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Mitochondrial cytochrome oxidase I gene sequences support an Asian origin for the Portuguese oyster *Crassostrea angulata*

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Abstract The Portuguese oyster *Crassostrea angulata* (Lamarck, 1819) was long assumed to be native to the northeastern Atlantic, however, a number of lines of evidence now indicate that it is a close relative, or identical, to the Asian Pacific oyster *C. gigas* (Thunberg, 1793). Three hypotheses have been proposed to explain how this strikingly disjunct geographic distribution may have come about: ancient vicariance events, recent anthropogenic introduction to Asia and recent anthropogenic introduction to Europe. We have performed a molecular phylogenetic analysis of *C. angulata* based on mitochondrial DNA sequence data for a 579-nucleotide fragment of cytochrome oxidase I. Our results show that Portuguese oyster haplotypes cluster robustly within a clade of Asian congeners and are closely related, but not identical, to *C. gigas* from Japan. The mitochondrial data are the first to show that Portuguese oysters are genetically distinct from geographically representative samples of Japanese Pacific oysters. Our phylogenetic analyses are consistent with a recent introduction of *C. angulata* to Europe either from a non-Japanese Asian source population or from a subsequently displaced Japanese source population. Genetic characterization of Pacific oysters throughout their Asian range is necessary to fully reveal the phylogenetic relationships among Portuguese and Pacific oysters.

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Introduction

It has become abundantly clear over the past decade that human-mediated transoceanic exchange on a massive scale is presently ongoing among global nearshore faunas (Carlton 1985, 1987, 1989; Hallegraeff and Bloch 1992; Carlton and Geller 1993; Paine 1993; Cohen et al. 1995; Geller 1996). The present-day distribution of some geographically extensive marine taxa may have been achieved by undocumented anthropogenic transfer, rather than by incremental spontaneous dispersal events over evolutionarily significant time frames (Carlton 1989). The scope of this process may be seriously underestimated where human-mediated introductions predate biological surveys (Carlton 1989) and where the species involved are inconspicuous or are difficult to distinguish using morphological characters (Geller 1996).

The Portuguese oyster *Crassostrea angulata* (Lamarck, 1819) represents a putative case of undocumented introduction which may have occurred soon after the genesis of global shipping routes (Ranson 1960; Menzel 1974; Edwards 1976; Buroker et al. 1979). This species was long assumed to be native to the northeastern Atlantic, however, Ranson (1948, 1960, 1967), on the basis of larval shell morphology, claimed that the Portuguese oyster is a geographically isolated population of the Asian Pacific oyster *C. gigas*. Subsequent investigators have compiled an impressive list of similarities between these two geographically distant taxa that support Ranson's proposal. These congeners are indistinguishable in terms of enzyme polymorphisms (Mathers et al. 1974; Buroker et al. 1979; Mattiucci and Villani 1983) and karyotype (Thiriot-Quiévreux 1984). Reciprocal crosses exhibit normal or near-normal rates of fertilization and development (Imai and Sakai 1961; Menzel 1974), and Walne and Helm (1979) noted normal growth and viability in F_1 and F_2 hybrids. Reports of successful hybridization in bivalves must be viewed with some caution, however, as hatchery contamination is commonplace (Gaffney and Allen 1993).

There is also evidence supporting the distinctiveness of Portuguese and Pacific oysters. Hèral and Deslous-Paoli (1991), on the basis of physiological reproductive characteristics, have contended that Pacific and Portuguese oysters are two separate species. Some ultrastructural studies of spermatogenesis in *Crassostrea gigas* (Brandiff et al. 1978; Komaru et al. 1994), and in *C. angulata* (Gutíerrez et al. 1978), have reported a difference in sperm acrosomal morphology, although this is not supported by Sousa and Oliveira (1994). Minor differences have also been reported for adductor muscle protein patterns (Moré et al. 1971) and for antigen profiles (Numachi 1962), but it is not clear if these distinctions are genetic in origin.

