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

Vu Van In [a,d], Wayne O'Connor [c], Vu Van Sang [a], Phan Thi Van [b], Wayne Knibb [d,*]

[a] Northern National Broodstock Center for Mariculture, Research Institute for Aquaculture No.1, Cat Ba Islands, Hai Phong, Vietnam
[b] Research Institute for Aquaculture No.1 (RIA1), Dinh Bang, Tu Son, Bac Ninh, Vietnam
[c] Industry and Investment NSW, Department of Primary Industry, Port Stephens Fisheries Institute, Taylors Beach, NSW 2316, Australia
[d] University of the Sunshine Coast, Maroochydore, Queensland 4558, Australia

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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 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 precedent with other hatchery bred highly fecund oyster species, is a little unexpected. It is speculated that local hatchery practises 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.

1. Introduction

Edible oyster aquaculture in Vietnam, largely based on introduced species, has expanded very rapidly from no production in 2007 to over 7000 t/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.

C. gigas and *C. angulata* are morphological similar (O'Connor and Dove, 2009). However, in the past, *C. angulata* and *C. gigas* were

* Corresponding author at: Faculty of Science, Health and Education, University of the Sunshine Coast, Locked Bag 4, Maroochydore QLD 4558, Australia.
E-mail addresses: vuvanin@ria1.org (V.V. In), wknibb@usc.edu.au (W. Knibb).

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considered to be different species by Thunberg 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, 2002). More recently, they were thought to be a single species due to their phenotypic similarity and ability to produce fertile hybrid offspring (Huvet, 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; O'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 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 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 practises 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 to 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?

2. Material and methods

2.1. Biological samples

Samples were taken from animals from four groups at three growout sites of Vietnam (Vandon, Catba and Nhatrang (Fig. 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 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.

2.2. 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 V 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 260/280 nm. Good DNA templates were then diluted in molecular grade water (Amresco) to 25 ng/μl^{-1}.

2.3. 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 25 μl 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).

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.

2.4. 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 12.5 μL PCR reactions containing