

IDENTIFICATION OF *CRASSOSTREA ARIAKENSIS* AND RELATED OYSTERS BY MULTIPLEX SPECIES-SPECIFIC PCR

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ABSTRACT Genetic markers are needed for rapid and reliable identification of oysters. In this study, we developed multiplex genus- and species-specific PCR markers for the identification of oysters from China. We used the mitochondrial cytochrome oxidase I (COI) and nuclear 28S ribosomal RNA genes for marker development. DNA sequences from different species were obtained from GenBank or by direct sequencing. Sequences were aligned, and genus- and species-specific nucleotides were identified. Primers were designed for genus/species-specific amplification to generate fragments of different sizes. A multiplex set of genus- and species-specific primers from the 28S gene was able to separate *C. ariakensis* and *C. hongkongensis* from other species and assign oysters to four genera. A set of species-specific COI primers provided positive identification of all five *Crassostrea* species from China, *C. ariakensis*, *C. hongkongensis*, *C. angulata*, *C. gigas*, and *C. sikamea* in a single PCR. The multiplex PCR assays do not require fluorescence-labeling or post-PCR enzyme digestion, providing a simple, fast and reliable method for the identification of oysters from China.

KEY WORDS: Oyster identification, species-specific PCR, cytochrome oxidase I, 28S rRNA, Ostreidae, Suminoe oyster, *Crassostrea ariakensis*

INTRODUCTION

Shell morphology of oysters is plastic and sensitive to environmental influence. It cannot be used as reliable characters for species identification. However, much of the oyster classification to date is based on shell characteristics. This has led to many errors and confusions in oyster classification. The problem is particularly pronounced in China, where a large number of oyster species occur (Zhang & Lou 1956, Li & Qi 1994, Guo et al. 1999, Wang et al. 2004). For example, three different species has been described under the name of *Crassostrea rivularis* (Gould, 1861) in China, and the species status of some species is questionable (Li & Qi 1994, Wang et al. 2004). Some of the confusions have recently been resolved using DNA sequence data (Lam & Morton 2003, Wang et al. 2004). However, DNA sequencing is time consuming and not suited for routine identification. Simple and effective genetic markers are needed.

Genetic markers have been developed and used for the identification of some oyster species (Liu & Dai 1998, Boudry et al. 1998, Klinbunga et al. 2003, Klinbunga et al. 2005, Cordes et al. 2005, Wang & Guo 2008). The available markers are either insufficient in species coverage or technically too complicated for routine identification of oysters in China. The development of simple, fast, and effective assays for species identification is essential in understanding the distribution of oysters in China, especially that of *C. ariakensis* (Fujita, 1913) and related species. *C. ariakensis* is being considered for possible introduction to Chesapeake Bay in the United States. Genetic markers are needed to identify and study *C. ariakensis* and to prevent accidental introduction of unwanted species should the proposed introduction is approved.

Among different types of genetic markers, multiplex species-specific PCR is probably the most effective, efficient, and yet practical approach for species identification. It can potentially identify several species in one reaction without further manip-

ulation. Multiplex species-specific PCR has been used for the identification of other bivalve species (Hare et al. 2000, Larsen et al. 2005). Species-specific PCR depends on the identification of species-specific DNA sequences as priming sites. We previously sequenced the cytochrome oxidase I (COI), 16S and 28S ribosomal RNA genes in over 150 oysters collected from China's coast (Wang 2004). From the sequence data, we were able to identify five *Crassostrea* and some *Ostrea* and *Saccostrea* species. In this study, we used those and other sequences from GenBank, and designed multiplex genus- and species-specific PCR primers for species identification. The multiplex PCR assays can identify all five *Crassostrea* species from China in one PCR and assign most oysters to their genera.

