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Resolution of the controversial relationship between Pacific and Portuguese oysters internationally and in Vietnam

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1 Abstract

Oyster aquaculture is a new and fast growing sector in Vietnam, but confusion exists about the identity of the species presently under culture, whether they are Pacific oysters *(Crassostrea gigas*), Portuguese oysters (*Crassostrea angulata*), hybrids thereof, or other species. This study was carried out to identify which oyster or oysters are most commonly cultured in Vietnam and, additionally, once the species identity was resolved, to assess three farmed Vietnamese stocks for levels of genetic variation and suitability for captive breeding programs.

To resolve the taxonomy issues, we searched for nucleotide differences (characteristic attributes) in published mitochondrial DNA cytochrome c oxidase subunit 1 (COI) sequences that, for the first time, would categorically separate and distinguish in particular *C. angulata* from *C. gigas*. On review of 300 published haplotypes of *C. angulata* and *C. gigas* based on a 293 bp nucleotide-fragment of published COI sequences, we found that there were five distinct nucleotides that are categorically different between *C. angulata* and *C. gigas* and that could be considered as diagnostic nucleotides. Using these five diagnostic nucleotides, we confirmed that the samples from northern Vietnam are *C. angulatam*, not *C. gigas*. Similarly, we identified other oyster species in Vietnam from Nhatrang as *C. sikamea* and *C. madrasensis*. DNA microsatellite data (following) can also

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support understanding of the taxonomy, directly by comparing allele types and frequencies between putative species, but also indirectly because as nuclear DNA, microsatellite genotypes may reveal if hybridization is occurring (as evidenced by deviations from Hardy-Weinberg equilibrium). No evidence, considering Hardy-Weinberg deviations, for interspecific hybridization was found.

To address the diversity issues, three hatchery bred populations of *C. angulata* were screened for allelic variation at nine DNA microsatellite loci. All three lines had high allelic diversity, moderate effective population sizes (N_e), and little evidence of kinship, which, by precent with other hatchery bred highly fecund oyster species, is a little unexpected. It is speculated that local hatchery practices may involve sharing stock among hatcheries which then may contribute to the maintenance of moderate to high levels of diversity during hatchery reproduction of this highly fecund species.

Keywords: Taxonomy, Portuguese oyster, *Crassostrea angulata*, Pacific oyster, *Crassostrea gigas*, COI, microsatellite markers

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2 Introduction

Edible oyster aquaculture in Vietnam, largely based on introduced species, has expanded very rapidly from no production in 2007 to over 7,000 tonnes/annum in 2010 with the majority coming from Quangninh and Haiphong provinces in northern Vietnam. Currently, the phylogenetic status of the introduced oysters in Vietnam is poorly understood. Locally, in Vietnam, edible oysters are widely referred to as Pacific oysters, *Crassostrea gigas* but there is some doubt over the species identity since oyster lines for the majority of production were imported from Taiwan, where the Portuguese oyster, *Crassostrea angulata*, is produced (Batista et al., 2005, Boudry et al., 1998). In addition to this Taiwanese stock, spat from Southern China was also imported to satisfy the increasing demand for production. These oysters may be *C. gigas, C. angulata* or hybrids of them, as China is known as the major producer of Pacific oyster (Lapegue et al., 2004, Boudry et al., 2003) but also was reported to have mixed populations of *C. angulata* and *C. gigas* (Batista et al., 2005). Critically, as production continues to expand, and selective breeding has begun, there is a need to better understand the taxonomy of Vietnam's hatchery oyster stocks.

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C. gigas and *C. angulata* are morphological similar (O'Connor and Dove, 2009). However, in the past, *C. angulata* and *C. gigas* were considered to be different species by Thunberg

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in 1793 and Lamarck in 1819 as they apparently were distributed in two separate areas: *C. angulata* in Europe and *C. gigas* in Asia (Huvet et al., 2000b). *C. angulata* was first found along the coasts of Southern Europe, while *C. Gigas* originates from and is distributed in Asia (Soletchnik et al., 2002). More recently, they were thought to be a single species due to their phenotypic similarity and ability to produce fertile hybrid offspring (Huvet et al., 2002, Huvet et al., 2004).

Previously, mtDNA sequences, especially cytochrome c oxidase (COI), have been widely used for the identification of oyster species due to morphological plasticity and similarities between the species. Specifically, COI sequences have been used to differentiate a variety of oyster species including flat oysters, *Ostrea edulis* (Boudry et al., 1998, Foighil et al., 1998, Huvet et al., 2000a), the native *O. angasi* in Oyster Harbour, Western Australia (Morton et al., 2003), *C. iredalei* and *Saccostrea cucullata* in Thailand (Klinbunga et al., 2003), and Malaysian *Crassostrea* spp: *C. iredalei, C. belcheri* and *C. madrasensis* (Mustaffa et al., 2010). COI has also been used to identify new oyster species such as *C. hongkongensis* in China (Lam and Morton, 2003).

It has been suggested *C. gigas* and *C. angulata* could be separated using percent COI DNA sequence differences (Wu and Yu, 2009), even though the differences are only a few

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percent, but others (Boudry et al., 2003, Liu et al., 2011) have cautioned that such slight differences are lower than those that normally evident between species, so that the whole matter of the taxonomic status of these two species remains controversial.

DNA sequences or allozymes other than mtDNA sequences have also been used in attempts to distinguish between *C. gigas* and *C. angulata* but generally have not succeeded (David and Savini, 2011), Boudry et al., 1998 for allozymes; Cordes et al. (2008) for nuclear rRNA ITS1 regions; Huvet et al. (2000a) and Reece et al. (2008) for microsatellite DNA markers; Larsen et al. (2005); David and Savini (2011) for nuclear genes: ITS1, ITS2 18S and 28S rDNA. Even the use of mtDNA other than COI (such asrrnL and MNR, 16S rDNA, 12S rDNA, internal transcribed spacer or ITS region) failed to distinguish between *C. gigas* and *C. angulata*as as suggested by Masaoka and Kobayashi (2005), David and Savini (2011), Lam and Morton (2003).

Beyond the taxonomic uncertainty of the oysters being cultured in Vietnam, there are additional concerns that with captive reproduction of such fecund species, that lines could go through population bottlenecks and lose genetic diversity, yielding inbred and poor quality spat. To develop and sustain an oyster industry in the long term, and to employ in selective breeding, it is desirable to use outbred stocks and to sustain their genetic

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diversity in the future. Maintaining a wide range of genotypes could give a hatchery population more flexibility of response to a constantly changing environment (Boudry, 2008, Taris et al., 2006). Genetic diversity is the initial requirement for a genetic improvement program, however it may be eroded by the process of selection, particularly mass selection where just a few elite families can be selected, and husbandry practices due to a limited number of broodstock individuals needed to produce the next generation and high variation in individual reproductive success (Boudry, 2008, Nguyen, 2009, Taris et al., 2006). With a high rate of inbreeding, individuals mate with their close relatives which results in a higher incidence of recessive deleterious genotypes. Previous studies reported loss of genetic variation in many hatchery populations, especially for broadcast spawners like oysters where a female oyster can release millions of egg in only one spawning event (O'Connor et al., 2008). The Vietnamese hatchery oyster stocks that have been established and captively bred for almost seven generations (seven years from 2008-2014), so there is concern about loss of variation and inbreeding.

Here we investigate three main issues 1) can DNA sequence data separate *C. gigas* and *C. angulata* or do we need other analyses, 2) are the oysters being farmed in Vietnam *C. gigas?* and 3) are the current hatchery stocks sufficiently genetically diverse and adequate to form the basis of a long term selective breeding program?

