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Artificial interspecific backcrosses between the hybrid of female *Crassostrea hongkongensis* × male *C. gigas* and the two parental species

Yuehuan Zhang [a,b], Yang Zhang [a,b], Jun Li [a,b], Shu Xiao [a,b], Zhiming Xiang [a,b], Zhaoping Wang [c], Xiwu Yan [d], Ziniu Yu [a,b,*]

[a] Key Laboratory of Tropical Marine Bio-resources and Ecology, Guangdong Provincial Key Laboratory of Applied Marine Biology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, China
[b] South China Sea Bio-Resource Exploitation and Utilization Collaborative Innovation Center, China
[c] Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao 266003, China
[d] Engineering Research Center of Shellfish Culture and Breeding of Liaoning Province, College of Fisheries and Life Science, Dalian Ocean University, Dalian 116023, China

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ABSTRACT

To explore the possibility of performance improvement in the hybrids of *Crassostrea hongkongensis* ♀ × *Crassostrea gigas* ♂, backcross progeny and F_2 hybrids were produced and evaluated. Three sets of replicates were successfully generated; each consisted of single crosses (HH, GG and HG), two female backcrosses between female hybrids and two male pure species (Hg/H and Hg/G), two male backcrosses between male hybrids and two female parents (H/Hg and G/Hg) and F_2 hybrids (Hg/Hg). Fertilization, survival, growth and gonad development of these experimental groups were evaluated. All backcross groups exhibited high fertilization levels; however, the female backcrosses (Hg/H and Hg/G) and F_2 hybrids exhibited a low hatching rate. Survival and growth superiority of the backcross progeny was observed from the larval stage to the adult stage, although outbreeding depression of the F_2 hybrids occurred. Interestingly, all backcross progeny and F_2 hybrids were fertile with functional gametes and normal sex ratios when fully matured. The genetic recombination of the backcross progeny and Mendel's genetic segregation of the F_2 hybrids were detected with the nuclear gene marker in the backcross. Our results revealed that artificial interspecific backcrosses are successful, and the backcross progeny are viable, fertile, and fast-growing, and therefore hold promise for potential utilization in aquaculture as a new oyster stock resource.

Statement of relevance: All authors agree that the author list is correct in its content and order, and we have no conflict of interest to declare.

1. Introduction

A minimum of five *Crassostrea* oyster species occur naturally along the coast of China: *Crassostrea gigas*, *Crassostrea angulata*, *Crassostrea hongkongensis*, *Crassostrea ariakensis* and *Crassostrea sikamea* (Wang et al., 2008). Several of these species overlap in distribution. In northern China, *C. ariakensis* coexists with *C. gigas*. In central and southern China, *C. ariakensis*, *C. hongkongensis*, *C. angulata*, and *C. sikamea* can be found in the same estuaries in various richness (Guo, 2009).

Among the five cupped oysters, *C. hongkongensis* (the Hong Kong oyster) and *C. gigas* (the Pacific oyster) are two of the three economically and ecologically important species in China. These oysters are native to the coastal waters of the South China Sea and the Yellow Sea, respectively (Zhang et al., 2012). *C. hongkongensis* is one of the most important endemic and cultured oyster species due to its high market value, with a cultivation history of nearly one thousand years and an annual production of over 1.3 million metric tons along the coastal waters of the South China Sea (Boudry et al., 2003; Huo et al., 2014; Lam and Morton, 2003; Li and Yu, 2010; Wang et al., 2004). *C. hongkongensis* is distributed from the Fujian to Guangxi provinces, with populations centered in the Guangdong province (Guo et al., 2006). *C. gigas* is the most important species due to its good economic traits and worldwide aquaculture. *C. gigas* has a very long aquaculture history in northern China; today, it is mainly farmed in the Liaoning and Shandong provinces, with an annual production of 0.8 million metric tons (Gaffney and Allen, 1993; Guo et al., 1999, 2006).