MATERIALS AND METHODS

Primer Design

We used mitochondrial COI and nuclear 28S genes to design genus- and species-specific PCR primers. Sequences from all known oyster species were downloaded from GenBank and combined with sequences that we obtained from 150+ oysters from China. When available, all different haplotypes of the same species were included. The 28S sequences included these for *C. ariakensis* (AY632553), *C. gigas* (AY632555), *C. sikamea* (AY632554), *C. hongkongensis* (AY632552), *C. nippona* (AB110095), *C. belcheri* (Z29545), *C. rhizophorae* (AF137049), *C. virginica* (AF137050), *Saccostrea cucullata* (Z29553), *S. commercialis* (Z29552), *Ostrea edulis* (Z29551), *O. angasi* (AF137046), *O. algoensis* (AF137041), *O. chilensis* (AF137045), and *O. denselamellosa* (AF137043). The COI sequences included that for *C. ariakensis* (AY632564, AY632559), *C. angulata* (AF152567), *C. gigas* (AF152565), *C. hongkongensis* (AY160746, AY632556), *C. sikamea* (AF152568), *C. virginica* (AF152566), *C. nippona* (AF300616), *C. iredalei* (AY038078), *C. belcheri* (AY038077), *S. cucullata* (AY038076), *S. kegaki* (AB076910), *O. edulis* (AF1206510),

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O. angasi (AF1122870), *O. aoupouria* (AF1122880), and *O. chilensis* (AF1122890).

Sequences were aligned using BioEdit to identify genus and species-specific nucleotide positions. For each of the two genes, we included two universal outer primers that could amplify in all oyster species (Folmer et al. 1994, Park & Ó Foighil 2000). The inner primers were genus or species-specific and designed to generate different size fragments in different species or genera. To enhance specific amplification, a mismatch was introduced at the 3' end of some primer (Little 1997, Ye et al. 2001). No mismatch was used at sites where two or more nucleotides at the 3' end of the primers were specific.

Species Collection and DNA Extraction

Fourteen oyster species were used for marker validation in this study (Table 1). Ten to 100 samples were tested for each species. Whenever possible, individuals from different populations of the same species were included. Most of the species were obtained from places where they have well-recognized species identity. Two samples of *C. ariakensis* were included: one from Rutgers University (originated from Japan via Washington State) and China. *Crassostrea gigas* samples were from cultured populations in China and the USA. For *C. ariakensis*, *C. angulata*, *C. gigas*, *C. hongkongensis*, *C. sikamea*, and *S. echinata* samples from China, at least eight individuals in each species were previously identified using 16S, COI, and 28S

sequences (Wang 2004), and others were obtained from the same populations and samplings. All *C. ariakensis* and *C. hongkongensis* from China were identified according to Wang et al. (2004). Other species were from wild populations and identified by people who supplied them.

PCR Amplification

DNA was extracted from ethanol fixed adductor muscle using the Qiagen DNeasy kit. PCR was performed in 25 μ L using a PE GeneAmp 9700 thermal cycler. PCR conditions were optimized by testing different primer and Mg⁺⁺ concentrations, and annealing temperatures. The optimized PCR contained: 2.0 mM MgCl₂, 150 μ M of each dNTP, 0.6 μ M each outer primer, 0.4 μ M each COI and 28S inner primer, 1 unit *Taq* polymerase (Promega, USA), 20 ng template DNA, 2.5 μ L of $\times 10$ buffer, and water to 25 μ L. The 28S fragments were amplified using the following protocol: an initial denaturing at 94°C for 4 min; 30 cycles of denaturing at 94°C for 40 s, annealing at 60°C for 40 s and extension at 72°C for 1.25 min; plus a final extension at 72°C for 10 min. The COI fragments were amplified with an initial denature at 95°C for 2 min; 30 cycles of 95°C for 1 min, 51°C for 1 min and 72°C for 1 min; and a final extension at 72°C for 5 min. The two sets of universal (or outer) primers were used as positive controls for COI and 28S genes. A negative control (no template) was included during each PCR run. All PCR products were separated on 1.5% agarose gels containing 0.2- μ g/mL ethidium bromide, and visualized on a UV transilluminator. Different species were identified by the length of their PCR products.

TABLE 1.
Oyster species and sample size used for the validation of multiplex genus- and species-specific PCR.