3 Material and methods

3.1 Biological samples

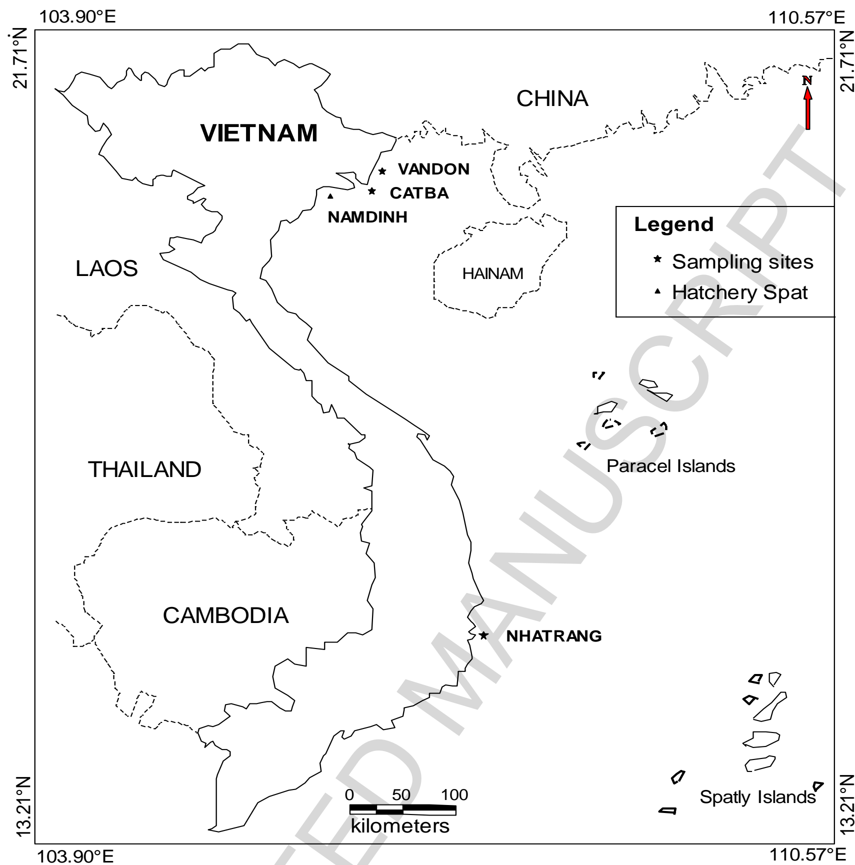

Figure 1. Hatchery and sampling sites

Samples were taken from animals from four groups at three growout sites of Vietnam (Vandon, Catba and Nhatrang (Figure 1, Supplementary Table 1). The samples were coded as follows: 1) RIA1 (RIA1 line cultured in Vandon), 2) China (stock were cultured in Vandon from spat imported from Southern China), and 3) Namdinh (Namdinh stock cultured in

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Catba from spat purchased from Namdinh hatcheries) and 4) Nhatrang (local oysters farmed in Nhatrang).

All samples from four different populations (Ria1, Namdinh, China and Nhatrang) were collected in December 2013, preserved in 70% ethanol and shipped to University of the Sunshine Coast (USC), where they were stored at -20°C until required. The minimum sample size for analysis of DNA microsatellite loci and COI was 32 and 24 individuals for each population, respectively.

3.2 DNA extraction

DNA extraction was according to the NaCl extraction protocol of Lopera-Barrero et al., 2008. The integrity of the DNA was verified by horizontal electrophoresis in a 0.9% agarose gel, at 110 volts for 40 min in a 0.6xTBE buffer (500 mM Tris-HCl, 60 mM boric acid, and 83 mM EDTA). The gel was dyed with ethidium bromide, verified and captured in GeneSnap with the Syngene System Bio-Rad). Moreover, the quality and quantity of the DNA were then evaluated using a Nanodrop 2000 (Thermo Scientific, USA) at the absorbance of 260/280nm. Good DNA templates were then diluted in molecular grade water (Amresco) to 25 ng/µl^{-1}.

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3.3 Mitochondrial DNA sequencing and analysis

The mitochondrial cytochrome C oxidase subunit I (COI) was amplified using universal primers (LCO1490 and HCO2198) developed by Folmer et al. (1994) in 25µl reactions using MyTaq DNA polymerase (Bioline). 96 DNA samples from four different cultured lines (19-20 samples per each population) were used for COI amplification and sequencing using an ABI 3730XL DNA analyser (Supplementary Table 2). Samples were sequenced in both directions and only consensus sequences used and raw sequencing files (ab1 files) are available on request. Sequencher 5.0 (Gene Codes Corporation, 2011) was used to edit and trim COI sequences. Mega 6.06 (Tamura et al., 2013) was used for sequence alignment, analysis of nucleotide differences, identification of haplotypes and building phylogenic tree.

All available published COI sequences of *C. gigas* and *C. angulata* from GenBank were downloaded as Fasta files and analysed to determine whether there were any fixed nucleotide differences among these two species for their COI sequences using Mega 6.06 (Tamura et al., 2013) and GeneDoc 2.7 (Nicholas et al., 1997). A phylogenic tree was built using all available COI sequences from *C. gigas* and *C. angulata* on Mega 6.06 (Tamura et al., 2013).

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To identify the oysters sampled from Nhatrang in central Vietnam, published COI sequences of other species in the *Crassostrea* genus (namely, *C. sikamea, C. ariakensis, C. hongkongensis, C. brasiliana, C. virginica, C. belcheri, C. nippona, C. iredalei* and *C. madrasensis*) and in the *Ostrea* genus (*O. edulis, O. chilensis* and *O. aupouria*) from GenBank were added and aligned with COI references on Mega 6.06 (Tamura et al., 2013) and then a tentative phylogenetic tree was built.

3.4 Validation of microsatellite primers

From previously published microsatellite primers, 48 primer pairs were selected (based on the polymorphic information content, number of alleles, expected heterozygosity), and used to amplify the farmed oyster individuals in Vietnam in 12.5µL PCR reactions containing 1xMyTaq reaction buffer, 0.125 mg ml^{-1} of bovine serum albumin, 30 ng of template DNA, 0.02 µM of forward and reverse primers where forward primers labelled with fluorescent dyes (FAM, NED, PET or VIC), and 1 unit of MyTaq DNA polymerase (Bioline). The reaction was amplified in an Eppendorf Mastercycler gradient thermal cycler using the following steps: an initial denaturing for 5 min at 95°C followed by 30 cycles of 95°C for 30 secs, annealing for 15s, extension at 72°C for 10 secs and final extension at 72°C for 2 mins. PCR products were qualified on 3% agarose gels run for 2 h at 140 volts to detect levels of polymorphism (variation of alleles on a locus) and quality of amplified

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products. On the basis of potential polymorphisms evident from agarose gel electrophoresis, 12 putatively polymorphic microsatellites were tested on eight different individual samples using fluorescent labelled primers and genotyped in an AB 3500 Genetic Analyser, Hitachi. Nine out of 12 published primers were qualified for this study based on good amplification, repeatability and reliability of scoring and degree of allelic variation (Supplementary Table 3).

3.5 Genotyping

PCR products were genotyped using an AB 3500 Genetic Analyser. For preparation prior to genotyping, PCR products were diluted 10 to 50 fold depending on the yield of PCR amplified products. One µl of each diluted PCR product was then transferred to 9µl of a combination of Liz 600 (size standard V2.0) and HiDi formamide from Life Technologies (7 µl Liz for 500 µl HiDi), then vortexed and briefly centrifuged before denaturation for 5 min at 95°C, cooling of DNA samples for 2-3 mins in an ice before transferring to the genotyping machine. Genotyping was conducted using the 500bp fragment analysis program. All genotypes from all individuals are lodged in Supplementary Table 4.