Available evidence strongly suggests that Portuguese and Pacific oysters are at least sister taxa, if not the same species. Three hypotheses have been proposed to explain how this strikingly disjunct geographic distribution may have come about. Stenzel (1971) and Lawrence (1995) argue that Pacific and Portuguese oysters are both descended from a Miocene fossil taxon, *Crassostrea gryphoides*, prevalent in the Eurasian Tethyan Seaway. They claim that Portuguese and Japanese oyster populations have been separated since before closure of the Seaway and that their demonstrated similarities reflect the conservative nature of their evolution rather than recent common ancestry. The other two hypotheses both assume that undocumented human-mediated introduction events have occurred, but they differ in the polarity of the proposed transfer events. Menzel (1974) argued that the ancestral stock was originally restricted to the Atlantic and was introduced to Japan during the sixteenth or seventeenth centuries by Portuguese traders. The majority view, however, is that *C. angulata* are descendants of Pacific oysters first brought to Europe by traders (Ranson 1960; Edwards 1976; Buroker et al. 1979) and that prehistoric oyster mounds in Japan demonstrate its antiquity there (Korringa 1976). More recently, the application of molecular phylogenetic methods has clearly established the Asian affinities of the Pacific oyster (Banks et al. 1993; Littlewood 1994; Ó Foighil et al. 1995).

We present here a phylogenetic analysis of *Crassostrea angulata* based on mitochondrial DNA sequence data. Our aim was to test the putative identity of *C. angulata* as a historically recent isolate of Japanese *C. gigas* by incorporating samples from the four historically recognized geographic races (Hokkaido, Miyagi, Hiroshima and Kyushu) of the Pacific oyster in Japan (Imai and Sakai 1961; Ahmed 1975; Quayle 1988). Our results show that the Portuguese oyster clusters within a clade of Asian congeners and is closely related, but not identical, to present day *C. gigas* from Japan. The ancestral Asian population that gave rise to *C. angulata* may have been displaced within Japan, or may be located in another part of Asia, most likely Taiwan (Boudry et al. 1998; J-H Cheng, Tungkang, Taiwan, personal communication).

Materials and methods

Ethanol-fixed samples of the Portuguese oyster, and of three Asian congeners, were fowarded by colleagues from wild populations and from hatchery broodstocks. *Crassostrea angulata* (Lamarck, 1819) were obtained from natural Sado estuary populations, in the south of Portugal, in August 1994. Samples of *C. gigas* (Thunberg, 1793) from each of the four historic stocks of this species in Japan were procured, two from wild populations (Hokkaido, Kyushu) and two from research hatchery broodstock (Hiroshima, Miyagi). Hokkaido *C. gigas* were sampled in June 1994 from the rocky intertidal at Kakijima, Atsukeshi on the southeast coast of Hokkaido. Kyushu *C. gigas* specimens were collected in the summer of 1992 from a wild population in Fukiage-cho, a fishing village on the west coast of Kyushu remote from oyster aquacultural locations. Hiroshima *C. gigas* broodstock, were obtained from the Haskin Shellfish Research in New Jersey. They were F_2 descendants of wild spat collected in Hiroshima Bay in 1988 and transferred to the Haskin Laboratory in 1990 (S. K. Allen, personal communication). Miyagi *C. gigas* broodstock, originally from Washington State, were also obtained from the Haskin Laboratory as were samples of *C. ariakensis* (Fujita, 1913). The *C. sikamea* (Amemiya, 1928) samples were progeny of commercial stock maintained in Tomales Bay (California) and in Yaquina Bay (Oregon) and confirmed as *C. sikamea* using diagnostic PCR/RFLP characterization of nuclear ITS1 ribosomal gene fragments (Gaffney and Wilbur, unpublished). Tissue samples from two outgroup oyster taxa were sampled from wild populations: *Crassostrea virginica* (Gmelin, 1791) in Delaware Bay in October 1996; and the flat oyster *Ostrea chilensis* (Philippi, 1845) from the shallow sublittoral at Moturekareka Island, Hauraki Gulf, New Zealand in November 1995.

DNA templates for thermal cycle amplification were prepared from mantle tissues of individual oysters as detailed by Ó Foighil et al. (1995). A 659-nucleotide (nt) portion of oyster cytochrome oxidase I (COI) was amplified for five specimens each of the ingroup samples of *Crassostrea angulata* and of the Asian congeners (total of 20 individuals from the four Japanese *C. gigas* samples) and for one specimen each of the two outgroup taxa using the Folmer et al. (1994) primer set (5′-GGTCAACAAATCATAAAGATATTGG-3′; 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′). A negative control (no template) was included in each run of 35 cycles of amplification (1 min 94 °C denaturing, 30 s 45 °C annealing, 1 min 72 °C extension). Double-stranded products were isolated on 1% agarose gels, excised under long-wavelength UV light (312 nm), and extracted using a GeneClean (Bio 101) NaI/glass powder kit. For most samples, both strands of the amplified fragments were directly cycle-sequenced using the original amplification primers and electrophoresed on ABI-automated DNA sequencers at either the University of Delaware or the University of Michigan. The Hokkaido *C. gigas* samples and the *Ostrea chilensis* specimen were manually sequenced at the University of South Carolina. Due to the shorter reads generated for the manual sequences, and to the excision from analysis of 35 nt of the 3′ end of

