

ITS LENGTH POLYMORPHISM IN OYSTERS AND ITS USE IN SPECIES IDENTIFICATION

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ABSTRACT In an effort to develop genetic markers for oyster identification, we studied length polymorphism in internal transcribed spacers (ITS) between major ribosomal RNA genes in 12 common species of Ostreidae: *Crassostrea virginica*, *C. rhizophorae*, *C. gigas*, *C. angulata*, *C. sikamea*, *C. ariakensis*, *C. hongkongensis*, *Saccostrea echinata*, *S. glomerata*, *Ostrea angasi*, *O. edulis*, and *O. conchaphila*. We designed two pairs of primers and optimized PCR conditions for simultaneous amplification of ITS1 and ITS2 in a single PCR. Amplification was successful in all 12 species, and PCR products were visualized on high-resolution agarose gels. ITS2 was longer than ITS1 in all *Crassostrea* and *Saccostrea* species, whereas they were about the same size in the three *Ostrea* species. No intraspecific variation in ITS length was detected. Among species, the length of ITS1 and ITS2 was polymorphic and provided unique identification of 8 species or species pairs: *C. ariakensis*, *C. hongkongensis*, *C. sikamea*, *O. conchaphila*, *C. virginica*/*C. rhizophorae*, *C. gigas*/*C. angulata*, *S. echinata*/*S. glomerata*, and *O. angasi*/*O. edulis*. The ITS assay provides simple, rapid and effective identification of *C. ariakensis* and several other oyster species. Because the primer sequences are conserved, the ITS assay may be useful in the identification of other bivalve species.

KEY WORDS: species identification, ribosomal RNA, ITS, genetic marker, oysters, Ostreidae, Suminoe oyster, *Crassostrea ariakensis*

INTRODUCTION

Oysters cannot be reliably identified using morphological characteristics. Shell coloration and morphology in oysters are highly variable and sensitive to environmental influence. The use of morphological characteristics has led to numerous errors in oyster identification and classification (Harry 1985, Li & Qi 1994). Genetic markers are needed for oyster identification. Oysters often occur sympatrically or are transported around for aquaculture purposes. Accurate identification of oyster species is essential for oyster research and aquaculture. The proposed introduction of *Crassostrea ariakensis* (Fujita 1913) to Chesapeake Bay adds urgency to the development of effective methods for oyster identification, because *C. ariakensis* coexists with several closely related species in Asia.

Several types of genetic techniques have been studied for oyster identification. They include cytogenetic analysis (Thiriot-Quiévreux & Insua 1992, Li & Havenhand 1997, Leitão et al. 1999, Xu et al. 2001, Wang et al. 2004a), DNA sequencing (Banks et al. 1993, Littlewood 1994, Ó Foighil et al. 1995, Ó Foighil et al. 1998, Wang et al. 2004b), restriction fragment length polymorphism (RFLP) (Cordes et al. 2005, Klinbunga et al. 2005) and multiplex species-specific polymerase chain reaction (PCR) (Wang & Guo, 2008). Cytogenetic analyses revealed considerable variation among species, but chromosomal differences in general are not useful or practical for species identification. DNA sequencing offers unprecedented power of revealing interspecific differences, but it is also time-consuming and not readily accessible. RFLP markers require post-PCR digestion with restriction enzymes, which may create additional variation. Multiplex species-specific PCR is more powerful if a large number of species are covered. Simple and effective methods for oyster identification are still needed.

In previous studies, we noticed that some oyster species differed in the length of ITS between major ribosomal genes (Xu et al. 2001, Wang et al. 2004a). To determine the extent of ITS length polymorphism and whether it can be used for oyster identification, we designed a set of primers and studied ITS length polymorphism in 12 species of Ostreidae. Here we report that ITS length is a stable character of species and can be used for the identification of *C. ariakensis* and seven other species and species groups.

MATERIALS AND METHODS

Primer Design

We downloaded major ribosomal RNA sequences from all available oyster species from GenBank, along with sequences from some other bivalve species. We aligned the sequences to identify conserved sequences flanking ITS1 and ITS2. We designed two pairs of primers targeting ITS1 between 28S and 5.8S, and ITS2 between 5.8S and 18S rRNA genes (Fig. 1, A). The primer sequences for ITS1 are 5'-GTTTCCGTAGGT-GAACCTGC (28S forward) and 5'-ACACGAGCCGAGT-GATCCAC (5.8S reverse). The primer sequences for ITS2 are 5'-TCTCGCCTGATCTGAGGTCG (5.8S forward) and GCAGGACACATTGAACATCG (18S reverse).