3.6 Data analysis and statistical method

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Output (.fsa files) from the genotyper were analysed and scored using GeneMarker 2.6.3 (Schmidt, 2011). Genotyping data was checked for null alleles on Microchecker 2.2.0.3 (Van Oosterhout et al., 2004). Genalex 6.5 (Peakall and Smouse, 2012) was used to estimate F_{st} and analyse allele diversity, molecular variance and departures from Hardy-Weinberg equilibrium. Colony 2.0.2.3 (Jones and Wang, 2010) was used to determine effective population size and kinship.

4 Results

4.1 Published sequences indicate fixed nucleotide differences between *C. gigas* and *C. angulata*

Analysis on 288 published COI sequences consisting of 222 sequences of *C. gigas* and 56 sequences of *C. angulata* obtained from GeneBank (full data in Supplementary Table 2) indicated there were five fixed nucleotide differences between *C. angulata* and *C. gigas* COI sequences, namely, at position 88 -T vs C, position 105 -C vs T, position 138 -T vs C, position 193 -T vs C and position 264 - G vs A), Table 1. Interestingly, the *C. angulata* haplotypes A (Genebank assess no: AJ553907.1), B (AJ553908.1) often used as COI references for *C. angulata* carry all these five exclusive nucleotides for *C. angulata*, meanwhile the most common *C. gigas* haplotypes C (AJ553909.1) and E (AJ553911.1) used as references contain all five exclusive nucleotides for *C. gigas*.

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Table 1. Five exclusive nucleotides in COI sequences between *C. gigas* and *C. angulata*.

```
                                60         .         80         .        100
                               TCTTATCATGGAGTTTGTATAGACCTTGCAATTCTAAGCCTTCACCTTGCTGGT
AJ553909|_C_gigas_hap_C       :......................................................:108
AJ553911|_C_gigas_hap_E       :......................................................:108
AB748797|_C_gigas_A-Cr06      :......................................................:108
KJ801546|_C_gigas_hap_AS1     :......................................................:108
KP099047|_C_gigas_hap_41      :......................................................:108
KJ801547|_C.gigas_hap_AS2     :......................................................:108
AB748801|_C_angulata_A-Cr01   :.................C..G............T................C...:108
AB748805|_C_angulata_G-Cr03   :.................C...............T................C...:108
AF152567|_C_angulata          :.................C...............T................C...:108
AB748802|_C_angulata_A-Cr05   :.................................T................C...:108
AB748809|_C_angulata_G-Cr07   :.................................T................C...:108
AJ553907|_C_angulata_hap_A    :.................C...............T................C...:108
AJ553908|_C_angulata_hap_B    :.................C...............T................C...:108
                                   .       120         .         140        .        160
                               ATTAGCTCTATTTTCAGGTCAATTAATTTCATAGTAACGATTAGAAATATGC
AJ553909|_C_gigas_hap_C*      :......................................................:162
AJ553911|_C_gigas_hap_E*      :......................................................:162
AB748797|_C_gigas_A-Cr06*     :......................................................:162
KJ801546|_C_gigas_hap_AS1*    :......................................................:162
KP099047|_C_gigas_hap_41      :......................................................:162
KJ801547|_C_gigas_hap_AS2     :......................................................:162
AB748801|_C_angulata_A-Cr01*  :.............................T........................:162
AB748805|_C_angulata_G-Cr03*  :.............................T........................:162
AF152567|_C_angulata*         :.............................T........................:162
AB748802|_C_angulata_A-Cr05*  :.............................T........................:162
AB748809|_C_angulata_G-Cr07*  :..C..........................T........................:162
AJ553907|_C_angulata_hap_A*   :.............................T........................:162
AJ553908|_C_angulata_hap_B    :.............................T........................:162
                                          .        180         .        200         .
                               TCTGTTGGGGGCCATTTACTAGCACTATTCCCTTGATCTATTAAGGTTACT
AJ553909|_C_gigas_hap_C*      :......................................................:216
AJ553911|_C_gigas_hap_E*      :.......................G..............................:216
AB748797|_C_gigas_A-Cr06*     :......................................................:216
KJ801546|_C_gigas_hap_AS1*    :......................................................:216
KP099047|_C_gigas_hap_41      :......................................................:216
KJ801547|_C.gigas_hap_AS2     :......................................................:216
AB748801|_C_angulata_A-Cr01*  :.............................T..C.....................:216
AB748805|_C_angulata_G-Cr03*  :.............................T..C.....................:216
AF152567|_C_angulata*         :.............................T........................:216
AB748802|_C_angulata_A-Cr05*  :.............................T........................:216
AB748809|_C_angulata_G-Cr07*  :.............................T........................:216
AJ553907|_C_angulata_hap_A*   :.............................T..C.....................:216
AJ553908|_C_angulata_hap_B    :.............................T..C.....................:216
                                  220         .        240         .         260        .
                               TTCTTGCTTTTGACTACTCTCCCAGTGTTAGCTGGAGGTCTTACTATACT
AJ553909|_C_gigas_hap_C*      :......................................................:270
AJ553911|_C_gigas_hap_E*      :......................................................:270
AB748797|_C_gigas_A-Cr06*     :....................A.................................:270
KJ801546|_C_gigas_hap_AS1*    :......................................................:270
KP099047|_C_gigas_hap_41      :......................................................:270
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KJ801547|_C.gigas_hap_AS2    :......................................................:270
AB748801|_C_angulata_A-Cr01* :..............................................G.......:270
AB748805|_C_angulata_G-Cr03* :..............................................G.......:270
AF152567|_C_angulata*        :..............................................G.......:270
AB748802|_C_angulata_A-Cr05* :..............................................G.......:270
AB748809|_C_angulata_G-Cr07* :..............................................G.......:270
AJ553907|_C_angulata_hap_A*  :..............................................G.......:270
AJ553908|_C_angulata_hap_B   :..............................................G.......:270
```

Note: Grey shadings highlight categorical nucleotide differences between *C. gigas* and *C. angulata*

4.2 Identification of species in northern Vietnam

Every DNA COI sequence of 72 oyster samples from northern Vietnam (i.e. from the Ria1, Namdinh and Chinese hatchery lines) carry all of the five diagnostic nucleotides indicative of *C. angulata* (Table 2). Additionally, three new *C. angulata* haplotypes were found, consisting of haplotype i1 (6 samples in Namdinh), haplotype i2 (3 samples in Namdinh and 6 samples in China line) and haplotype i3 (one sample in China line) (Table 2).