▶

Fig. 1 *Crassostrea* spp., *Ostrea chilensis*. Alignment of nine oyster COI gene fragment genotypes (579 nucleotides) obtained in the present study with that of a homologous 588-nucleotide portion of the chiton *Katherina tunicata* COI [positions 67–654 of the chiton mitochondrial genome (Boore and Brown 1994)]. GIGAS, ANGUL, SIKAMEA, ARIAKEN, VIRGIN and CHILENS, respectively, indicate genotypes encountered in *Crassostrea gigas*, *C. angulata*, *C. sikamea*, *C. ariakensis*, *C. virginica* and *Ostrea chilensis* samples. *Dots* indicate nucleotide identity to the first sequence presented, *C. gigas*, and inferred changes relative to *C. gigas* are shown. *Dashes* indicate inferred nucleotide insertions/deletions relative to the chiton (KATHERIN). The number of individuals encountered per haplotype for each population sample is indicated in parentheses at the end of each sequence

the fragment to remove a small number of ambiguous positions, our final data set consisted of 579 homologous nucleotides of COI for all of the study taxa.

Translations to inferred amino acid sequences were performed using the *Drosophila yakuba* mitochondrial genetic code (Clary and Wolstenholme 1985). Once aligned, sequences were analyzed using a maximum parsimony (PAUP 3.1) approach (Swofford 1993) with *Ostrea chilensis* designated as an outgroup. Branch support levels were estimated using Bremer support values (Bremer 1995), calculated using Treerot (Sorenson 1996), and also by bootstrapping.

Results

Figure 1 shows the genotypic variation encountered among the study taxa. A total of nine haplotypes were detected among the 37 oyster specimens sequenced for 579 homologous nucleotide positions of COI. Four of these haplotypes occurred in the Portuguese *Crassostrea angulata* sample. Samples of Asian congeners exhibited

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                                                                                                          100
GIGAS    GCTGTTCTTG CGGGAACTAG GTTTAGGTCT CTTATTCGTT GGAGACTTTA TAACCCTGGA GCTAAGTTTT TAGACCCCGT GACTTATAAT GCAGTTGTAA
ANGUL1   .......... .......... .......... .......... ....G..... ......C... .......... .......... .......... ..........
ANGUL2   .......... .......... .......... .......... ....G..... ......C... .......... .......... .......... ..........
ANGUL3   .......... .......... .......... .......... ....G..... ......C... .......... .......... .......... ..........
ANGUL4   .......... .......... .......... .......... ....G..... ......C... .......... .......... .......... ..........
SIKAMEA  A......... .......... .......... .......... ....G..A.. C........G ..C....... ....T..A.. .......... ..........
ARIAKEN  ..C....... TA........ ...C..A... .......... .A..TT.G.. ..C...A..G .......... .......T.. A..C.....C ..........
VIRGIN   ..A...T.A. .T..G..C.. T......... .........C. .A..T..... ..CT.....G .....A.... ....G..T.. TGTG.....C ..T..G....
CHILENS   ......T.G. .T........ .C....A... T.A....... .A...T.G.T .......... .....A.... ....T..T.. ATG...C... ..C..A..T.
KATHERIN ....GGT.A. TA..G...GC T..A..A.TA ..A......G CAGAG..AGG .C.A..A..G ...TTA..GG GG..TGA.CA ACTG...... .TTA....C.

