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## The taxonomic status and origin of the Portuguese oyster

### *Crassostrea angulata* (Lamarck, 1819)

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#### Abstract

The taxonomic status of the Portuguese oyster *Crassostrea angulata* (Lamarck, 1819) and the Pacific oyster *C. gigas* (Thunberg, 1793) has often been a matter of controversy. Based on larval shell morphology, experimental hybridization and electrophoretic studies of enzyme polymorphism several authors have considered these two species as being synonymous. During the recent years, several genetic studies based on mitochondrial DNA and microsatellites data have provided evidences that the two taxa are genetically distinct although closely related. Furthermore, karyotype analysis has also supported the close genetic similarity of these taxa in comparison with other cupped oyster species. However, a recent comparative analysis of restriction enzymes banding patterns highlighted differences between all chromosomes pairs of *C. angulata* and *C. gigas* with the exception of chromosomal pair 10. In addition, significant phenotypic differences between the two taxa were observed in terms of aquaculture production and eco-physiological characteristics. The two cupped oyster *C. angulata* and *C. gigas* were long assumed to be native to the northeastern Atlantic and Asia, respectively. Different hypotheses have been proposed to explain the apparent geographically distant distribution of these close related taxa. Phylogenetic analysis firmly places both Portuguese and Pacific oysters within an Asian *Crassostrea* clade supporting the hypothesis of the introduction of *C. angulata* from Asia to Europe. Pure populations of *C. angulata* were observed in Taiwan as well as presumed mixed populations of *C. angulata* and *C. gigas* in Northern China. Remarkably, using equivalent amount of research on both taxa, the level of genetic variability of *C. gigas*

(samples collected in different parts of the world) appears to be lower than that of *C. angulata* (samples collected in Portugal) based on cytochrome oxidase C subunit I haplotypes. This difference could be related to the dissemination of *C. gigas* mainly from Miyagi Prefecture to different regions in Japan and other places in the world where the Pacific oyster was introduced. These findings suggest that (1) *C. angulata* and *C. gigas* are very close related, but they cannot be considered synonymous and (2) the Portuguese oyster has an Asian origin.

#### Introduction

Cupped oysters belonging to the genus *Crassostrea* are among the most important commercial aquatic species in the world. These species have been harvested from the wild and cultivated since centuries. Among them we can find the Portuguese oyster *Crassostrea angulata* (Lamarck, 1819) and the Pacific oyster *C. gigas* (Thunberg, 1793). The two cupped oysters *C. angulata* and *C. gigas* were long assumed to be native of the northeastern Atlantic and Asia, respectively. The Portuguese oyster was a species of major economic importance in Europe from the late 19<sup>th</sup> century up to the early 1970's, sustaining the European oyster production for almost one century. Major mortalities between 1967 and 1973 almost wiped out the Portuguese oyster from Europe (Comps, 1988). The Pacific oyster was introduced in different regions and is presently farmed throughout the Americas, Africa, Australia, Europe, and Asia and is the marine organism with the highest annual aquacultural production in the world (FAO, 2005).

The taxonomic status of the Portuguese and the Pacific oysters has often been a matter of controversy. The main reason of their distinction into two different species was their separated geographical

distribution whereas some data (morphological, experimental hybridization or genetic) have suggested the two taxa as being synonymous. The objective of the present paper is to provide a review of the published studies about *C. angulata* and *C. gigas* that can help to clarify their relative taxonomic status and the origin of the Portuguese oyster. It is also discussed the role that *C. angulata* may have for the future development of the oyster production industry.

### **Morphological analysis**

Classification of oysters based only on morphological analysis can be problematic, especially in the genus *Crassostrea*, due to the high variation and plasticity that is strongly influenced by environmental conditions (e.g., Galtsoff, 1964). Although some adult shell features can be used in the classification of *Crassostrea* species their utility is limited, especially in case of closely related taxa. Other morphological characteristics, like the morphology of late larval shells (Hu *et al.*, 1993) and anatomic features (Wang *et al.*, 2004), are usually considered more useful taxonomic characteristics. According to several authors the adult shells of *C. angulata* and *C. gigas* are indistinguishable (Menzel, 1974; Biocca and Matta, 1982) albeit no morphometric analysis were performed and the criteria's used to differentiate them were not specified. Furthermore, Ranson (1960) reported no differences between the morphology of larval shells of *C. angulata* and *C. gigas*, whereas he could separate other species using the same approach. Based on these observations Ranson stated that the Portuguese and Pacific oysters are the same species. Evseev *et al.* (1996) described the anatomy of *C. gigas* and found anatomical differences in the arrangements of the interlamellar septa when compared with the observations for the same features by Nelson (1960, in Evseev *et al.*, 1996) of *C. angulata*. A direct comparison of the anatomy of the two taxa using animals from different populations is still lacking in order to clarify these differences. Nevertheless, both taxa are morphologically very similar which suggests that they are very close related.