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Table 2. Alignment of oysters sampled from lines of Ria1, Namdinh and China with COI references of *C. gigas* and *C. angulata*.

```
                                 60         .        80         .       100         .
                               CTTATCATGGAGTTTGTATAGACCTTGCAATTCTAAGCCTTCACCTTGCTGGTAT
AJ553909.1|_C_gigas_hap_C     :.......................................................:110
AJ553907.1|_C_angulata_hap_A  :................C...............T................C.....:110
Nandinh_(3_7_10-12-13-15)*    :................C..G............T................C.....:110
Namdinh_(5_21_22)**           :................C..G............T................C.....:110
China_(1-2-3_20_21_22)**      :................C..G............T................C.....:110
China_5***                    :................................T................C.....:110
RIA1_(8,9,11,13,17,19)a       :................C..G............T................C.....:110
Namdinh_(4_6_14_16)a          :................C..G............T................C.....:110
China_(4_7_10_11_12)a         :................C..G............T................C.....:110
China_(16_17_18_23_24)a       :................C..G............T................C.....:110
Ria1_(2_4-5-16)b              :................C...............T................C.....:110
Ria1_(20-22-23-24)b           :................C...............T................C.....:110
Namdinh_(1_2_8_11)b           :................C...............T................C.....:110
Namdinh_(17_18_20)b           :................C...............T................C.....:110
China_(8_15)c                 :................C...............T................C.....:110
Ria1_(1-3_6_7_10_12)c         :................................T................C.....:110
Ria1_(14_15_18)c              :................................T................C.....:110
Namdinh_(9_19_23_24)c         :................................T................C.....:110
China_(6_9_13_14_19)c         :................................T................C.....:110

                                      120         .       140         .       160
                               TAGCTCTATTTTCAGGTCAATTAATTTCATAGTAACGATTAGAAATATGCGATCT
AJ553909.1|_C_gigas_hap_C     :.......................................................:165
AJ553907.1|_C_angulata_hap_A  :...........................T...........................:165
Nandinh_(3_7_10-12-13-15)*    :...........................T...........................:165
Namdinh_(5_21_22)**           :...........................T...........................:165
China_(1-2-3_20_21_22)**      :...........................T...........................:165
China_5***                    :...........................T...........................:165
RIA1_(8-9_11_13_17_19)        :...........................T...........................:165
Namdinh_(4_6_14_16)a          :...........................T...........................:165
China_(4_7_10_11_12)a         :...........................T...........................:165
China_(16_17_18_23_24)a       :...........................T...........................:165
Ria1_(2_4-5-16)b              :...........................T...........................:165
Ria1_(20-22-23-24)b           :...........................T...........................:165
Namdinh_(1_2_8_11)b           :...........................T...........................:165
Namdinh_(17_18_20)b           :...........................T...........................:165
China_(8_15)c                 :...........................T...........................:165
Ria1_(1-3_6_7_10_12)c         :...........................T...........................:165
Ria1_(14_15_18)c              :...........................T...........................:165
Namdinh_(9_19_23_24)c         :...........................T...........................:165
China_(6_9_13_14_19)c         :...........................T...........................:165

                                   .       180         .       200         .       220
                               GTTGGGGGCCATTTACTAGCACTATTCCCTTGATCTATTAAGGTTACTTCATTCT
AJ553909.1|_C_gigas_hap_C     :.......................................................:220
AJ553907.1|_C_angulata_hap_A  :..........................T..C.........................:220
Nandinh_(3_7_10-12-13-15)*    :.................G........T..C.........................:220
Namdinh_(5_21_22)**           :..........................T..C.........................:220
China_(1-2-3_20_21_22)**      :..........................T..C.........................:220
China_5***                    :..........................T............................:220
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```
RIA1_(8-9_11_13_17_19)       :..........................T..C.........................:220
Namdinh_(4_6_14_16)a         :..........................T..C.........................:220
China_(4_7_10_11_12)a        :..........................T..C.........................:220
China_(16_17_18_23_24)a      :..........................T..C.........................:220
Ria1_(2_4-5-16)b             :..........................T..C.........................:220
Ria1_(20-22-23-24)b          :..........................T..C.........................:220
Namdinh_(1_2_8_11)b          :..........................T..C.........................:220
Namdinh_(17_18_20)b          :..........................T..C.........................:220
China_(8_15)c                :..........................T..C.........................:220
Ria1_(1-3_6_7_10_12)c        :..........................T............................:220
Ria1_(14_15_18)c             :..........................T............................:220
Namdinh_(9_19_23_24)c        :..........................T............................:220
China_(6_9_13_14_19)c        :..........................T............................:220

                                       .       240         .       260         .
                              TGCTTTTGACTACTCTCCCAGTGTTAGCTGGAGGTCTTACTATACTTTTGACTGA
AJ553909.1|_C_gigas_hap_C    :.......................................................:275
AJ553907.1|_C_angulata_hap_A :...........................................G...........:275
Nandinh_(3_7_10-12-13-15)*   :.......................................... G...........:275
Namdinh_(5_21_22)**          :...........................................G...........:275
China_(1-2-3_20_21_22)**     :...........................................G...........:275
China_5***                   :..................................C........G...........:275
RIA1_(8-9_11_13_17_19)       :...........................................G...........:275
Namdinh_(4_6_14_16)a         :...........................................G...........:275
China_(4_7_10_11_12)a        :...........................................G...........:275
China_(16_17_18_23_24)a      :...........................................G...........:275
Ria1_(2_4-5-16)b             :...........................................G...........:275
Ria1_(20-22-23-24)b          :...........................................G...........:275
Namdinh_(1_2_8_11)b          :...........................................G...........:275
Namdinh_(17_18_20)b          :...........................................G...........:275
China_(8_15)c                :...........................................G...........:275
Ria1_(1-3_6_7_10_12)c        :...........................................G...........:275
Ria1_(14_15_18)c             :...........................................G...........:275
Namdinh_(9_19_23_24)c        :...........................................G...........:275
China_(6_9_13_14_19)c        :...........................................G...........:275
```

*: new *C. angulata* haplotype 1; **: new *C. angulata* haplotype 2; ***: new *C. angulata* haplotype 3.
The different letters (a, b, c) after the sample numbers indicate the different haplotypes.
The numbers following the name of lines e.g. Namdinh_(3_7_10_12_13_15) mean that they are the sample numbers belonging to Namdinh line.

The constructed COI phylogenic tree (Figure 2) shows that the *C. angulata* sequences, including those from the northern Vietnamese samples (from the three hatchery lines, Ria1, Namdinh and China), cluster separately from *C. gigas*.