Species	Origin	Number
<i>C. ariakensis</i>	Weifang, Shandong Province, China	50
	Rutgers, NJ, USA	50
<i>C. angulata</i>	Putian, Fujian Province, China	50
	Xiamen, Fujian Province, China	50
	Rutgers, NJ, USA	50
<i>C. gigas</i>	Qingdao, Shandong province, China	50
	Rutgers, NJ, USA	50
<i>C. hongkongensis</i>	Shenzhen, Guangdong province, China	50
	Beihai, Guangxi province, China	50
<i>C. sikamea</i>	Zhoushan, Zhejiang province, China	50
	Oregon, USA	30
<i>C. virginica</i>	Delaware Bay, Arnolds Point Shoal, USA	20
	Grand Isle, LA, USA	20
<i>C. rhizophorae</i>	Florida, USA	20
<i>S. echinata</i>	Zhanjiang, Guangdong province, China	20
<i>S. commercialis</i>	Australia	10
<i>O. equestris</i>	North Carolina, USA	10
<i>O. conchaphila</i>	Washington State, USA	10
<i>O. edulis</i>	Maine, USA	10
<i>O. angasi</i>	Australia	10
<i>H. hyotis</i>	Hainan, China	10

RESULTS

Separation of Genera Using 28S Gene

The 28S gene is highly conserved among species of Ostreidae. Not many nucleotide positions are species-specific or can be used for species-specific amplification, but several nucleotide positions are genus-specific. A *Crassostrea*-specific primer, 28Ca1489r (Table 2), was designed to target nucleotide positions 465 and 466, which are occupied by CA in all *Crassostrea* species (except *C. virginica* and *C. rhizophorae*) and AT in *Saccostrea* and *Ostrea* species (Fig. 1). Nucleotide position 140 also separates *Crassostrea* (T) from *Saccostrea* and *Ostrea* species (A), which was used to design a *Saccostrea/Ostrea*-specific primer, 28Soa166r (Fig. 1). The primer also covered a diagnostic nucleotide position at 145: G in *Saccostrea/Ostrea* and C in *Crassostrea* species. A mismatch at position 141 was introduced to reduce nonspecific amplification in *Crassostrea* species (Table 2). A *Saccostrea*-specific primer, 28Sa1923, was designed by targeting C at position 893 (G for other genera) and an insertion of T at position 896 (Fig. 1; Table 2).

Only two species have distinctive nucleotides in the 28S fragment that can be used for primer design. *Crassostrea ariakensis* has a unique nucleotide C at position 628, which is G in all other species; it also has a C at position 630, which is occupied by T in other *Crassostrea* species or G in *Saccostrea* species (Fig. 1). Consequently, a *C. ariakensis*-specific primer, 28Scar650, was designed targeting position 628 and 630 (Table 2). Position 771 is occupied by T in *C. hongkongensis*, *S. cucullata*, *O. denselamillosa*, and by C in all other species. This

TABLE 2.

Genus and species-specific PCR primers and expected product size. A mismatch nucleotide (**bold**) is introduced at the 3' end of some primers to promote specific amplification, and the correct base is presented in parentheses.

Primer	Specificity	Primer Sequence	Size (bp)
28S ribosomal RNA gene			
28forward	All		
28SOa166r	<i>Saccostrea</i> , <i>Ostrea</i>	ATCAAGAGGACTTGGGCTCCCGCAG(C)T	166
28cal489r	<i>Crassostrea</i>	AGCGTTGACCGCGAACGGCCCCCTG	489
28car654r	<i>C. ariakensis</i>	GCGTTCGGGAGGCTATAACTCCCGAG	654
28Chk798r	<i>C. hongkongensis</i>	CACAGCTCACGCATCCCGGTCCAGC(T)A	798
28sal923r	<i>Saccostrea</i>	CACITTCATTTTCGCTTTAGGTTTCGAAAG	923
28reverse	All		1200
Cytochrome oxidase I (COI) gene			
COforward	All		
COCar183r	<i>C. ariakensis</i>	AAAAAAGATTATAACTAATGCATGTCG(T)G	183
COCan222r	<i>C. angulata</i>	AGTTACCAAACCCCAATTATCAG(C)G	222
COGgi269r	<i>C. gigas</i>	TCGAGGAAATTGCATGTCTGCTACA(T)A	269
COChk387r	<i>C. hongkongensis</i>	GGAGTAAGTGGATAAGGGTGGATAG	387
COcsi546r	<i>C. sikamea</i>	AAGTAACCTTAATAGATCAGGGAAC(A)C	546
COreverse	All		697