### **Growth and ecophysiological comparisons**

Significant phenotypic differences between Portuguese and Pacific oysters have been reported in several studies in the last decades. Production yield is an important economic trait in shellfish farming that takes into account survival and growth. A higher production yield in the natural environment has been observed for *C. gigas* when compared with *C. angulata* (Parache, 1989; Soletchnik *et al.*, 2002). Héral *et al.* (1986) observed that the production was always higher for *C. gigas* than for *C. angulata* at different values of overall stocking biomass in the Marennes-Oléron bay, France. These differences were mainly attributed to the faster growth of *C. gigas*, but are also related in some cases with a higher mortality observed in *C. angulata* (His, 1972;

Bougrier *et al.*, 1986; Soletchnik *et al.*, 2002). Some ecophysiological parameters have been compared between the two taxa in order to better understand the differences observed in growth rate. His (1971) observed that the Pacific oyster had a higher clearance rate than the Portuguese oyster. This author also observed that below 10°C the difference in valve activity between these taxa increased and that only *C. gigas* was active at 2°C. It was also observed that juveniles of the Pacific oyster had higher oxygen consumption rates than juveniles of the Portuguese oyster (Goulletquer *et al.*, 1999). Haure *et al.* (2003) did not observe the same differences in the clearance and oxygen consumption rates, but only in terms of feeding time activity. These ecophysiological results suggest that *C. angulata* and *C. gigas* have different strategies for the allocation of the available energy, which could explain their dissimilar growth.

### **Parasitic-disease susceptibility**

Major mortalities between 1967 and 1973 almost led to the disappearance of the Portuguese oyster from Europe (Renault, 1996). The “gill disease” has been identified as one of the cause of *C. angulata* high mortalities, that started in France in 1966 and the disease was characterized by the appearance of gill lesions (Arvy and Franc, 1968; Alderman and Gras, 1969; Marteil, 1969; Comps, 1970). This mortality episode was followed by a second period of mass mortalities, but this time without distinctive clinical signs (Comps, 1988). Different agents were implicated in the mortalities such as fungi, protozoans and viruses (Arvy and Franc, 1968; Besse, 1968; Gras, 1969; Comps and Duthoit, 1976). Irido-like viruses are regarded as one of the most probable causes of the mortalities of *C. angulata* and they were designated as gill necrosis virus (Comps and Duthoit, 1976) and haemocyte infection virus (Comps, 1988). However, these viruses were not isolated neither experimental transmission studies were performed to demonstrate their pathogenicity (Comps, 1988). It was also reported that the oyster stocks were very high in some regions prior to the mortalities, increasing the risk and impact of presumed pathogens (Héral *et al.*, 1986). Large-scale introduction of a replacement species in France, the Pacific oyster *C. gigas*, was decided in 1970 in order to overcome the crisis (Grizel and Héral, 1991). It was point out that smaller scale introductions that started in 1966, may have led to the introduction of the putative agent that caused the mortalities (Renault, 1996). The irido-like viruses associated with the mortalities were also observed in the Pacific oyster (Comps and Bonami, 1977; Comps and Duthoit, 1976), but no losses were detected in this species during a period when the Portuguese oyster suffered mass mortalities (Comps, 1988). Hence, these results suggest that *C. angulata* and *C. gigas* have different susceptibility to the putative disease responsible for the mortalities that almost wiped out the Portuguese oyster from Europe.

Recent unpublished studies revealed different

levels of prevalence and intensity of a parasitic copepod of *C. angulata* and *C. gigas* that is found mainly in the gills. These results also suggested that there is a host response to the copepod that may explain the differences observed in prevalence and intensity.