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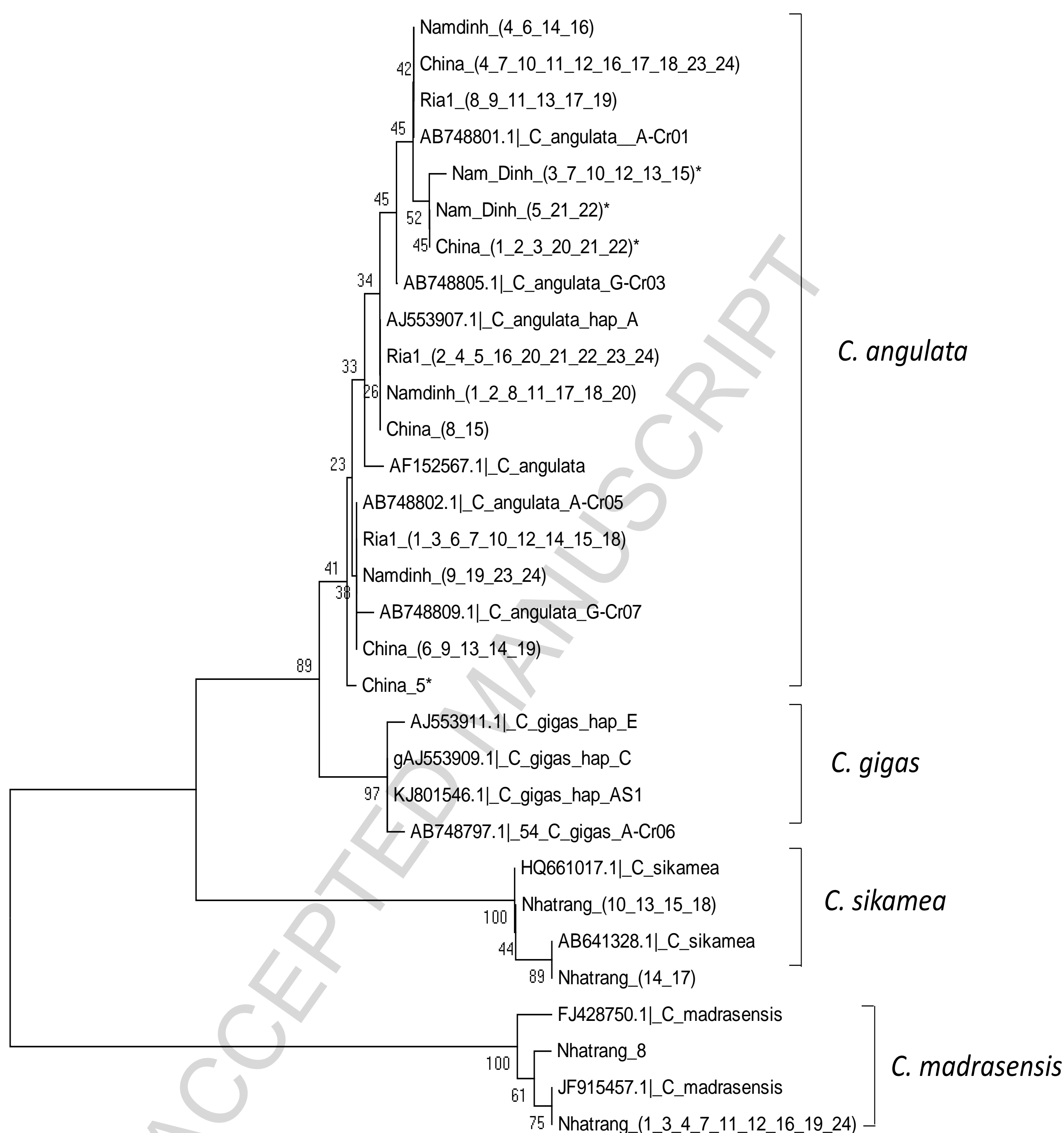
Figure 2. Phylogenic tree using MtDNA COI sequences.



Tree produced using phylogeny reconstruction analysis with Neighbour-joining statistical method, Bootstrap test with 500 replications, and mode with maximum composite

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likelihood on Mega6.0. Number on the branches indicate bootstrap values. Numbers inside the brackets are sample numbers
*: new *C. angulata* haplotypes.

4.3 Identification of hatchery oyster species in central Vietnam (Nhatrang)

Four different haplotypes were evident from the 16 Nhatrang samples (Table 3); they are obviously different from both *C. angulata* and *C. gigas*. Two haplotypes (haplotype 1 and 2, Table 3) corresponded to *C. sikamea* (AB641328; HQ661017), and the other two haplotypes corresponded to *C. madrasensis* (FJ428750: 01 sample; and JF915457: 9 samples, Table 3). Figure 2 shows that the local hatchery oysters from Nhatrang are obviously different from *C. gigas* and *C. angulata*, and there appear to be two species present: *C. sikamea* and *C. madrasensis*.

Table 3. Alignment of oyster sampled from Nhatrang with *Crassostrea* references.

```
                                     .        20         .        40         .
                              GTAGAAAACGGAGTTGGGGCAGGGTGAACAATTTACCCTCCTTTATCAACTTACT
AJ553909.1|_C_gigas_hap_C    :....................................................... : 55
AJ553907|_C_angulata_hap_A   :....................................................... : 55
AB641328.1|_C_sikamea        :..G....GG.............................C.....G..G....... : 55
Nhatrang_14-17(1)            :..G....GG.............................C.....G..G....... : 55
HQ661017.1|_C_sikamea        :..G....GG...................................G..G....... : 55
Nhatrang_10-13-15-18(2)      :..G....GG...................................G..G....... : 55
FJ428750.1|_C_madrasensis    :........T..G........T..A.....T........G..GC..........T. : 55
Nhatrang_8 (3)               :........T..G........T..A.....T........G..GC..........T. : 55
JF915457.1|_C_madrasensis    :........T..G........T..A.....T........G..GC..........T. : 55
Nhatrang_1-3-4-7 (4)         :........T..G........T..A.....T........G..GC..........T. : 55
Nhatrang_11-12-16-19-24 (4)  :........T..G........T..A.....T........G..GC..........T. : 55

                                60         .        80         .        100        .
                              CTTATCATGGAGTTTGTATAGACCTTGCAATTCTAAGCCTTCACCTTGCTGGTAT
AJ553909|_C_gigas_hap_C      :....................................................... :110
AJ553907|_C_angulata_hap_A   :................C...............T................C..... :110
AB641328.1|_C_sikamea        :................C..G............T....TT.A.....A........ :110
```

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```
Nhatrang_14-17 1)           :................C..G............T....TT.A.....A........ :110
HQ661017.1|_C_sikamea       :................C..G............T....TT.A.....A........ :110
Nhatrang_10-13-15-18 (2)    :................C..G............T....TT.A.....A........ :110
FJ428750.1|_C_madrasensis   :..........T...........T.....T...T....G..G..TT.G........ :110
Nhatrang_8 (3)              :..........C.................T...T....G..G..TT.G........ :110
JF915457.1|_C_madrasensis   :..........C.................T...T....G..G..TT.G........ :110
Nhatrang_1-3-4-7 (4)        :..........C.................T...T....G..G..TT.G........ :110
Nhatrang_11-12-16-19-24 (4) :..........C.................T...T....G..G..TT.G........ :110

                                    120       .       140       .       160
                             TAGCTCTATTTTCAGGTCAATTAATTTCATAGTAACGATTAGAAATATGCGATCT
AJ553909|_C_gigas_hap_C     :....................................................... :165
AJ553907|_C_angulata_hap_A  :...........................T........................... :165
AB641328.1|_C_sikamea       :...........................T........A...........A...... :165
Nhatrang_14-17 (1)          :...........................T........A...........A...... :165
HQ661017.1|_C_sikamea       :...........................T........A...........A...... :165
Nhatrang_10-13-15-18 (2)    :...........................T........A...........A...... :165
FJ428750.1|_C_madrasensis   :............T..A........C..T........C..C...........G... :165
Nhatrang_8 (3)              :............T..A........C..T.....G..C..C...........G... :165
JF915457.1|_C_madrasensis   :............T..A...........T.....G..C..C...........G... :165
Nhatrang_1-3-4-7 (4)        :............T..A...........T.....G..C..C...........G... :165
Nhatrang_11-12-16-19-24 (4) :............T..A...........T.....G..C..C...........G... :165

                                 .       180       .       200       .       220
                             GTTGGGGGCCATTTACTAGCACTATTCCCTTGATCTATTAAGGTTACTTCATTCT
AJ553909|_C_gigas_hap_C     :....................................................... :220
AJ553907|_C_angulata_hap_A  :..........................T..C......................... :220
AB641328.1|_C_sikamea       :...............T.G..G..G..T..C.......................TC :220
Nhatrang_14-17 (1)          :...............T.G..G..G..T..C.......................TC :220
HQ661017.1|_C_sikamea       :...............T.G..G..G..T..C.......................TC :220
Nhatrang_10-13-15-18 (2)    :...............T.G..G..G..T..C.......................TC :220
FJ428750.1|_C_madrasensis   :.....C..G............T.......C..G...........C..A.....T. :220
Nhatrang_8 (3)              :.....C..G............T.......C..G...........C..A.....T. :220
JF915457.1|_C_madrasensis   :.....C..G............T.......C..G...........C..A.....T. :220
Nhatrang_1-3-4-7 (4)        :.....C..G............T.......C..G...........C..A.....T. :220
Nhatrang_11-12-16-19-24 (4) :.....C..G............T.......C..G...........C..A.....T. :220