position was targeted for a *C. hongkongensis*-specific primer that did not match most *Ostrea* and *Saccostrea* species at other positions (Fig. 1). An additional mismatch was introduced at the position 772 to reduce nonspecific amplification (Table 2).

Multiplex PCR with all seven 28S primers (two outer and 5 inner) produced fragments of expected size in all genera and

species. First, the outer primers produced a fragment of about 1,200 bp in all species except *O. angasi*. The *Crassostrea*-specific primer, 28Cal489r, produced a fragment of the expected 489 bp in all *Crassostrea* species, except *C. virginica* and *C. rhizophorae*, but not in any *Saccostrea* and *Ostrea* species (Fig. 2). The *Saccostrea/Ostrea* primer, 28Soa166r, generated a fragment of

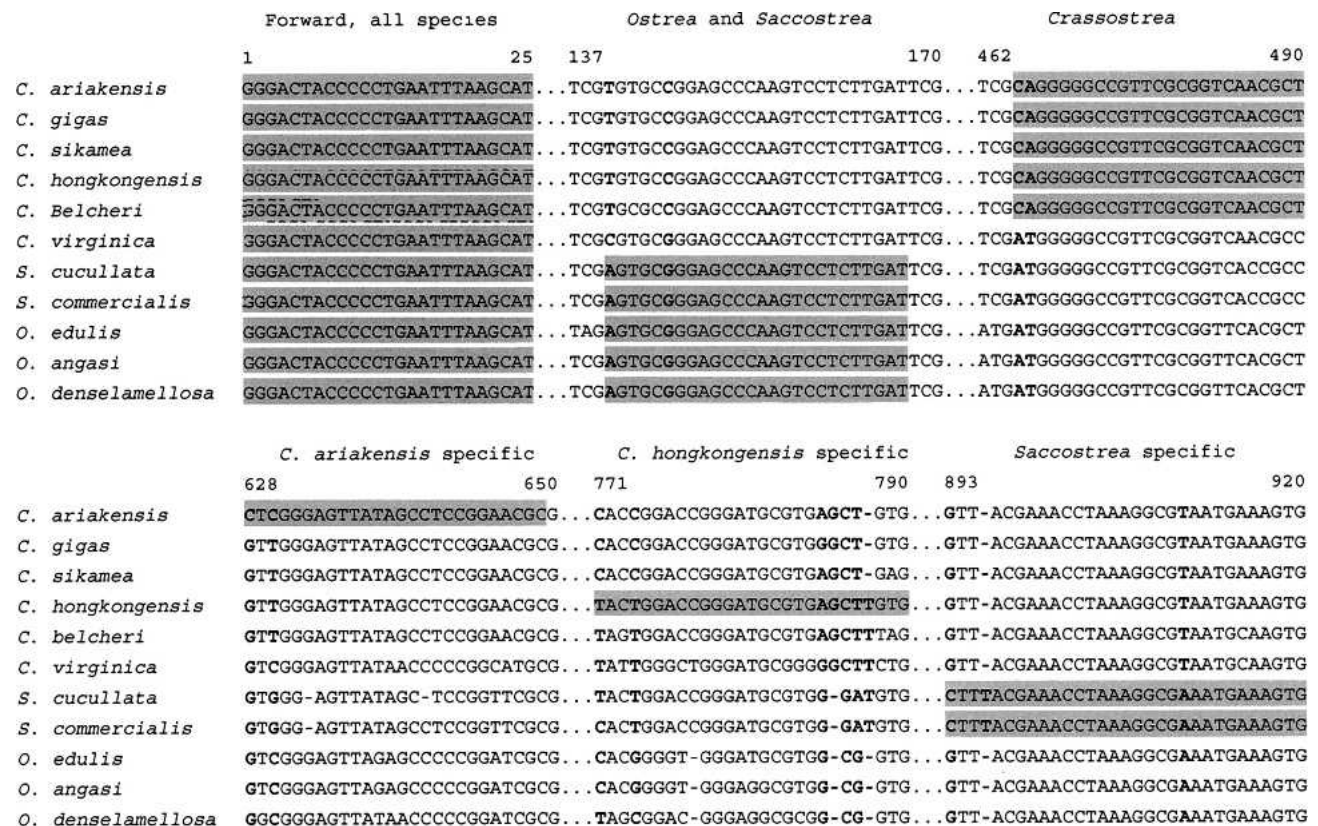


Figure 1. Sequence alignment of partial 28S gene of oyster species showing the site of genus- and species-specific primers (in shade). Diagnostic nucleotide positions are in bold.

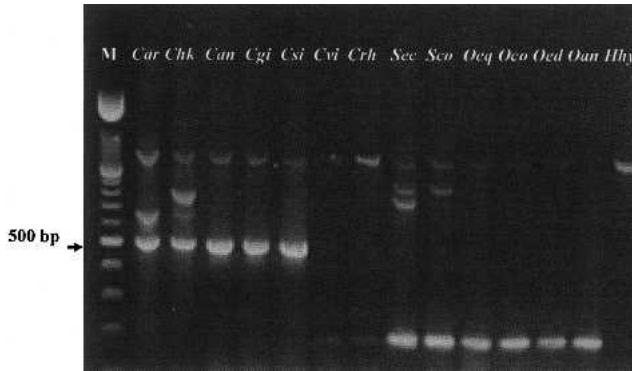


Figure 2. Multiplex genus and species-specific PCR with 7 primers from the 28S rRNA gene: M, 100 bp DNA ladder; Car, *C. ariakensis*; Chk, *C. hongkongensis*; Can, *C. angulata*; Cgi, *C. gigas*; Csi, *C. sikamea*; Cvi, *C. virginica*; Crh, *C. rhizophorae*; Sec, *S. echinata*; Sco, *S. commercialis*; Oeq, *O. equestris*; Oco, *O. conchaphila*; Oed, *O. edulis*; Oan, *O. angasi*; and Hhy, *H. hyotis*.

expected size in all *Saccostrea* and *Ostrea* species. The *Saccostrea*-specific primer 28Sa1923 produced expected fragments in the two *Saccostrea* species and none in non-*Saccostrea* species. The *C. ariakensis*-specific primers amplified only *C. ariakensis*. The *C. hongkongensis*-specific primer