                                                                                                          200
GIGAS    CTAGGCATGC GTTGGTTATG ATTTTTTTCT TTGTTATACC TGTAATAATT GGGGGGTTTG GTAACTGGCT TATCCCTTTG ATGCTTCTAG TAGCAGACAT
ANGUL1   .......... .......... .......... .......... C..G...... .......... .......... .......... .......A.. ..........
ANGUL2   .......... .......... .......... .......... C..G...... .......... .......... .......... .......A.. ..........
ANGUL3   .......... .......... .......... .......... C..G...... .......... .......... .......... .......A.. ..........
ANGUL4   .......... .......... .......... .......... C..G...... .......... .......... .......... .......A.. ..........
SIKAMEA  .......... A......... .......... .......G.. A..G...... .......... .......... .........A .......... .......T..
ARIAKEN  .C..A..... A..A.....A ..C.....T. .......... G.......C. .......... .A..T..... ......A..A .......A.. .......T..
VIRGIN   .G..T..C.. ...A..A... .....C.... .......G.. A......... ..T....... ....T..... ...T..GC.T ..A...GA.. .G........
CHILENS  .A.T...... C..A..G... .....C..T. ....A..... ...T...... ..T....... ....T..A.. AG.G..G..A .....C.A.. .GC.T..T..
KATHERIN .AGCT..... T..T.....A ........T. .G.....G.. .A........ .......... .......... AG.G.....A ...T.AGGG. .GC.G.....

                                                                                                          300
GIGAS    GCAATTTCCT CGATTAAATG CATTTAGATT TTGAGTTTTG CCAGGGTCTC TTTATCTTAT GCTTATGTCT AACATTGTAG AAAACGGAGT TGGGGCAGGG
ANGUL1   .......... .......... .......... .......... .......... .......... .......... .....C.... .......... ..........
ANGUL2   .......... .......... .......... .......... .......... .......... .......... .....C.... .......... ..........
ANGUL3   .......... .......... .......... .......... .......... .......... .......... .....C.... .......... ..........
ANGUL4   .......... .......... .......... .......... .......... .......... .......... .....C.... .......... ..........
SIKAMEA  A.....C... ..G.....C. .......... ......C... ........A. .......... .......... ..T.....G. ....G..... ..........
ARIAKEN  ...G...... ..G....... .......... C..G...... .....C..A. .......... .......... ..TC...... ...GT..G.. C........A
VIRGIN   ...G..C... .......... .T........ C.....A... ........AT .GCTG..A.. AT.G.....A ..T..GTCT. .G.GA..... ....T....A
CHILENS  ...G...... ...A.G.... .T..C..G.. C..G..A..A ..G.TA..CT .A..CT.... AG.AG.T... GCAT....T. .G..T..T.. ....A....C
KATHERIN .GCT..C..C ..G......A ATA.A..T.. ....C..... ..TCC.G.AT .A.G....T. .T.AGCT..A GGGGCG..T. .G.GG..G.C ....A.T...

                                                                                                          400
                                                                                                          400
GIGAS    TGAACAATTT ACCCTCCTTT ATCAACTTAC TCTTATCAT- --GGAGTTTG TATAGACCTT GCAATTCTAA GCCTTCACCT TGCTGGTATT AGCTCTATTT
ANGUL1   ..G....... .......... .......... .........- --........ C..G...... ......T... .......... ...C...... ..........
ANGUL2   .......... .......... ......C... .........- --........ C......... ......T... .......... ...C...... ..........
ANGUL3   ..G....... .......... .......... .........- --........ C......... ......T... .......... ...C...... ..........
ANGUL4   .......... .......... .......... .........- --........ C......... ......T... .......... ...C...... ..........
SIKAMEA  .......... .......... G..G...... .........- --........ C..G...... ......T... .TT.A..... A......... ..........
ARIAKEN  .......... .......... .......... .........- --..G..C.. C......... .......... .......TT. A.....A... ..G.......
VIRGIN   .....C.... .......GC. G.......TT .....C...- --........ C..G..TT.. ..C...T... .GT.A...T. A..A...... ..G.......
CHILENS  .....TG.C. .......AC. ........TT ..A..C..C- --..GA.G.. C..G..T..G ......T... .AT.G..... A......... .....A....
KATHERIN .....TG.G. .T........ GG.GGGGA.T GTGGGG...G CT..T.GA.C .G.T...T.A ..T...T.TT CTT.A..TT. A.....AG.A TCG.......