                                      .       240       .       260       .
                             TGCTTTTGACTACTCTCCCAGTGTTAGCTGGAGGTCTTACTATACTTTTGACTGA
AJ553909|_C_gigas_hap_C     :....................................................... :275
AJ553907|_C_angulata_hap_A  :...........................................G........... :275
AB641328.1|_C_sikamea       :.A.....A........T..............G..C........G........... :275
Nhatrang_14-17 (1)          :.A.....A........T..............G..C........G........... :275
HQ661017.1|_C_sikamea       :.A.....A........T..............G..C........G........... :275
Nhatrang_10-13-15-18 (2)    :.A.....A........T..............G..C........G........... :275
FJ428750.1|_C_madrasensis   :.......A..C.....T........G..A..G..C...........CC.T..... :275
Nhatrang_8 (3)              :.......A..C.....T........G..A..G..C...........CC.T..... :275
JF915457.1|_C_madrasensis   :.......A..C.....T.....A..G..A..G..C...........CC.T..... :275
Nhatrang_1-3-4-7 (4)        :.......A..C.....T.....A..G..A..G..C...........CC.T..... :275
Nhatrang_11-12-16-19-24 (4) :.......A..C.....T.....A..G..A..G..C...........CC.T..... :275
```

Number in the brackets indicates number of different haplotypes. †: GenBankaccess number.

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4.4 DNA microsatellite (taxonomic) differences between *C. gigas* and *C. angulata*

Available published data (Supplementary Table 3) indicated for the same DNA microsatellite locus, that the range of allele sizes found for both species overlap, except possibly for one loci (L48).

4.5 Genetic diversity among *C. angulata* lines in Vietnam considering both mtDNA and DNA microsatellite variation

4.5.1 Haplotypic diversity among lines

There were six haplotypes among the 72 samples from all the hatchery lines (Ria1, Namdinh and China), of which three are new haplotypes (haplotype i1, 2 and 3) that are different from all published haplotypes in GenBank (Table 4). Namdinh and China lines both have five haplotypes, including two of the three new haplotypes while RIA1 has the least haplotypes (three) and none of them are the newly found haplotypes. There were marginally more haplotypes in the combined samples across hatcheries than in the separate hatchery samples whether considering all 72 combined samples or taking a reduced set of 24 samples comprising eight random samples from each hatchery line (to standardize sample sizes -Table 4). However, there is no statistically significant differences between the number of different haplotypes in each of the hatchery line with either Pooled 72 or Pooled 24 samples.

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Table 4. Counts of mtDNA haplotype types in hatchery lines

Haplotype	Ria1 (24)	ND (24)	China (24)	Pooled (24)	Pooled (72)
Haplotype_I1	0	6†	0	4	6
Haplotype_I2	0	3	6	2	9
Haplotype_I3	0	0	1†	1	1
Haplotype A	9	7	2	7	18
Haplotype A-Cr01	6	4	10	5	20
Haplotype G-Cr07	9	4	5	5	18
Number of different haplotypes‡	3	5	5	6	6

sample sizes are given in parentheses.
† private haplotypes.
‡ for example, Ria 1 had only three different types of haplotypes

4.5.2 Microsatellite diversity among populations

Considering all nine DNA microsatellite loci, the number of alleles ranged from 4 to 19 alleles per locus per hatchery line (Table 5). The total number of different alleles was very similar among the three hatchery lines and the small differences were not statistically significantly different using Chi Square tests. However, each of the hatchery lines had statistically significantly fewer allele totals than that recorded for the “pooled 96” sample set using Chi Square tests; however the “pooled 96” data set, representing an amalgam of all the samples from all the hatchery lines, had three times greater sample size that each of the hatchery lines. Another pooled data set, “pooled 32” representing an amalgam of

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10-11 samples per hatchery line had total allele counts not statistically significantly different from any of the individual hatchery lines.

Table 5. Counts of different types of DNA microsatellite alleles among loci from Ria1, Namdinh and China lines

	Ria1 (32)	**Namdinh (32)**	**China (32)**	**Pooled 32[†] (32)**	**Pooled 96[‡] (94)**
CgEH42	17†	14	14	18	25
CgEH149	6	9	10	8	13
CgEH143	17	15	15	15	24
ucdCg153	9	12	10	13	16
L48	16	12	19	15	27
Kaki18	12	15	14	17	23
L8	10	13	13	13	17
Cgsili57	5	5	4	4	5
CgEH173	5	7	5	5	7
Total no. of different alleles	**97**	**102**	**104**	**108**	**157**
Av. No. different alleles per locus (Mean ±SE)	**10.78±1.57**	**11.33±1.12**	**11.52±1.61**	**12.0±1.61**	**17.44±2.50**
No. private alleles	**17**	**23**	**22**	**na**	**na**

sample sizes are given in parentheses.

†This means there were e.g. 12 different CgEH42 alleles found in 32 sample from Ria1

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Though allele counts were not very different among hatchery lines, there was a substantial proportion of “private alleles”, i. e. alleles that are only present in one hatchery line (Table 5). Analysis of molecular variance showed that 5.227 % of the total variance is among populations, which while small, was statistically significant ($P < 0.01$); pairwise F_{st} values were all minor but all statistically significant (China-Namdinh: 0.061, $P < 0.01$; China-RIA1: 0.047, $P < 0.01$; Namdinh-RIA1: 0.048, $P < 0.01$). Calculated Nei’s genetic distances suggested China and RIA 1 were most closely related (Nei’s distances were for China-Namdinh: 0.352; China-RIA1: 0.266, $P < 0.01$; Namdinh-RIA1: 0.313).

Each hatchery line often statistically significantly deviated from Hardy-Weinberg equilibrium, always by showing more homozygotes than expected (Supplementary Table 5).

Pedigree analyses using “Colony” software and considering all hatchery lines together, detected only one full sib pair (i.e. brothers or sisters) which was for two individuals from Namdinh. No full sib relatives were detected between hatchery lines. N_e was estimated to range from 25-68, 22-62 and 43-119 respectively for the China hatchery line, the Namdinh line and the RIA1 line.

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5 Discussion

5.1 Nucleotides that categorically distinguish *C. gigas* and *C. angulata*

The ability of mtDNA COI DNA percent sequence divergence data to separate *C. gigas* and *C. angulata* is controversial. On one hand, Boudry et al. (2003), Huvet et al. (2000a), Huvet et al. (2000b) and O'Foighil et al. (1998) suggest that it is an effective tool to differentiate between these two species. However, due to close genetic relatedness, the divergences for these two taxa was very slight e.g. 2-3% (Boudry et al., 2003) and 2.22 – 3.37% (Wang et al., 2010), far lower than 13-14% divergence between *C. agulata* and *C. hongkongensis* or 12-13% divergence between *C. gigas* and *C. nippona* (Boudry et al., 2003). In addition, Liu et al. (2011) reported 2.2-3.2% genetic distances between *C. gigas* and *C. angulata* while the variation of COI between other species ranges from 9-30% within the oyster genus or 25-32.5% between species from different genera based on pairwise divergence. Thus, the COI sequence divergence between *C. gigas* and *C. angulata* is lower than that normally evident between oyster species (Liu et al., 2011, Yu et al., 2010) raising concerns about the species status of *C. angulata* and *C. gigas* – are they one species with slightly divergent populations or two species?

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O'Foighil et al. (1998) were the first authors to attempt to differentiate the two taxa by looking for fixed nucleotide differences at particular positions. They reported 11 fixed nucleotide differences between *C. angulata* and *C. gigas*, and concluded that they were two species, not a single species as reported by many previous reports. However, the limitation of the work by O'Foighil et al. 1998 is that a small number of haplotypes were analysed (because few were available at that time) preventing a firm conclusion about the existence of exclusive nucleotides between *C. angulata* vs *C. gigas*. O'Foighil et al. 1998 used one *C. gigas* haplotype (AF152565) - identical to the most common known *C. gigas* haplotype C (Huvet et al., 2000b) and four *C. angulata* haplotypes: angul1, angul3, angul4 and angul2. Subsequently, Huvet et al., 2000b tested an analogous approach and suggested there could be specific mtDNA haplotypes unique to each species, such as haplotype A for *C. angulata* and haplotype C for *C. gigas*. However, the problem is that not all oyster individuals carry these haplotypes so they do not categorically separate the species, rather oysters may carry one of various haplotypes and to date 85 different haplotypes for *C. gigas* and 18 different haplotypes for *C. angulata* have been reported (Huvet et al., 2000b).