amplified in *C. hongkongensis* and *S. echinata*, but the latter species can be easily identified by the absence of the *Crassostrea*-specific band, and the presence of the *Saccostrea* and *Ostrea/Saccostrea* bands (Fig. 2). *Hyotissa hyotis* can be identified by the absence of *Crassostrea* as well as *Saccostrea/Ostrea*-specific bands. In summary, the 28S primers can separate oysters from the four

genera and provide positive identification of *C. ariakensis* and *C. hongkongensis*.

Identification of Five Crassostrea Species Using COI

Compared with the 28S gene, COI is highly variable among species within each genus. Each of the five common *Crassostrea* species from China can be identified by multiple unique nucleotide positions, and only the highly diagnostic sites (with the maximum unique nucleotides) that showed no intraspecific variation were selected for primer design. A *C. ariakensis*-specific primer, COCar183r, was designed using 5 diagnostic nucleotide positions at 157, 160, 169, 178, and 184 (Fig. 3). A mismatch at position 158 was added to reduce nonspecific priming (Table 2). Similarly, a *C. angulata*-specific primer was designed using diagnostic nucleotides at position 196 and 199, which separate it from the closely related *C. gigas* (Fig. 3), and a mismatch at position 197 (Table 2). A *C. gigas*-specific primer was designed based on the unique base at position 243, and a *C. sikamea*-specific primer was designed using diagnostic bases at positions 520 and 526 (Fig. 3). A mismatch was added in each primer at the second bases from the 3' end. The *C. hongkongensis*-specific primer contained six diagnostic bases at positions 364, 367, 370, 373, 379, and 388; and no mismatch was used (Fig. 3).