C. hongkongensis is native to the coastal waters of the South China Sea and exhibits adaptations or tolerance to estuary salinity fluctuations and the high temperature of the subtropical zone; these traits may be useful for stock improvement (Zhang et al., 2012). For example, the tolerance to high temperature found in *C. hongkongensis* would be a desirable trait for *C. gigas*, which often suffers from summer mortality. Furthermore, successful transfer of the tolerance for higher salinity of *C. gigas* to *C. hongkongensis* could potentially expand its culture

* Corresponding author at: South China Sea Institute of Oceanology, Chinese Academy of Sciences, 164 West Xingang Road, Guangzhou 510301, China.
E-mail address: carlzyu@scsio.ac.cn (Z. Yu).

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boundaries to higher salinity waters in China. Thus, hybridization between *C. hongkongensis* and *C. gigas* is potentially useful for the genetic improvement of these two important aquaculture species. We previously attempted this hybridization, but the results were not ideal due to slow growth and partial male fertility.

Generally, interspecific backcrosses can maintain some desired genetic complexes that are already present in the adapted genotypes while allowing recombination between exotic and adapted genomes. Such genomic rearrangements may disrupt epistatic interactions that confer fitness in specific environments and gene interactions that are independent of the environment (Lynch, 1991). Thus, backcrosses can potentially broaden the genetic basis of a starting population for use in a selective breeding program by combining beneficial alleles. However, research addressing the success and fitness of backcrossed hybrids in aquatic animals is scarce (Arnold, 1997; Edmands, 1999). Several studies of interspecific backcrosses have focused on salmon (Johnson and wright, 1986), bass (Gomelsky et al., 2004), catfish (Argue et al., 2003, 2014), tilapia (Rajaee et al., 2014; Tave et al., 1990), and carp (Anil et al., 2014), which occasionally produce spontaneous polyploidy and gynogenesis progeny during the backcrossing process (Arai, 2001; Argue and Dunham, 1999; Bartley et al., 2001; Hulata, 1995, 2001).

To further explore the possibility of the application of diploid hybrids, we conducted an interspecific backcross between partially fertile hybrids and two parental species. The phenotypic characteristics (including fertilization, survival, growth and gonad development) of the backcross progeny and their F_2 generation were analyzed and compared in this study.

2. Materials and methods

2.1. Production of backcross progeny

Sexually mature yearling *C. hongkongensis*, *C. gigas* and their hybrid (*C. hongkongensis* ♀ × *C. gigas* ♂) oysters were derived from the previous report (Zhang et al., 2012). Due to a high degree of sterility of the hybrids, fertile individuals were chosen through gamete check under a microscope (Zhang et al., 2014). Gametes from mature broodstocks were obtained by dissection. After gamete collection, a piece of adductor muscle from each selected oyster was fixed in 95% ethanol for subsequent confirmation with genetic markers (H. Wang and Guo, 2008; Y. Wang and Guo, 2008).

Three sets of replicates were successfully performed. Each set of replicate consisted of a cross of a single male with a single female (HH — *C. hongkongensis* ♀ × *C. hongkongensis* ♂, GG — *C. gigas* ♀ × *C. gigas* ♂ and HG — *C. hongkongensis* ♀ × *C. gigas* ♂), two male backcrosses between male hybrids and female pure species (H/Hg — *C. hongkongensis* ♀ × hybrids ♂, G/Hg — *C. gigas* ♀ × hybrids ♂), two female backcrosses between female hybrids and male pure species (Hg/H — hybrids ♀ × *C. hongkongensis* ♂), and F_2 hybrids (Hg/Hg — hybrids ♀ × hybrids ♂) that were conducted.

Egg suspensions were passed through an 80-μm nylon screen to remove tissue debris, and then the eggs were captured and washed on a 20-mm screen. For each species, eggs from one female were divided equally into three 2-l beakers and examined under a microscope to ensure that no uncontrolled fertilization had occurred, as indicated by the absence of polar bodies. Subsequently, the eggs in the three beakers were fertilized with sperm from one *C. hongkongensis*, *C. gigas* and hybrid male, respectively. Thus, 3 × 3 interspecific crosses and backcrosses were created without GH crosses (*C. gigas* ♀ × *C. hongkongensis* ♂) because *C. gigas* eggs cannot be fertilized with *C. hongkongensis* sperm (Zhang et al., 2012, 2014). Fertilization was conducted within 60 min post-gamete collection to a density of approximately 20–25 sperm surrounding an egg in each group in filtered seawater with a salinity of 25 ppt. The experiment was repeated three times using three sets of parents. Fertilized eggs were sampled and held in beakers to evaluate the fertilization success and survival to D stage. The remaining fertilized eggs were counted and pooled separately into a 60-l bucket for hatching with gentle aeration at a density of 30–40 eggs/ml. Zygotes developed in filtered seawater were maintained at 26.3 to 27.5 °C with a salinity of 24 to 27 ppt.