                                                                                                          500
GIGAS    TCAGGTCAAT TAATTTCATA GTAACGATTA GAAATATGCG ATCTGTTGGG GGCCATTTA- -----CTAGC ACTATTCCCT TGATCTATTA AGGTTACTTC
ANGUL1   .......... ......T... .......... .......... .......... .........- -----..... .......... .......... ..........
ANGUL2   .......... ......T... .......... .......... .......... .........- -----..... ......T... .......... ..........
ANGUL3   .......... ......T... .......... .......... .......... .........- -----..... .......... .......... ..........
ANGUL4   .......... ......T... .......... .......... .......... .........- -----..... .......... .......... ..........
SIKAMEA  .......... ......T... .....A.... .......A.. .......... .........- -----T.G.. G..G..T..C .......... ..........
ARIAKEN  .T........ .......... .....T.... .......... ......C..T ..G.....G- -----T.G.. G.....T..A ..G.....C. .A..C..A..
VIRGIN   .......T.. ......T... .....C.... ....C..... .......... ..T..CA..- -----T.... ...T...... ..G..A.... .A..G..A..
CHILENS  .......... ......T... ..T....... C......A.. .G.A....AC ..T......- -----..G.. G..G..T... .....C.... .A.....G..
KATHERIN .AG..G.TG. ......T..T ACT..A...G T......A.. .AGA.AA... ATA..A...G AGCGAT..C. TT.G..TGT. ..G...G.A. .AA.....G.

                                                                                               588
GIGAS    ATTCTTGCTT TTGACTACTC TCCCAGTGTT AGCTGGAGGT CTTACTATAC TTTTGACTGA TCGTCATTTT AATACCTCTT TTTTTGAC (20)
ANGUL1   .......... .......... .......... .......... ........G. .......... .......... .......... ....C...  (1)
ANGUL2   .......... .......... .......... .......... ........G. .......... .......... .......... ....C...  (1)
ANGUL3   .......... .......... .......... .......... ........G. .......... .......... .......... ....C...  (2)
ANGUL4   .......... .......... .......... .......... ........G. .......... .......... .......... ....C...  (1)
SIKAMEA  ...TC.A... ..A....... .T........ ......G..C ........G. .......... C......... ..C..G.... .C.....T  (5)
ARIAKEN  ...T..A... ..A..A..C. .T..G..AC. .......... .....C..G. .......... C..G...... ..C..G.... .......T  (5)
VIRGIN   ....C....G C.T..C..G. .A..T..TC. ......C..G ........G. .CC.T..... ...A.....C .....A..G. ..CC..TA  (1)
CHILENS  ...T..AT.G ..A.....A. .T..T..... ......G... T.G..A...T .AC.C..A.. ...G...... .....A..C. .C......  (1)
KATHERIN TA.T...T.A ...CT.T.CT .G..T..T.. .........A A....A...T .G..A..... ....A..... .....T.... .......T
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single species-specific genotypes, including all 20 specimens of the Pacific oyster *C. gigas* obtained from Hokkaido, Miyagi, Hiroshima and Kyushu. Including the outgroup taxa, 217 positions are variable in the oyster COI data set (37, 17, 163, respectively, for 1st, 2nd and 3rd codon positions) and 87 of these are informative under conditions of parsimony. No insertion/deletion events were detected, and Table 1 presents pairwise genetic distances among the nine genotypes. Note that the smallest genetic distances among the oyster taxa are between Pacific and Portuguese oysters.

Two most parsimonious phylogenetic trees (337 steps, CI = 0.8605; RI = 0.6328) were repeatedly obtained by exhaustive PAUP analyses of the oyster COI data set. They differed in one minor detail, the relative placement of two of the four *Crassostrea angulata* haplotypes (ANGUL2, ANGUL4) in the terminal tips of the trees. A strict consensus tree is shown in Fig. 2, and essentially the same topology was also produced by maximum-likelihood analysis. Note that the *C. angulata* genotypes are nested in a robust clade containing the Asian congeners *C. gigas*, *C. sikamea* and *C. ariakensis* and that Pacific and Portuguese oysters form a well-supported sub-clade. *C. angulata* and *C. gigas* are clearly sister taxa, but the characterized haplotypes in these two oysters differ in their COI gene fragment sequences by a minimum of 12 substitutions. Eleven of these steps are due to inferred synonymous transitions and one results from an inferred non-synonymous transversion (position 188, Fig. 1) in which a glutamine is substituted for a leucine. We also sequenced a 444-nt fragment of the mt 16S large ribosomal subunit gene for our samples of these two oyster taxa, and they differed by three additional transitions (data not shown). It is likely that Pacific and Portuguese oysters contain fixed substitutions in many of their mitochondrial genes.