Oyster Species

We used 12 species of Ostreidae that were available to us to test the ITS primer pairs and determine possible length polymorphism (Table 1). Each species was represented by at least five individuals. Whenever possible, individuals from diverse geographic populations were included to test possible intraspecific variation. The 36 *C. virginica* (Gmelin, 1791) samples covered various sites along the Atlantic and Gulf coasts of the United States. The *C. rhizophorae* (Guilding, 1828) samples

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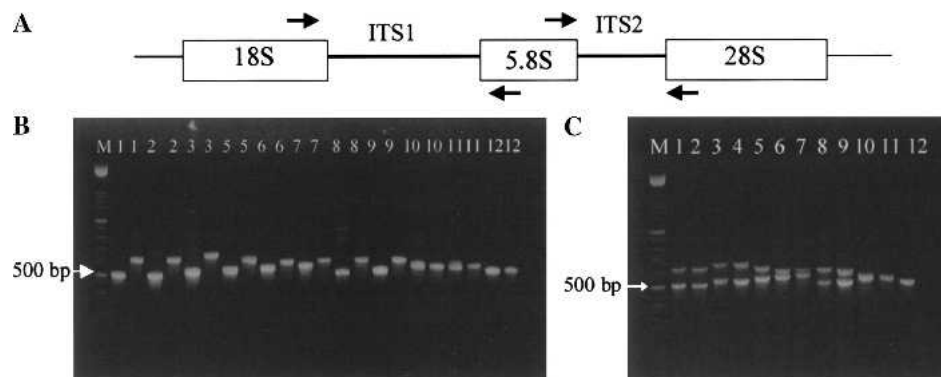


Figure 1. Amplification of ITS1 and ITS2 in 12 species of Ostreidae. (A) a schematic presentation of primer positions for ITS1 and ITS2; (B) separate amplification of ITS1 (first) and ITS2 (second) in 12 species; (C) Simultaneous amplification of ITS1 and ITS2 in one PCR. Species codes are: M, 100 bp marker; 1, *Crassostrea virginica*; 2, *C. rhizophorae*; 3, *C. gigas*; 4, *C. angulata*; 5, *C. sikamea*; 6, *C. ariakensis*; 7, *C. hongkongensis*; 8, *Saccostrea echinata*; 9, *S. glomerata*; 10, *Ostrea angasi*; 11, *O. edulis*; and 12, *O. conchaphila*. (Note: *C. angulata* is absent in B).

were from the first generation progeny of a Caribbean population produced at the Harbor Branch Oceanographic Institute, FL. Four geographic populations of *C. gigas* (Thunberg, 1793) were represented: one was a Rutgers stock (originated

from Japan via Washington State), and the other three were wild and hatchery stocks from Shandong, China. *C. angulata* (Lamarck, 1819) samples were collected from three sites in southern China. *C. sikamea* (Amemiya, 1828) samples were from Washington state (originated from Japan) and Xiamen, China. Two populations of *C. ariakensis* were used, one from Rutgers University (originated from Japan via Washington state) and the other from Weifang, China. *C. hongkongensis* (Lam & Morton 2003) samples were from Guangdong and Guangxi, China. *Saccostrea echinata* (Quoy & Gairnard, 1835) samples came from Fujian and Guangdong, China. *S. glomerata* (Gould, 1850) and *Ostrea angasi* (Sowerby 1871) samples were obtained from Australia. *O. edulis* (Linnaeus 1758) samples were from Maine, and *O. conchaphila* (Carpenter 1857) came from Washington state. The identity of most samples from China was confirmed using DNA sequences in a previous study (Wang 2004).

TABLE 1.

Species and geographic populations of Ostreidae represented in the ITS study.