Multiplex PCR with the seven COI primers, two universal outer primers and five specific-specific inner primers, was successful in all species after some optimization. The two outer primers amplified an about 700 bp fragment in all species, and the species-specific inner primers amplified only in the targeted species. As expected, the species-specific fragments were at the expected size for each of the targeted species: approximately

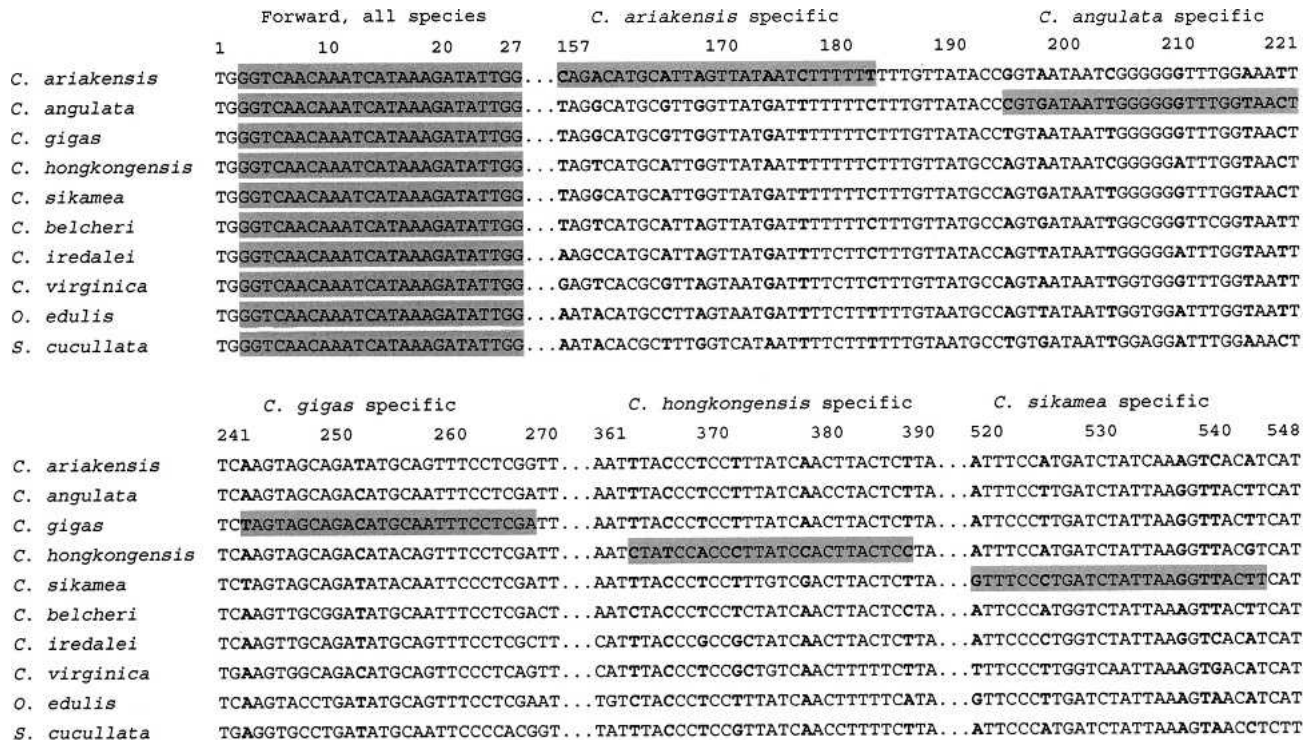


Figure 3. Sequence alignment of COI genes of oyster species showing the site of species-specific primers (in shade) for five *Crassostrea* species. Targeted species-specific nucleotides are in bold.