#### Genetic differentiation and variability

The high genetic similarity between *C. angulata* and *C. gigas* observed by several authors using allozymes markers supported the hypothesis that the Portuguese and Pacific oysters should be classified as the same species (Mathers *et al.*, 1974; Buroker *et al.*, 1979; Biocca and Matta, 1982; Mattiucci and Villani, 1983). However, studies on the mitochondrial cytochrome oxidase subunit I (COI) gene have shown clear genetic differences between the two taxa (Boudry *et al.*, 1998; O'Foighil *et al.*, 1998). An average of 2.3% differences in mitochondrial COI nucleotide sequence suggests that populations of *C. angulata* and *C. gigas* may have diverged several hundred thousand years ago (Hedgecock *et al.*, 2004). Huvet *et al.* (2000) revealed low but significant genetic differences between Portuguese and Pacific oysters populations using microsatellites markers (mean Wright's fixation index;  $F_{st} = 0.022$ ). These authors reported that the genetic differentiation observed in pairs of populations of the two different taxa were twice as large as in pairs of populations of the same taxon. A more recent study based on a satellite DNA highlighted the genetic similarity between *C. angulata* and *C. gigas* (López-Flores *et al.*, 2004). All these genetic studies clearly indicate close phylogenetic ties between the Portuguese and Pacific oysters. Nevertheless, studies using mitochondrial and microsatellites markers have shown that there are low but clear genetic differences between them. It is noteworthy that similar mitochondrial differences were also observed between populations of the American oyster *C. virginica* from the Atlantic and Gulf coasts of America (estimated to differ by about 2.6% in nucleotide sequence), but they are not considered two different species (Reeb and Avise, 1990).

Using equivalent amount of research on both taxa, the level of mitochondrial genetic variation of *C. gigas* (samples collected in different parts of the world) appears to be lower than that of *C. angulata* (samples collected in Portugal) based on PCR-RFLP haplotypes of a COI fragment (Boudry *et al.*, 1998; Lapègue *et al.*, 2004) (figure 1). For the same mitochondrial gene, O'Foighil *et al.* (1998) observed no polymorphism in individuals of *C. gigas* with different origins whereas a high polymorphism was observed in *C. angulata* from Sado estuary, Portugal. The work of Buroker *et al.* (1979) has also showed a higher genetic variation (estimated by the mean heterozygosity) in *C. angulata* from Sado estuary (Portugal), when compared with other *Crassostrea* species. Nevertheless, Huvet *et al.* (2000) using microsatellites markers observed similar levels of

genetic variation in both taxa. The low mitochondrial variability of *C. gigas* when compared with *C. angulata* could be related with the dissemination of *C. gigas* basically from Miyagi Prefecture to different regions in Japan (O'Foighil *et al.*, 1998) and other places in the world where the Pacific oyster was introduced.

#### Cytotaxonomy

Oysters of the genera *Crassostrea* have a diploid chromosome number of  $2n = 20$  which is a common feature in the *Ostreidae* family (Thiriot-Quiévreux, 2002). Comparative standard karyological analysis of *C. angulata* and *C. gigas* supported the close genetic similarity of these two taxa when compared with other cupped oyster species (Leitão *et al.*, 1999a). A study in which the G-banding technique was applied to chromosomes of *C. angulata* and *C. gigas* confirmed the high similarity between karyotypes of both taxa, but showed differences which agreed with their taxonomic separation (Leitão *et al.*, 1999b). A more recent study by Leitão *et al.* (2004) confirmed the previous findings, through the application, this time, of a restriction endonucleases banding technique that put in evidence differences between the banding pattern in all chromosomes pairs of *C. angulata* and *C. gigas* with the exception for chromosome pair 10. From a cytogenetics point of view the two taxa are indeed very similar, but can be distinguished.

#### Reproductive isolation

Pre- and post-zygotic mechanisms that can contribute to the biological isolation of *Crassostrea* species have been addressed in several studies. There are apparently no pre-zygotic barriers to interspecific hybridization between geographically or ecologically isolated species since interspecific fertilization often occurs with at least moderate success (Gaffney and Allen, 1993). On the other hand, asymmetric fertilization success was observed between sympatric species of the genus *Crassostrea* (Banks *et al.*, 1994). High fertilization rates in crosses of *C. angulata* with *C. gigas* have been reported by different authors (Imai and Sakai, 1961; Menzel, 1974; Huvet *et al.*, 2001). Molecular analysis of six-hour old embryos revealed no evidence of preferential fertilization between gametes from the same taxon when spermatric competition was allowed between taxa (Huvet *et al.*, 2001). Asynchronous spawning can also lead to reproductive isolation (pre-zygotic isolation). In the American oyster *C. virginica* (Gmelin, 1791) genetic variation in the timing of gonadal maturation and spawning was observed among distinct populations (Barber *et al.*, 1991). The results of Huvet (2000) and Soletchnik *et al.* (2002) based on the analysis of sexual maturation of *C. angulata* and *C. gigas* suggests that asynchronous spawning may occur. In addition, differences between these two taxa in the minimum temperature at which eggs or sperm are released were reported