Species	Number	Population/Origin
<i>Crassostrea virginica</i>		
Gmelin	4	Long Island Sound, USA
	4	Delaware Bay, USA
	3	Quinby Inlet, VA, USA
	3	Chincoteague Bay, VA, USA
	3	Pensacola Bay, FL, USA
	3	Pass Christian, MS Sound, USA
	3	Biloxi Bay, MS Sound, USA
	4	Grand Isle, LA, USA.
	3	San Antonio Bay, TX, USA
	3	Bill Days Reef, TX, USA
	3	South Pass Reef, TX, USA
<i>C. rhizophorae</i> Guilding	5	HBOI, FL, USA
<i>C. gigas</i> Thunberg	7	Rutgers, NJ, USA
	3	Penglai, Shandong, China
	3	Weihai, Shandong, China
	3	Rushan, Shandong, China
<i>C. angulata</i> Lamarck	3	Xiamen, Fujian, China
	1	Putian, Fujian, China
	1	Wenzhou, Zhejiang, China
<i>C. sikamea</i> Amemiya	3	Washington State, USA
	2	Xiamen, Fujian, China
<i>C. ariakensis</i> Fujita	3	Rutgers, NJ, USA
	2	Weifang, Shandong, China
<i>C. hongkongensis</i> Lam and Morton	4	Zhanjiang, Guangdong, China
	1	Beihai, Guangxi, China
<i>Saccostrea echinata</i> Quoy et Gairnard	3	Xiamen, Fujian, China
	2	Zhanjiang, Guangdong, China
<i>S. glomerata</i> Gould	5	Australia
<i>Ostrea angasi</i> Sowerby	5	Australia
<i>O. edulis</i> Linnaeus	5	Maine, USA
<i>O. conchaphila</i> Carpenter	5	Washington State, USA

PCR Condition and Optimization

Genomic DNA was isolated from 30–50 mg of adductor muscle using CTAB (hexadecyltrimethylammonium bromide) buffer and phenol/chloroform protocol as described in Doyle & Doyle (1987). Primers for ITS1 and ITS2 were tested separately first, to identify ITS1 and ITS2 fragments. The two primers pairs were then combined to amplify ITS1 and ITS2 in a single-tube PCR. PCR condition was optimized by using different annealing temperature, and different concentrations of MgCl₂. Different proportions of ITS1 and ITS2 primers were tested for balanced amplification of the two fragments. The optimal PCR condition was identified as: 10 mM Tris-HCl, pH8.3, 50 mM KCl, 1.5 mM of MgCl₂, 0.2 mM each of dNTP, 0.4 mg/mL BSA, 0.05 μM of internal primers, 1 μM of external primers, 0.24 U Taq DNA polymerase (Promega), 10–25 ng of genomic DNA in a total volume of 10-μL. PCR was conducted on a PE 9700 thermocycler (PE Biosystems) using the follow profile: an initial 5 min denature at 95°C; 30 cycles of 1 min denature at 95°C, 1 min annealing at 60°C and 1 min extension at 72°C; and a final extension at 72°C for 5 min, and followed by holding at 4°C. Negative controls, containing no template, were included in each run. PCR products were separated on 1.5% (w:v) high resolution agarose gels and visualized by ethidium bromide

(0.5 µg/mL) staining and UV illumination. A 100-bp ladder (Roche) was used as the size standard.

We used one individual from each of the 12 species for PCR optimization. After optimization, we analyzed all individuals listed in Table 1 to detect interspecific and intraspecific differences in ITS length.

RESULTS

PCR Amplification

Amplification of ITS1 and ITS2 were successful in all 12 species tested. ITS1 primers were slightly more efficient, as they produced stronger bands than ITS2 primers under the same condition. In all *Crassostrea* and *Saccostrea* species studied, ITS2 are longer than ITS1 (Fig. 1, B). ITS1 varied from about 500–550 bp, and ITS2 ranged from 550–600 bp. In the three *Ostrea* species, ITS1 and ITS2 appeared to be the same size at around 550 bp.

Simultaneous amplification of ITS1 and ITS2 required optimization. Among different annealing temperatures tested, 60°C gave the best results. At low annealing temperatures (50°C and 55°C), the two primer pairs produced weak bands. Cycle number and magnesium chloride did not significantly affect amplification. Different ratios of internal and external of primers (1:1, 1:5, 1:20, and 1:50) were tested, and the external: internal primer ratio of 1:20 produced the best results with clear and sharp bands.