183 bp in *C. ariakensis*, 222 bp in *C. angulata*, 269 bp in *C. gigas*, 387 bp in *C. hongkongensis*, and 546 bp in *C. sikamea* (Fig. 4). All five species can be positively identified in one PCR. The *C. hongkongensis*-specific primer occasionally produced a faint band in the Atlantic species, *Ostrea equestris*, but the band intensity was clearly different (Fig. 4). None of the other eight species showed any cross-amplification.

Intraspecific Variation

To test the reproducibility of the assays and possible intraspecific variation, 10 to 100 individuals from each species were tested. All individuals of the five *Crassostrea* species listed in Table 2 were positively identified, and no intraspecific variation in PCR product size was observed, suggesting the assays are reproducible and robust. All other species were identified by their genus-specific band patterns. Photos are not shown as they are identical to Figures 2 and 4.

DISCUSSION

Several genetic techniques have been used for oyster identification, including random amplified DNA polymorphism (RAPD, Liu & Dai 1998), restriction fragment length polymorphism (RFLP, Boudry et al. 1998, Klinbunga et al. 2003, Klinbunga et al. 2005), PCR fragment length polymorphism (Wang & Guo 2008) and DNA sequencing (Littlewood 1994, Ó Foighil et al. 1995, Ó Foighil et al. 1998, Ó Foighil & Taylor 2000, Lapegue et al. 2002, Inmaculada & Roberto 2004). DNA sequencing is undoubtedly the most powerful approach, but it is also the most costly and time-consuming. DNA sequencing is best suited for establishing species identity and phylogenetic analysis but not appropriate for routine identification of large numbers of oysters. RAPD markers do not require prior knowledge of DNA sequence and can be used in any species, but it suffers from poor reproducibility. RFLPs are widely used for species identification. They offer clear identification of species when differences in DNA sequence are recognized by restriction enzymes. However, not all differences in DNA sequence can be cleaved by restriction enzymes, and it often takes several genes or enzymes (therefore steps) to identify species in a mixed population.

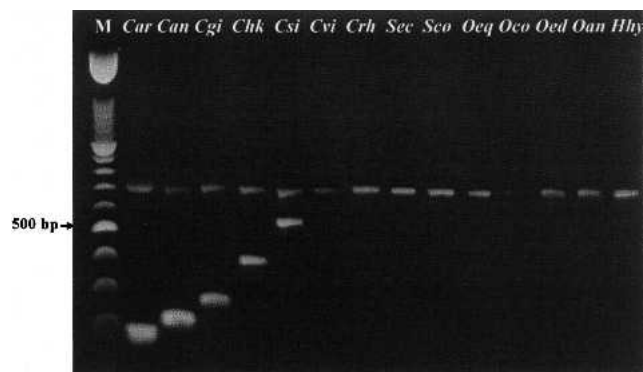


Figure 4. Multiplex species-specific PCR with 7 primers from the COI gene: M, 100 bp DNA ladder; Car, *C. ariakensis*; Can, *C. angulata*; Cgi, *C. gigas*; Chk, *C. hongkongensis*; Csi, *C. sikamea*; Cvi, *C. virginica*; Crh, *C. rhizophorae*; Sec, *S. echinata*; Sco, *S. commercialis*; Oeq, *O. equestris*; Oco, *O. conchaphila*; Oed, *O. edulis*; Oan, *O. angasi*; and Hhy, *H. hyotis*.