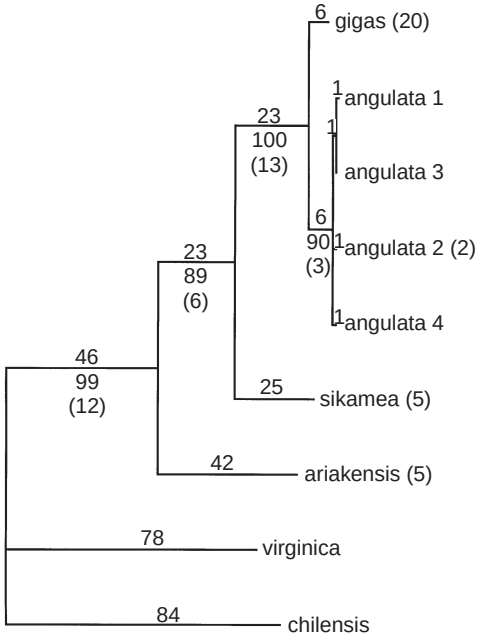

Fig. 2 *Crassostrea* spp., *Ostrea chilensis*. Strict consensus of the two most parsimonious trees (337 steps) obtained by an exhaustive search for optimal trees (PAUP) using the nine characterized oyster genotypes of a 579-nucleotide COI mitochondrial gene fragment (see Fig. 1). *O. chilensis* (chilensis) was employed as an outgroup. The two most parsimonious trees differed only in relative positioning of two of the four terminal *C. angulata* (angulata 2, angulata 4) haplotypes. Respective numbers of steps are indicated above each branch, and the bootstrap values (500 branch and bound iterations) and Bremer support values (in parentheses) supporting each node are presented below the branches. In parentheses after haplotype labels, numbers of individuals sequenced if >1

Discussion

The mitochondrial COI sequence data yield intriguing new insights into the phylogenetic relationships of Pacific and Portuguese oysters. On one level, the molecular results are congruent with data on larval morphology (Ranson 1948, 1960, 1967), allozymes (Mathers et al. 1974; Buroker et al. 1979; Mattiucci and Villani 1983) and mating experiments (Imai and Sakai 1961; Menzel 1974), which indicate close phylogenetic ties between these two taxa. At the same time, the mitochondrial data, together with the complementary results of Boudry et al. (1998), are the first to demonstrate clear genetic distinctions between *Crassostrea angulata* and Japanese populations of *C. gigas*. These results underline the superior resolution of mitochondrial molecular characters in distinguishing closely related oyster lineages (Reeb and Avise 1990). Assessment of their systematic significance will require genetic characterization of *C. gigas* throughout its natural range in Asia.

Our molecular phylogenetic results have relevance to the three competing hypotheses proposed to explain the disjunct geographic distributions of Pacific and Portuguese oysters. The vicariance hypothesis (Stenzel 1971;

Table 1 *Crassostrea* spp., *Ostrea chilensis*. Pairwise number of sequence differences for the study oyster taxa COI gene fragments (579 nucleotides). *Below diagonal* all changes; *above diagonal* mean distances

	1	2	3	4	5	6	7	8	9
1 *C. gigas*		0.024	0.024	0.022	0.021	0.093	0.145	0.214	0.237
2 *C. angulata1*	14		0.007	0.002	0.003	0.092	0.154	0.211	0.242
3 *C. angulata2*	14	4		0.005	0.003	0.092	0.150	0.214	0.242
4 *C. angulata3*	13	1	3		0.002	0.093	0.152	0.212	0.244
5 *C. angulata4*	12	2	2	1		0.092	0.150	0.211	0.242
6 *C. sikamea*	54	53	53	54	53		0.150	0.225	0.240
7 *C. ariakensis*	84	89	87	88	87	87		0.235	0.254
8 *C. virginica*	124	122	124	123	122	130	136		0.266
9 *O. chilensis*	137	140	140	141	140	139	147	154	