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This study builds on the work of O'Foighil et al. (1998) and analyses 222 COI references of *C. gigas* and 56 COI sequences of *C. angulata* from GenBank to reveal there are actually at least five nucleotides completely exclusive between the two taxa. Four out of the five exclusive nucleotides found in this study (namely, at position 88 -T vs C, position 105 -C vs T, position 138 -T vs C, and position 264 - G vs A on 293 bp residue) likely correspond to four distinct nucleotides discovered by O'Foighil et al. (1998) (namely, at position 368 –T vs C, position 384 –C vs T, position 417 –T vs C, 559 –G vs A on 588 bp residue).

If we assume our conclusion of these fixed differences is correct, and they are completely diagnostic of the two species, then we can resolve some possible confusion of species identities listed in Genbank. For example, GenBank access no: AJ553910.1 lists *C. gigas* as the species, but it has, according to our work, nucleotides exclusive for *C. angulata* and this 'error' would lead to confused phylogenic trees. Moreover, six COI sequences under name of *C. angulata* in GenBank that were sampled from the south of China obviously belong to *C. gigas* as they carry all of the five exclusive nucleotides for *C. gigas* (details in Supplementary Table 2).

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In this study, we confirm with extensive data that *C. angulata* and *C. gigas*, though very closely genetic related, can be categorically distinguished using mtDNA COI sequences differences at particular nucleotide positions. This is the first report on diagnostic nucleotides for identifying *C. angulata* and *C. gigas* based on analysis of large number of COI sequences.

5.2 Is the oyster cultured in Vietnam *C. gigas*?

In this study, we aligned COI sequences from 72 samples taken from three Vietnamese hatcheries (Ria1, Namdinh and China) with published COI sequences of *C. angulata* and *C. gigas* as references, and found all five exclusive nucleotides for *C. angulata* were present in all Vietnamese samples from the three lines. We conclude that cultured oysters from northern Vietnam are *C. angulata*, not *C. gigas*. Hybrids between the two taxa can be fertile (Huvet et al., 2002), and even a natural hybrid stock was established in some places e.g. Tokyo Bay (Iwasaki et al., 2009). If there had been mixing (hybridization) of the two species in Vietnam, then we would conclude all females in the hybrid crosses must have been *C. angulata*. This seems unlikely on the basis of probability and is not supported by the DNA microsatellite data (see following discussion).

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The DNA microsatellite results showed that primers from *C. gigas* will amplify sequences from the Vietnamese “angulata” samples suggesting genetic closeness; also the sizes of the amplified products from the Vietnamese “angulata” were in the same size range as those reported for *C. gigas*, again suggesting genetic closeness. No statistically significant excess of heterozygotes were detected from the microsatellite data for Vietnamese samples; heterozyote excess may have been expected if the Vietnamese samples were hybrids between *C. gigas* and *C. angulata*. Indeed, only cases of statically significant excesses of homozygotes were detected (discussed following).

Overall, the present findings are agreement with the conclusions of a previous study that an oyster population in Taiwan, the origin of the Vietnamese RIA1 line, is a pure breeding *C. angulata* population (Hsiao et al., 2009). Putative *C. angulata* was found in many places in the South of China Sea (Liu et al., 2011, Wang et al., 2010, Xia et al., 2009) including Fujian and Guangdong provinces from where the Chinese line in this study originated.

There is accumulating bio-geographic evidence that *C. gigas* may favour sea water temperatures cooler than those in the north of Vietnam (which are 20-35^{o}C), and so North Vietnam is not suitable for *C. gigas* to normally grow. Indeed attempts to grow out *C. gigas* in Vietnam during 2005/06 from broodstock imported from Australia failed (O'Connor and

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Dove, 2009). On the other hand, *C. angulata* favour warmer water temperatures than *C. gigas* (Orton and Awati, 1926) and so may have better tolerance to near tropical water temperatures found in northern Vietnam and southern China.

Of the three northern Vietnamese lines, the origin of two is known (China from China, and Ria1 from Taiwan), but the origin of the Namdinh line is unknown. A previous study using COI sequences by Binh and Quyen (2013) could not resolve the species identity of the Namdinh line, however, this matter was resolved in this study. Even so, the present study sheds no light on the precise origin of Namdinh line – it is possible it originated from either China or Taiwan.

5.3 What oyster species is being cultured in Nhatrang?

It appears that samples from Nhatrang belong to clades other than *C. angulata* or *C. gigas*. All COI sequences from Nhatrang aligned with those reported either for Kumamoto oyster *C. sikamea* or the Indian oyster, *C. madrasensis*, and were obviously different from *C. angulata* or *C. gigas*. This finding corresponds with the report of Dang and Quyen (2003) who concluded, using COI sequences, that *C. sikamea* was present in samples taken from Nhatrang (Khanh Hoa province). Hedgecock et al. (1999) reported wild *C. sikamea* occurred on the eastern and northern shores of Japan and it is assumed *C. sikamea,*

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originates from southern Japan and is now present in many places in the world including China, Korea, USA and some European countries (Yu and Li, 2012). *C. sikamea* looks like *C. gigas*; these species cannot be distinguished just by shell morphology (Hedgecock et al., 1999). Data from the present study shows *C. sikamea* and *C. gigas* are not as close, according to DNA sequence divergence, as *C. gigas* and *C. angulata.* Boudry et al. (1998) also reported a greater genetic distance between *C. sikamea* and *C. gigas* than between *C. angulata* and *C. gigas* using both large ribosomal subunit (16SrDNA) and COI fragments. Hybridization experiments align with the DNA taxonomy: sperm of *C. gigas* can fertilize *C. sikamea* eggs to generate viable hybrid offspring but the reciprocal cross does not produce offspring (Banks et al., 1994). Although *S. sikamea* is native to Japan, how it arrived in Vietnam is still unknown.

Another species found at Nhatrang, according to COI sequence analyses, is the Indian oyster,*C. madrasensis.* Wild *C. madrasensis* are found in estuaries, bays and backwaters along the southeast and southwest coasts of India (Sanil et al., 2012). This species was identified from the Malaysian peninsula water using mtDNA COI (Suzana et al., 2011). The origin of *C. madrasensis* in Vietnam remains unclear.

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5.4 Genetic diversity of *C. angulata* cultured lines in Vietnam considering mtDNA hapotypes and DNA microsatellite alleles

A total of three new *C. angulata* haplotypes were found: haplotype i1 and i2 in Namdinh and halotypes i2, i3 in the China line, but the frequencies of these haplotypes are low in these lines, namely 8.3%; 12.5% and 1.4% for *C. angulata* haplotypes i1, i2 and i3, respectively. Finding a new haplotype is relatively rare in *C. angulata* lines. Huvet *et al.*(2000b) used COI sequences (710 nucleotides) to analyse nine populations (five populations from Europe and three populations from Asia), but no new haplotypes other than those previous reported were observed even when a large geographic zone was considered. Boudry et al. (1998) found haplotype A was the most common one (76%) in European *C. angulata* samples studied, however in this study, only 25% (18 out of 72 samples of *C. angulata*) carry *C. angulata* haplotype A.