Theoretically, any species-specific single nucleotide polymorphisms (SNPs) can be genotyped and used for species identification. There are abundant SNPs among species. SNPs can be genotyped by several methods including single-base primer extension, allele-specific extension, and microarrays (Holloway et al. 1999, Hacia & Collins 1999, Vignal et al. 2002, Ye et al. 2001, Brightwell et al. 2002, Palmer et al. 2003). We tested single-base primer extension using the species-specific SNPs identified in oysters (results not shown here). Whereas it can be multiplexed and effective, primer extension requires automatic genetic analyzers that are not readily available to oyster researchers. Similarly, microarray is a powerful technology that can potentially identify all species in one reaction, but it is expensive to develop and use. In comparison, allele-specific PCR offers a simple and easily accessible approach to SNP genotyping. When allele-specific PCR is applied to species-specific SNPs or alleles, it provides an effective approach to species identification. Species-specific amplification can be detected on inexpensive and commonly available agarose gels. When multiplexed, species-specific PCR can separate several species in one PCR.

Multiplex species-specific PCR has been used for species identification in various taxa including some bivalves (Hare et al. 2000, Larsen et al. 2005). This is probably the first time that this technology is applied to oyster identification. This study demonstrates the multiplex genus- and species-specific PCR is a powerful technology for oyster identification. It can provide highly specific, reproducible, and efficient identification of oyster species. The 28S assay developed here provides positive identification of two species (*C. ariakensis* and *C. hongkongensis*) and quick assignment of oysters according to genera. The COI assay offers unambiguous and efficient identification of all five *Crassostrea* species, *C. ariakensis*, *C. angulata*, *C. gigas*, *C. hongkongensis* and *C. sikamea*, from China in a single PCR. Unlike the RFLP-based methods, the multiplex PCR assays developed here do not involve post-PCR digestion with restriction enzymes, which adds another step and additional source of variation.

The success of multiplex species-specific PCR depends on two factors: the availability of diagnostic SNPs and the ability to genotype them in multiplex. By choosing a highly variable gene such as COI, we were able to identify many diagnostic SNPs even among closely related species such as *C. gigas* and *C. angulata* (Fig. 3). The use of the highly conserved 28S gene allowed us to identify genus-specific SNPs that can be used to assign unknown species to specific genera. The two genes provide an effective combination for oyster identification. The genus- and species-specific primers identified here are highly specific because they are selected from the alignment of many sequences. All available haplotypes were used for alignment, which for COI included 13 haplotypes of *C. ariakensis* and 9 of *C. hongkongensis*. Only nucleotide positions that showed no intraspecific variation were targeted for primer design.

Primer specificity is critical for the successful amplification in multiplex PCR. When targeting a single base difference between species, PCR primers may cross-amplify in nontargeted species. An extra destabilizing mismatch can be introduced at the 3' end, so that only perfect matches at the last base can overcome the instability and be amplified (Newton et al. 1989, Ye et al. 1992, Little 1997, Ye et al. 2001, Chiu et al. 2001, Donohoe et al. 1999, Bathelier et al. 1998). Some of species-specific primers,

such as 28cal489r, contain more than one nucleotides at the 3' end that are unique to the targeted species, provide species-specific amplification without any additional mismatches. For others, we included intentional mismatches at the second base at the 3' end, following rules outlined by Little (1997). By creating these 3' mismatches, we were able to obtain species-specific amplification for five diagnostic primers in one multiplex PCR.

The development of simple and effective genetic markers is important for oyster research and aquaculture, as the rapid and accurate identification of oyster species remains a challenge. Oyster identification is especially difficult in China, where over 15 species of Ostreidae have been reported (Li & Qi 1994). Whereas most of the species are rare, five species of *Crassostrea* are common and of commercial importance. These five species are similar in shell morphology and often coexist in the same area. They cannot be reliably identified by shell morphology alone. The classification of *C. ariakensis/rivularis* is particularly confusing, as at least two other species *C. hongkongensis* and *C. gigas* have been reported as *C. rivularis* in the

literature (Zhang & Lou 1956, Qi 1989, Wang et al. 2004). Consequently, we have no reliable information on the distribution and biology of true *C. ariakensis* in China. A reclassification of oyster populations in China using genetic markers is needed. The multiplex genus- and species-specific assays developed in this study provide rapid, accurate, and efficient identification of all five *Crassostrea* species from China and the assignment of unknown species to different genera. These assays are being used to redefine *C. ariakensis* distribution in China (Wang et al. 2006).

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