by Lubet (1994) based on the work of Le Dantec (1968). It is noteworthy that there are evidences that eggs and sperm from one *Crassostrea* species can induce spawning in another one under laboratory conditions (Galtsoff and Smith, 1932), which can lead to synchronous spawning. Strong post-zygotic barriers to interspecific hybridization in the genus *Crassostrea* such as the viability and fertility of the hybrids are usually reported (Gaffney and Allen, 1993). Normal viability of the F1 hybrids between the Portuguese and Pacific oysters has been observed by several authors (Imai and Sakai, 1961; Menzel, 1974; Bougrier *et al.*, 1986; Huvet *et al.*, 2002). According to Menzel (1974) meiosis in the F1 hybrids of the Portuguese and Pacific oysters as well as mitosis in the F2's embryos appeared normal. However, Numachi (1966, in Gaffney and Allen, 1993) have previously reported that F1 hybrids of both taxa display normal viability and fertility, but the F2 progeny did not survive to settlement. These results were not corroborated by Huvet *et al.* (2002) that managed to produce F2 hybrids that showed normal fertilization rates, developmental yields, and settlement rates. A recent study provided evidence for the existence of hybridization between the Portuguese and Pacific oysters in the natural environment where the two taxa were put in contact recently (Huvet *et al.*, 2004).

The different studies about the mechanisms that can contribute to the biological isolation of *C. angulata* and *C. gigas* suggest that there are no major barriers that can prevent the genomes of these taxa from merging. More studies (*e.g.*, deficits of hybrid forms in the natural environment) are needed in order to confirm these findings.

### Origin of the Portuguese oyster

The different geographical distribution, *C. angulata* being present in the northeastern Atlantic and *C. gigas* in Asia, before the voluntary introduction of *C. gigas* in Europe (Grizel and Héral, 1991) in the early 1970s, lead to the question of the origin of these two close related taxa. Three hypothesis have been proposed to explain their geographically distant distributions: (i) *C. angulata* (Iberian peninsula and Morocco), *C. gigas* (China, Japan, to Sakhalin Island) and *C. cattuckensis* (India) derived from a fossil ancestor *C. gryphoides* and toward the end of the Miocene and later, tectonic events produced land barriers, isolating three populations, resulting in the distribution of the three species (Stenzel, 1971); (ii) *C. angulata* was transported from Europe to Asia some centuries ago by European merchants ships (Menzel, 1974); (iii) *C. angulata* was introduced in Europe by undocumented anthropogenic transfer during the earliest days of circumglobal navigation from the Far East (Ranson, 1960).

Based on mitochondrial DNA sequence data, O'Foighil *et al.* (1998) estimated a divergence time