Interspecific Differences

Length of both ITS1 and ITS2 were polymorphic among the 12 species studied. Some species can be clearly identified by their unique ITS size, whereas some closely related species cannot be separated. When ITS1 and ITS2 were simultaneously amplified and analyzed, they can distinguish eight species or species-groups. First, all three *Ostrea* species can be separated from *Crassostrea* and *Saccostrea* species, because they produced a single band for ITS1 and ITS2, and all oysters from other two

genus had two bands (Fig. 1, C). Within *Ostrea*, *O. angasi* and *O. edulis* cannot be separated by ITS length, but both of them were clearly different from *O. conchaphila*. The two species of *Saccostrea* can be separated from *Crassostrea* species. They also differed slightly in ITS length, but the difference was too small to allow reliable separation. Among the 7 *Crassostrea* species, *C. ariakensis*, *C. hongkongensis* and *C. sikamea* had unique ITS profiles and could be positively identified. *C. virginica* and *C. rhizophorae* were almost identical in their ITS length and could not be separated from each other. They could be identified as a pair. Similarly, there was no difference in ITS length between *C. gigas* and *C. angulata*, but they could be clearly identified as a pair. In summary, ITS1 and ITS2 profile provided identification of 8 species and species-pairs: *C. ariakensis*, *C. hongkongensis*, *C. sikamea*, *O. conchaphila*, *C. virginica*/*C. rhizophorae*, *C. gigas*/*C. angulata*, *S. echinata*/*S. glomerata*, and *O. angasi*/*O. edulis*. The assay also separated all three *Ostrea* species from members of the other two genera.

Intraspecific Variation

To determine if the size of ITS1 and ITS2 are variable within species, individuals representing diverse populations of each species were analyzed. In *C. virginica*, 36 oysters collected from 11 sites along the Atlantic and Gulf coasts did not show any detectable variation in ITS length. Similarly, 16 *C. gigas* that originated from Japan and China had indistinguishable ITS profiles. Five oysters were tested in the other species and none showed any intraspecific difference. The ITS profiles of five individuals from each species are presented in Figure 2.

DISCUSSION

This study shows that the size of ITS1 and ITS2 are conserved within species, but variable among some species of Ostreidae. Small intraspecific variation in ITS size has been observed at the DNA sequence level (Kenchington et al. 2002). At the level of agarose gel electrophoresis, however, all individuals of the same species are indistinguishable in their ITS

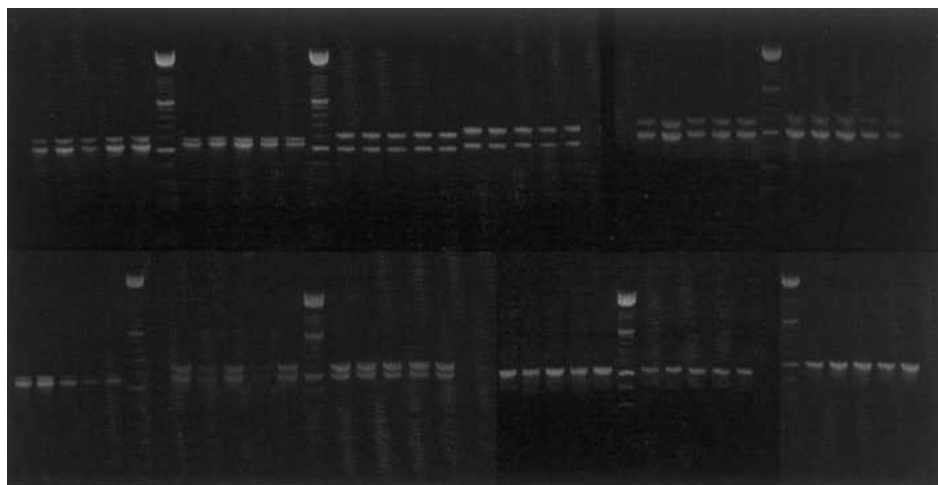


Figure 2. Amplification of ITS1 and ITS2 in five individuals from each of 12 species of Ostreidae showing no intraspecific variation. Species codes are: M, 100 bp marker; 1, *Crassostrea sikamea*; 2, *C. ariakensis*; 3, *C. virginica*; 4, *C. gigas*; 5, *C. hongkongensis*; 6, *C. rhizophorae*; 7, *C. angulata*; 8, *Saccostrea echinata*; 9, *S. glomerata*; 10, *Ostrea angasi*; 11, *O. edulis*; and 12, *O. conchaphila*. When two bands are present, the upper band is ITS2 and the lower band is ITS1.