Lawrence 1995) assumes that the last common ancestor of these taxa dates from some unknown period prior to final closure of the Eurasian Tethyan Seaway in the Messinian period (late Miocene) approximately 7 million years ago (Robba 1987; Por 1989). The most compelling evidence against this hypothesis is the absence of *C. angulata* from the European Pliocene and Quaternary fossil record (Ranson 1948, 1960; Edwards 1976). Our molecular data may also undermine the vicariance hypothesis by indicating that closure of the Eurasian Tethyan Seaway may significantly predate the last common ancestor of Pacific and Portuguese oysters. The most detailed estimates for fossil-calibrated molluscan mtDNA divergence rates are provided by Collins et al. (1996) for a protein coding gene and by Reid et al. (1996) for a ribosomal gene. Both studies utilized marine gastropod taxa, however, they obtained remarkably distinct estimates of molecular divergence rates for these two very different genes. The Collins et al. (1996) rate (2% per million years per lineage for *Nucella* species cytochrome *b* third-codon transitional differences) is more relevant to the oyster study because it is based on largely synonymous substitutions in a protein-coding gene. Ten transitions (all synonymous) were detected in our pairwise comparisons of 190 third-codon positions for *C. angulata* and *C. gigas* COI. Application of the *Nucella* spp. rate to the oyster data is complicated by pronounced phylogenetic and life-history differences between the snails and the oysters, and undoubtedly yields a very crude estimate of the ages of the oyster lineages. For instance, the *Nucella* spp. rate may significantly overestimate oyster divergence times because there are indications of an unusually high rate of mutation, attributed to the large number of germline cell divisions per generation, in the mitochondrial genomes of cupped oysters (Beckenbach 1994). Although it may well be a significant overestimate, our observed value of 5.26% produces an estimated divergence date of 1 to 2 million years ago for the Portuguese and Pacific oyster sequences, long after closure of the Tethyan Seaway.

Our phylogenetic analyses firmly place both Portuguese and Pacific oysters within an Asian *Crassostrea* clade and are consistent with previous molecular characterization of *C. gigas* and its Asian congeners (Banks et al. 1993, 1994; Littlewood 1994; Ó Foighil et al. 1995). These results establish the Asian affinities of both Portuguese and Pacific oysters; they are incompatible with Menzel's (1974) hypothesis for a European origin for these taxa but are most consistent with the remaining hypothesis of undocumented recent human introduction of *C. angulata* to Europe (Ranson 1960; Edwards 1976; Buroker et al. 1979). A prediction of the latter hypothesis is that Portuguese oysters should share haplotypes in common with Asian source populations because insufficient time (< 500 years) has elapsed for significant levels of new mutations to arise in the recently separated populations. The clear mitochondrial genetic divergence of Portuguese and Pacific oysters does not therefore allow us to identify a source population within Japan for *C. angulata*. The observed genetic distinction between the two closely related oyster taxa may reflect: (1) inadequate sampling of genotypic diversity, (2) a founder effect in the establishment of *C. angulata* populations, (3) a non-Japanese Asian source population, and/or (4) changes in the genetic structure of Japanese oyster populations subsequent to establishment of Portuguese oysters in Europe.

Mitochondrial restriction fragment length polymorphism (RFLP) surveys of natural cupped oyster populations reveal that one or two common haplotypes typically dominate such populations in association with a large number of rare haplotypes (Reeb and Avise 1990; Beckenbach 1994; Boom et al. 1994). Our small sample sizes therefore underestimate the amount of genetic diversity in the study populations, but we have probably sampled the predominant haplotype(s). This is especially clear for the *Crassostrea gigas* samples from the four Japanese stocks ($n = 20$), which, remarkably, all had identical genotypes. In contrast, considerable mitochondrial diversity has been detected in RFLP population genetic studies of transplanted Miyagi stock populations in Canada (Boom et al. 1994). Some of this difference may be methodological in origin, as the RFLP approach used by Boom et al. (1994) sampled the entire mitochondrial genome, including areas that may be experiencing higher mutation rates than the COI fragment we characterized. Data from a contemporaneous population genetic study (Boudry et al. 1998) utilizing RFLP analyses of homologous COI mitochondrial gene fragments, however, strongly indicate that our results are a valid reflection of genetic differences among Portuguese oysters and Japanese stocks of Pacific oysters.