of 1 to 2 million years for the Portuguese and Pacific oysters, long after closure of the Tethyan Seaway estimated in approximately 7 million years ago. Although this study may overestimate oysters divergence times, it clearly undermines Stenzel's hypothesis, suggesting that the closure of the Tethyan Seaway occurred before the appearance of the last common ancestor of the Portuguese and Pacific oysters. According to Ranson (1948, in Edwards, 1976) there are no evidences of the presence of *C. angulata* on the Miocene, Pliocene and Quaternary beds of Portugal, which supports the hypothesis of a recent introduction of *C. angulata* in to Europe from another region. On the other hand, Lawrence (1995) argued that the fossil record does not promote the notion that the Portuguese and Pacific oysters may have been imported into the eastern Atlantic by humans. In fact, *C. angulata* have been reported to exist for at least 2200 years BP in Spain (Ruiz *et al.*, 2004). It is also possible that other *Crassostrea* species, native of the eastern Atlantic, have derived from *C. gryphoides*. Those species, that are no longer present in Europe, could have been incorrectly identified in the late Holocene as *C. angulata*. Another possibility is that in some palaeontological studies, other species of oysters were mistakenly classified as *C. angulata* because of the high morphologic plasticity of oysters. The close genetic relationship between *C. angulata*, *C. gigas*, and other *Crassostrea* species from Asia (figure 2) such as the Kumamoto oyster *C. sikamea* and the Suminoe oyster *C. ariakensis* suggests that the Portuguese oyster has an Asian origin (Buroker *et al.*, 1979; O'Foighil *et al.*, 1998; Boudry *et al.*, 2003). Moreover, studies using mitochondrial markers reported the presence of pure populations of *C. angulata* in Taiwan (Boudry *et al.*, 1998) as well as presumed mixed populations of *C. angulata* and *C. gigas* in Northern China (Yu *et al.*, 2003; Lapègue *et al.*, 2004). Consequently, Taiwan has been proposed as a possible origin of European *C. angulata* populations (Boudry *et al.*, 1998; Huvet *et al.*, 2000). These findings suggest that the Portuguese oyster has an Asian origin and is probably a case of recent undocumented anthropogenic introduction.

### Final considerations

The current knowledge about the Portuguese and Pacific oysters show that they are very close related, but also that there are clear genetic and phenotypic differences between them. Consequently, these two taxa cannot be considered synonymous and more data about putative barriers to gene flow between the two taxa is needed in order to define their taxonomic status. Different lines of evidence suggest that the Portuguese oyster is of Asian origin and implicate Taiwan as the possible origin for the European *C. angulata* population (Boudry *et al.*, 1998). A better knowledge on the current

distribution of *C. angulata* in Asia can provide new insights about the origin of the Portuguese oyster, namely in regions where the most common historic commercial routes between Europe and Asia were established during the earliest days of circumglobal navigation.

Other cases of close related bivalves molluscs taxa that are genetic and phenotypically distinct, but interbreed producing sexually viable offspring, have been reported. One of the best studied cases are the mussels of the *Mytilus* complex composed by the close related but genetically distinct taxa *M. edulis*, *M. galloprovincialis* and *M. trossulus* (McDonald *et al.*, 1991). Two of these species, *M. edulis* and *M. galloprovincialis*, co-occur in western Europe and readily interbreed and produce hybrid zones with parental genotypes, high frequencies of F1 and F2 hybrids, and mussels of mixed genetic ancestry (Hilbish *et al.*, 2002; Bierne *et al.*, 2003). Another case of close related taxa that hybridize in the natural environment is the hard clams *Mercenaria mercenaria* and *M. campechiensis* that occur on the east coast of North America (Bert and Arnold, 1995). Despite the taxonomic difficulties posed by these taxa, hybridization between them and hybrid zones offer excellent opportunities to study speciation and processes, which contribute to reproductive isolation (Gardner, 1997). Evidence of natural hybridization between *C. angulata* and *C. gigas* throughout the study of wild cupped oyster population located in the south of Portugal where the two taxa are in contact due to recent anthropogenic transfer opened new perspectives to study the evolutionary history of the *Crassostrea* genus (Huvet *et al.*, 2004). The development of nuclear markers will be of crucial importance to study natural hybridization between these two cupped oysters.

The differences observed between the close related taxa *C. angulata* and *C. gigas* in growth, ecophysiologic characteristics, and parasitic-disease susceptibility also opens new perspectives to study the genetic basis of growth and disease resistance. On the other hand, *C. angulata* can also be seen as a valuable genetic resource for the development of selective breeding programs as well as in the context of production diversification and biodiversity preservation. The conservation of the remaining pure populations of *C. angulata* in Europe (Lapègue *et al.*, 2004) is of great importance since these genetic resources can be used for the development of the European oyster industry without the zoosanitary risks associated with the introduction of exotic oysters (Berthe and Boudry, 1999).