Notwithstanding finding new haplotypes, there were relatively few different haplotypes (3 - 5 haplotypes) in each of the hatchery lines in this study. Relatively low levels of haplotypic diversity (only 6 haplotypes) were reported in *C. angulata* populations by Boudry et al. (1998) and Huvet et al. (2000b), in spite of numerous sampling sites (25 locations) around the world including European and Asian locations. Accordingly, the parsimonious reasoning is that the low number of haplotypes is probably not evidence of

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inbreeding in the Vietnamese lines *per se*, but a reflection of the overall haplotype diversity in the species.

DNA microsatellite allelic variation of all three lines (Ria1, Namdinh and China), considering all nine loci, was relatively high, averaging about 11 alleles per locus. This finding aligns with previous accounts of relatively high nuclear allelic genetic variability in *C. angulata* populations compared with other Crassostrea species based on allozyme data (Buroker al., 1979). Allele numbers in the C*. angulata* hatchery lines were about twice that we found for hatchery lines of Sydney Rock Oysters in Australia (In *et al.*, 2016).

Why exactly there appears to be in our study greater diversity of DNA microsatellites alleles than mtDNA haplotypes is unclear, but Knibb et al. (2014) speculated, notwithstanding different mutation rates, that mitochondrial haplotypes in general may be less diverse than nuclear alleles in hatchery aquacultured stocks because there are two possible given nuclear chromosomes but only one effective mtDNA chromosome, so bottlenecks may reduce mtDNA diversity more than nuclear diversity. Also our sample sizes used for the mtDNA sequences were a little less than those for the DNA microsatellites.

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DNA microsatellite genotypes at several or more loci often showed statistically significant excesses of homozygotes which is sometimes considered evidence of mating of relatives, i. e. inbreeding and high levels of kinship. Alternatively, homozygote excess can be due to the presence of null alleles because null alleles may produce pseudo-homozygotes and scoring errors; several loci considered here seemed to have reasonable proportions of nulls. Many other studies using microsatellite markers have also reported null alleles, particularly in studies of mollusc species (Astanei et al., 2005, Carlsson et al., 2006, In et al., 2016, Li et al., 2003, Reece et al., 2004). Mixed populations, prior to intermating, could also lead to homozygote excess, but mixed populations *C. angulata* and *C. gigas* before mating would have been detected by the presence of *C. gigas* mtDNA haplotypes, and they were not present. Given the high allelic diversity, and given that estimated effective population sizes were moderate, and given only one full sib pair was detected in a hatchery line, the parsimonious explanation for the homozygote excess is the presence of nulls.

Genetic loss among hatchery lines has been reported in many fecund species including Eastern oyster, *C. virginica* (Carlsson et al., 2006, Yu and Guo, 2004), a 4th generation selected *C. gigas* line (Appleyard and Ward, 2006) and mass selected Sydney Rock oyster *S. glomerata* (In et al., 2016). A 22-44% loss of alleles was reported for hatchery bred

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cultured silver-lipped pearl oysters, *Pinctada maxima* and up to 40% of the progeny were produced from just a single family despite a large cohort of 28 parents (Lind et al., 2009). In *Ostrea edulis*, mass spawning resulted in only a limited number of parents contributing to the next generation, leading to a highly inbred population comprising mostly half or full-sib offspring (Launey et al., 2001). A substantial loss of allele number in F_1 progeny of hatchery bred abalone was reported for *Haliotis rubra* in Australia and *H. midae* in South Africa (Evans et al., 2004). Accordingly, a reasonably expectation would be for reduced variation in Asian hatchery lines of *C. angulata* compared with wild stock. Unfortunately, in this study, no wild Asian reference samples were available with which to compare the hatchery lines although data from wild European mtDNA haplotypes did not indicate loss of diversity in the Asian hatcheries (see data of Huvet et al. 2004). Without wild references, we can compare a synthetic pooled population, pooling across hatchery samples, which may approximate the wild stock if the hatchery lines are independently derived from the wild (see Knibb et al. 2014). However neither allele or haplotype numbers were statistically significantly lower in the hatchery lines than in the pooled samples (after adjustment to standardize sample sizes).

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Lack of evidence for diversity loss is rather contrary not only to theoretical genetic expectations but also to trends evident almost universally in other fecund hatchery bred species, including *S. glomerata* (In et al., 2016a) and banana shrimp (Knibb et al., 2014). It could be that *C. angulata* farmers often use different hatchery populations from different sources when breeding. If there really is an ongoing practice of sharing genetic material among lines, effectively line crossing, then one would predict possible conservation of genetic diversity (see In *et al.*, 2016, Knibb *et al.*, 2014). This supposition is perhaps supported by our observations of moderately sized effective population sizes (e.g. 43-119 for the RIA1 line) and rather contrasts those estimates for other highly fecund captive bred species such as an Ne of 9-36 for *C. gigas* (Appleyard and Ward, 2006), 21-43 for *S. glomerata* after three to seven generations of captive breeding (In et al., 2016) and 5-25 for white leg shrimp *Penaeus vannamei* after 14 generations (De Donato et al., 2005). Presumably, without ongoing mixing between hatcheries as suspected for *C. angulata*, regular hatchery breeding practices (using few broodstock), differential reproductive success or mass selection (for offspring of just a few elite families) would typically result in just a few families contributing to the next generation (Lallias et al., 2010; Knibb et al. 2014; Boudry et al., 2002, Goyard et al., 2003). The estimates of N_e in this study generally indicate the three hatchery lines are reaching the minimum N_e of 50 recommended by Bijma et al. (2000) and Ponzoni et al. (2010) required for a sustainable selective breeding

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program. This effective population size limits the level of inbreeding to around 1% per generation, which is an acceptable level for maintaining long term genetic response (Frankel and Soule, 1981, Granleeseet al., 2015).

5.5 Conclusions

a) Five diagnostic nucleotides in mtDNA COI sequences have been confirmed in this study and can be used to distinguish *C. angulata* from *C. gigas*.

b) The oysters cultured in northern Vietnam are *C. angulata*, not *C. gigas* whereas local hatchery oyster samples in Nhatrang of Vietnam are a mixture of two oyster species: *C. sikamea* or *C. madrasensis*.

c) All three hatchery oyster lines (Ria1, Namdinh and China) still maintain adequate genetic diversity to form the basis of a breeding program.

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Highlights

The relationship and taxonomy of two closely related species, namely Pacific and Portuguese oysters has been controversial. Here we show there are five diagnostic nucleotides in mtDNA COX1 sequences that can be used to distinguish these species. We also show that levels of diversity among Vietnamese hatchery Portuguese oyster stocks, assessed using DNA microsatellites and mtDNA haplotypes, was unexpectedly high.

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