size. The individuals that we used were from diverse populations. Some of the populations, such as *C. virginica* populations from the Atlantic and Gulf coasts, are known to be genetically different (Karl & Avise 1992). Geographically distant populations were represented in several other species. For *C. gigas*, *C. ariakensis* and *C. sikamea*, populations originated from Japan and China were studied. The fact that no intraspecific variation was observed in such diverse samples (Table 1) suggests that ITS size is a stable character for each species and can be used for the purpose of species identification. Although diverse geographic populations are represented, the sample size for most species is small and needs to be expanded. For most *Crassostrea* species tested here, the lack of intraspecific variation has been confirmed through routine use in our laboratory (see later).

Among species, variation in ITS size is clearly correlated with phylogenetic relationships (i.e., closely related species have the same or similar ITS sizes) whereas distant species differ considerably. All species-pairs that cannot be separated by ITS size, *C. gigas/angulata*, *C. virginica/rhizophorae*, *S. echinata/glomerata* and *O. edulis/angasi*, are closely related to each other (Littlewood 1994) and some are even considered as the same species. *C. gigas* and *C. angulata* are regarded as same species by some (Menzel 1974, Buroker et al. 1979), although they are clearly different at the DNA sequence level (Boudry et al. 1998, Ó Foighil et al. 1998). Kenchington et al. (2002) have suggested that *O. edulis* and *O. angasi* may be con-specific based on ITS sequences. There are also suggestions that *C. virginica* and *C. rhizophorae* are the same species (Littlewood 1994). Further, ITS size clearly sets the genus *Ostrea* apart from *Crassostrea* and *Saccostrea*. All these data suggest that variation in ITS size is not random, but a function of phylogeny. Whereas ITS sequences are highly variable (Harris & Crandall 2000, Kenchington et al. 2002, He et al. 2005), concerted evolution may favor the conservation of ITS size within species.

This study provides a simple and effective method for the identification of eight species and species groups. Oysters in mixed populations cannot be reliably identified using morphological characters. Simple and reliable methods of oyster are greatly needed for routine identification. The power of molecular techniques for identifying species has been unequivocally demonstrated in many species. Despite their proven effectiveness, molecular techniques are not widely used for oyster identification. This is largely because most current methods are still too labor-intensive or complicated for common use.

Compared with available techniques, the ITS assay developed in this study is relatively easy and inexpensive. PCR with ITS primers are highly specific and robust. The combination of ITS1 and ITS2 primers in one reaction provides two measurements of possible differences. The assay is simple and requires no post-PCR treatment. A limitation of the ITS assay is that it cannot identify closely related species. It is possible that the difference between the closely related species is too small to be detected on agarose gels. The use of sequencing gel or automated sequencers may greatly improve the resolution and provide separation of the closely related species. For *C. ariakensis* and other species, the assay running on regular agarose gel is sufficient. It offers reliable separation of *C. ariakensis* from *C. virginica* and common Asian species, providing a useful tool in *C. ariakensis* research. This assay has been used in our laboratory to separate *C. ariakensis* from *C. virginica*, *C. hongkongensis*, *C. sikamea*, and *C. gigas/C. sikamea*, or for hybrid detection (Bushek et al. 2008). Over hundreds of samples analyzed, we have not seen any intraspecific variation in ITS size variation in those species.

Another advantage of the ITS assay is its wide applicability. Because primers are designed using highly conserved sequences, the ITS primers will likely amplify in all oyster species and hence provide possible detection and identification of new or unknown species. The ITS assay is best suited for separating known species with characterized ITS sizes. Caution is needed when the assay is applied to situations where unknown or uncharacterized species are involved. All possible species should be characterized first, and any unexpected ITS length polymorphism should be interrogated to confirm species identity. During the primer design, sequence from other bivalves such as scallops and clams were also considered. It is possible the ITS assay developed here will work in other bivalve species, but further study is needed to determine its usefulness in other bivalves.

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