Another possibility is that the observed genetic divergence of Pacific and Portuguese oysters resulted from a founder effect, whereby a very small number of transplanted Japanese *Crassostrea gigas*, possessing rare haplotypes, established the European population. Cladistic analysis of mitochondrial RFLP variation in Pacific oysters (Boom et al. 1994) and in Gulf of Mexico and Atlantic Coast populations of American oysters (Reeb and Avise 1990) reveals that the great majority of haplotypes in these populations differ by one restriction site loss/gain from a small number of common haplotypes (Beckenbach 1994). Beckenbach (1994) proposed that this pattern reflects the interaction of a small effective population size (due to variable reproductive success of individual females) and a high rate of mitochondrial mutation in oysters. Common haplotypes occupy central positions in the cladograms, separated by single steps from the majority of the rare haplotypes which occupy terminal positions (Beckenbach 1994). The phylogenetic placement of Portuguese oyster haplotypes relative to those of Japanese Pacific oysters are not consistent with this pattern and indicate that the observed genetic divergence did not result from a founder effect. All *C. angulata* haplotypes are separated by 12 steps from Japanese *C. gigas* and constitute a distinct clade. The Portuguese/Pacific oyster mitochon-

drial comparison does, however, resemble the pronounced mitochondrial break in *C. virginica* Gulf of Mexico/Atlantic Coast populations (Reeb and Avise 1990) both in its phylogenetic tree topology (Beckenbach 1994) and in its genetic divergence levels [11 substitutions for *C. virginica* homologous COI gene fragments (Gaffney, unpublished)]. It is likely that the putative Asian source population for *C. angulata* was genetically differentiated from the Japanese populations of *C. gigas* we have sampled and that this factor, rather than a founder effect, is responsible for our results.

The simplest and most plausible interpretation of our data is that the putative Asian source population for the Portuguese oyster exists outside of Japan and will be encountered when the Pacific oyster is genetically characterized throughout its natural range. Contemporaneous mitochondrial genetic characterization of Taiwanese samples, using RFLP analyses of COI fragments (Boudry et al. 1998) and sequencing of 16S gene fragments (J-H Cheng, Tungkang, Taiwan, personal communication), strongly indicate that Taiwanese stocks represent plausible source populations. Boudry et al. (1998) have found that the predominant haplotypes from Taiwanese and from *Crassostrea angulata* samples share *Mse I* (5′-TTAA-3′) and *Taq I* (5′-TCGA-3′) restriction sites which differentiate them from Japanese Pacific oysters and are consistent with our observed substitutions at positions 367 and 585 (Fig. 1), respectively. In addition, our *C. angulata* sequence obtained for a 444-nt fragment of the mt 16S gene is identical to that of Taiwanese samples (J-H Cheng, personal communication) although it differs from Japanese samples by three transitions (data not shown).

Another, more remote, possibility is that the source population for *Crassostrea angulata* was indeed Japanese, but that this genetic stock has been displaced during the past half milleninum. Remarkably, there may be a recent precedent for this scenario as the Kumamoto oyster, *C. sikamea*, has apparently become displaced/extinct within the past 40 years in its home range in Kyushu and is now found only in North American culture operations (Banks et al. 1993, 1994). One of the contributing factors identified in the apparent extirpation of *C. sikamea* in Japan (Banks et al. 1994) has been the mass distribution of *C. gigas* seed, particularly from Miyagi populations, throughout Japan by oyster culturists (Ozaki and Fujio 1985). The process of homogenization or swamping by cultured oysters may be still in progress. Genetic distances (Nei's unbiased *D*) based on five polymorphic allozyme loci ranged from 0.045 to 0.064 among Hokkaido, Miyagi and Kyushu natural populations in 1979 (Fujio 1979). Less than a decade later, both northern (Hokkaido) and southern (Kyushu) populations were more similar (*D*-values 0.011 to 0.029 for the same loci) to Miyagi (central Japan) seed source populations (Ozaki and Fujio 1985). Culture-related transfer may also account for the surprising absence of polymorphism in our *C. gigas* specimens, especially in the natural population samples from opposite ends of Japan (Kyushu, Hokkaido). It is possible that we encountered the predominant Miyagi haplotype in our modest sample of five individuals each from the respective geographic ranges of the four historical Japanese stocks. A large scale population genetic survey of Japanese populations is required to address this issue.

The phylogenetic relationships of the Portuguese oyster *Crassostrea angulata* are unusually interesting because several independent lines of evidence including larval shell morphology, allozymes, breeding experiments and now mitochondrial gene sequences, strongly indicate that this oyster is of recent Asian origin. It may represent a case of undocumented anthropogenic introduction dating from the earliest days of circumglobal navigation. The mitochondrial data are the first to show the genetic distinctiveness of Portuguese oysters and present-day Japanese stocks of Pacific oysters. Ongoing genetic characterization of Pacific oysters throughout their Asian range promises to fully expose the phylogenetic relationships among Portuguese and Pacific oysters and to positively identify convincing Asian source populations for *C. angulata*.

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