2.2. Larval rearing, spat nursery and grow-out

At 30 h after fertilization, D larvae from each group were collected through a 40-μm screen and reared in a 60-l bucket at a density of 5 larvae/ml. Larvae were fed with *Isochrysis galbana* at days 0–6 and a mixture of *Platymonas subcordiformis* and *I. galbana* (1:1) after day 6. Feeding was gradually increased from 6000 to 80,000 cells ml^{-1} day^{-1}. The seawater was maintained at 26.2–27.8 °C and was completely changed every three days. When most larvae developed an eyespot and foot, strings of corrugated plastic plates were placed in the buckets as substrates. Newly settled spat were nursed in the buckets for two weeks to prevent contamination by wild spat.

Subsequently, all spat were transported to concrete tanks (8.0 × 6.0 × 1.5 m) and fed with *Chlorella vulgaris* at 80,000–10,000 cells ml^{-1} day^{-1}. Thirty percent of the water was changed once daily. At day 60, the spat were detached, randomly transferred into spat bags and hung on suspended long lines in a large shrimp pond filled with regular seawater without artificial feeding in Zhuanghe Gulf, Dalian (Liaoning province) (longitude, 122.99; latitude, 39.66). The spat bags were periodically changed from small to large mesh sizes. During the grow-out period from July to July of the following year, the water temperature varied from −1.0 to 30.5 °C and the salinity from 23 to 30 ppt.

2.3. Parentage identification

2.3.1. Ploidy analysis

At 30 h post-fertilization, 10,000–20,000 D larvae from each group were collected and pooled for flow cytometry analysis to determine their composite ploidy (Jankun et al., 2007). At day 15, 5000–10,000 eyespot larvae from each group were randomly sampled and pooled to measure their relative DNA content. In preparation for flow cytometry, larvae were concentrated into a 2 ml suspension and then pelleted by centrifugation at 1500 ×*g* for 10 s in a microcentrifuge. Supernatant seawater was withdrawn and 1.0 ml of DAPI/detergent/DMSO solution was added to the tube. Larvae were re-suspended by vortexing. Larval suspensions were frozen at −80 °C for at least 1 h. Subsequently, the larvae were thawed and disaggregated by repeated aspiration with a 2-ml syringe fitted with a 26G needle. Cell suspensions were passed through a 25 μm screen immediately before the assay. Ploidy levels were calculated relative to a diploid standard from the gill cells of hybrid spat (Eudeline et al., 2000).

To further observe the occurrence of spontaneous polyploidy, 100 gill fragments were randomly sampled from each group at day 360 for ploidy analysis. Adults were placed into separate tubes and crushed gently. Approximately 1.0 ml of DAPI solution was added to each tube, and the contents were vortexed and screened through a 25 μm Nytex screen prior to flow cytometry analysis. Finally, a comparison of mean relative DNA content among hybrids, backcrossed progeny and their parental species was tested by a χ^2 test using SPSS 18.0.

2.3.2. Molecular confirmation

For each experimental group, the parents, hybrids and backcross progeny were examined at day 360, while the number of each group was ranged from 67 to 210. DNA was extracted from ethanol-fixed samples from the progeny and their parents using the TIANamp Marine Animals DNA Kit (Tiangen). A set of species-specific COI (cytochrome oxidase I) primers was applied to identify the two oyster species (Y. Wang and Guo, 2008). The universal PCR primers for mitochondrial COI were: 5′-GGTCAACAAATCATAAAGATATTGG-3′ (LCO1490) and 5′-TAAACTTCAGGGTGACCAAAAAATCA-3′ (HCO-2198). A species-specific