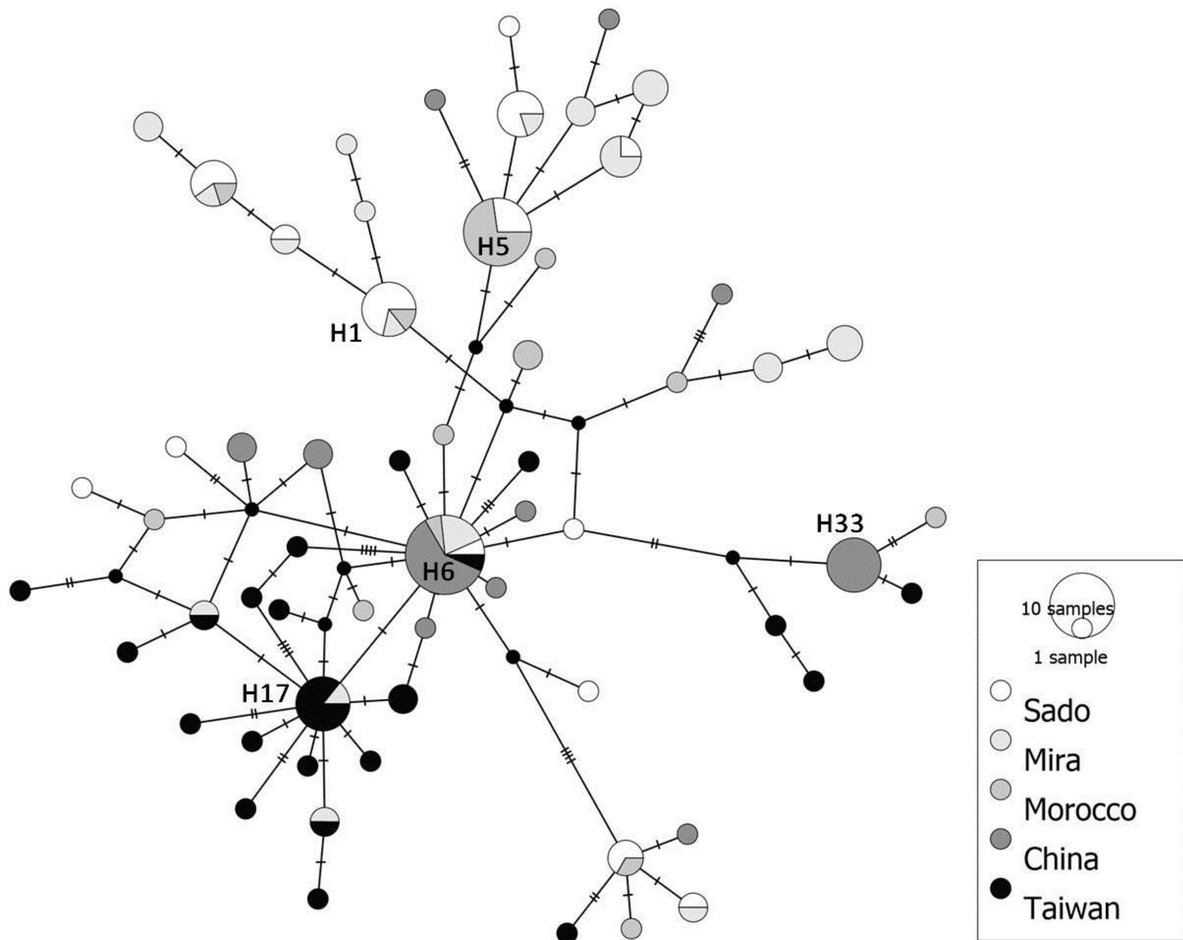
441

442

443

444

445



446

447

448 **Fig. 2.** Median joining network of *C. angulata* DNA haplotypes of a fragment (position
449 10617-11217 bp of EU672832) of the major noncoding region of the mitochondrial genome.
450 The size of circles corresponds to haplotype frequency and the different colours represent the
451 geographical sites where the haplotypes were observed. The number of perpendicular bars on
452 branches represents the number of mutational steps (indels and/or substitutions) between
453 haplotypes.