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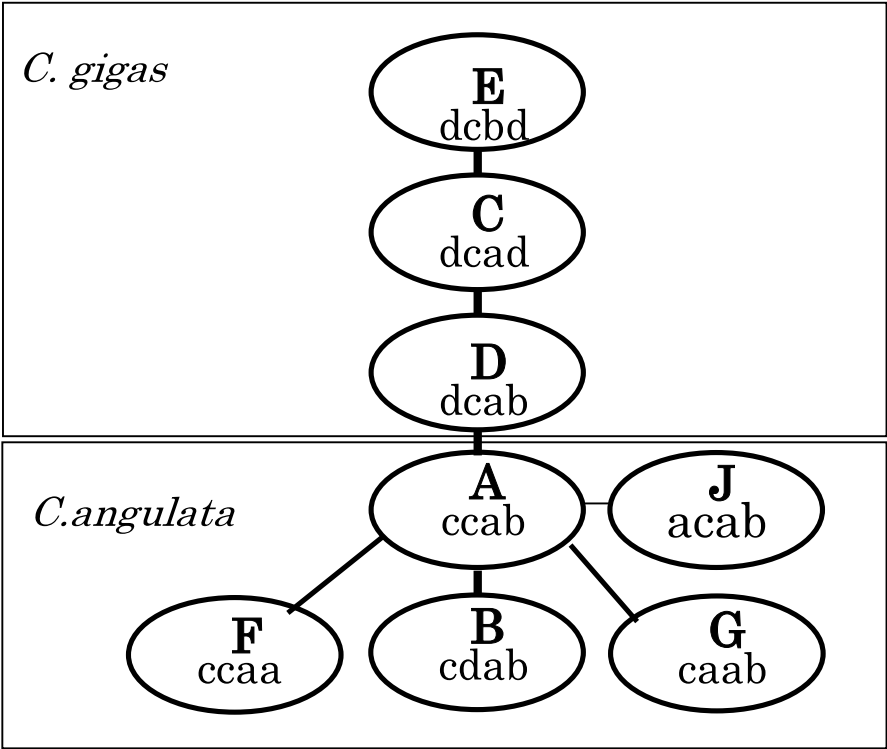


Figure 1. Haplotypes associated with populations of *Crassostrea gigas* (E, C and D) and *C. angulata* (A, J, F, G and B) obtained by PCR-RFLP from a fragment of COI. Each circle is separated from adjacent circles by a single restriction site. The haplotypes E, C, D, A, J and B were described by Boudry *et al.* (1998) and haplotypes F and G were described by Lapègue *et al.* (2004).

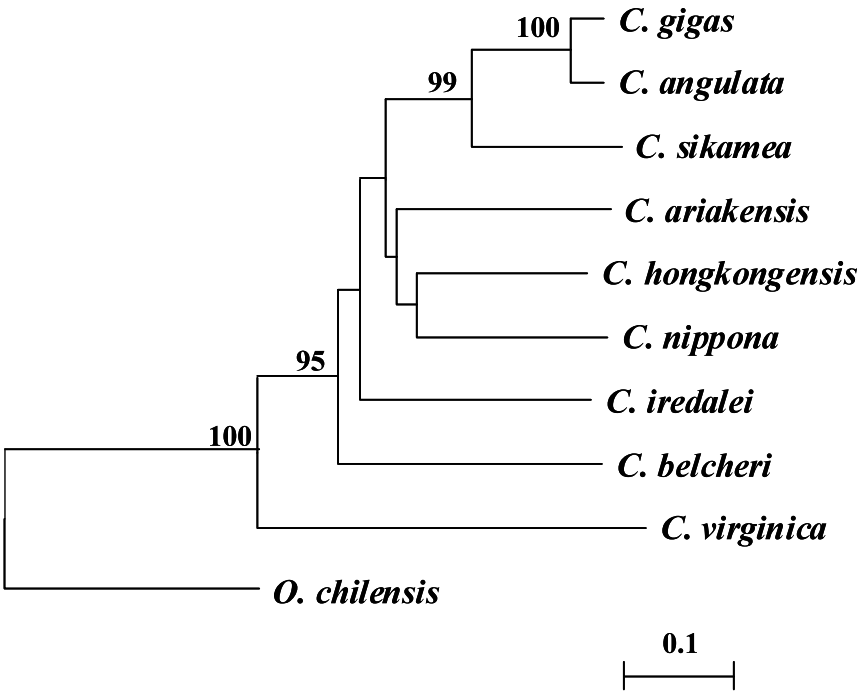


Figure 2. Phylogenetic tree based on neighbor-joining analyses of *Crassostrea* species of a partial COI sequence using Kimura two-parameter distances with 1000 bootstrap. *Ostrea chilensis* was used as outgroup. Numbers at specific nodes represent bootstrap values greater than 70 %.