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DISCRIMINATION OF NINE *CRASSOSTREA* OYSTER SPECIES BASED UPON RESTRICTION FRAGMENT-LENGTH POLYMORPHISM ANALYSIS OF NUCLEAR AND MITOCHONDRIAL DNA MARKERS

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ABSTRACT A molecular genetic identification key for nine species of *Crassostrea* oysters was developed based on restriction fragment-length polymorphism (RFLP) analyses of nuclear and mitochondrial DNA markers. Seven of nine species were unambiguously differentiated based on digestion of the ITS-1 nuclear marker with *Hae* III and *Hinf* I. Individual species exhibited one or two RFLP patterns for each restriction endonuclease, and only *C. madrasensis* and *C. iredalei* were indistinguishable electrophoretically. All nine species were unambiguously differentiated based on digestion of the COI mitochondrial marker with *Dde* I and *Hae* III. Species exhibited one or two RFLP patterns for each restriction endonuclease, and species pairs unresolved by the first restriction enzyme were completely resolved with the second. The resulting key distinguishes among many Indo-Pacific *Crassostrea* oysters that overlap across some or all of their ranges, and establish as an expandable framework for future additions of other species to the key.

KEY WORDS: *Crassostrea*, oysters, species identification, ITS-1, COI, PCR/RFLP

INTRODUCTION

Oysters are widely distributed in all tropical to temperate oceans from 64° N to 44 ° S latitudes (Harry 1985, Hedgecock 1995). Up to 30 nominal species have been recorded in China alone (Qi 1989, Bernard et al. 1993), with as many as nine of those species in the genus *Crassostrea* (Lam & Morton 2003 and references therein). A number of these species represent significant aquaculture industries including *C. gigas*, *C. sikamea*, *C. ariakensis* (Qi 1989) and the newly described *C. hongkongensis* (Lam & Morton 2003). *Crassostrea ariakensis* is of particular interest to scientists and managers in the United States, because of the proposed introduction of this species into the Chesapeake Bay as a means of replacing native populations of *C. virginica* ravaged by overfishing, habitat degradation, and disease.

Recent genetic studies have indicated that identifications of many oyster species based on morphological characters are prone to error (Boudry et al. 1998, Hedgecock et al. 1999, Francis et al. 2000, Klinbunga et al. 2003, Reece et al. 2008). This has caused confusion regarding the geographic distribution of various species including *C. ariakensis*. Carriker and Gaffney (1996) consider *C. ariakensis* to be synonymous with *C. rivularis*, and list its range as extending from Pakistan and India to China and Japan, although they suggest that the populations in Pakistan and India may be a different species. Assuming this geographic distribution is correct, *C. ariakensis* could be sympatric through all or part of its range with as many as 10 different congeners including *C. belcheri*, *C. iredalei*, *C. gryphoides, C. madrasensis*, *C. nippona*, *C. angulata*, *C. gigas*, *C. plicatula*, *C. sikamea*, and *C. hongkongensis*, making species identifications difficult because of a lack of distinguishing morphological characters among many of the species. Indeed, recent evidence indicates that *C. hongkongensis* has been traditionally misidentified as *C. gigas* in Hong Kong (Lam & Morton 2003, Boudry et al. 2003) and *C. ariakensis* on the mainland of southern China (Wang et al. 2004, Zhang et al. 2005). Given the proposed introduction of *C. ariakensis* into the Chesapeake Bay and the potential for the inadvertent introduction of multiple species, it has become imperative that a reliable means of identifying Indo-Pacific oysters of the genus *Crassostrea* be found.

Over the last decade a number of molecular markers have been developed to distinguish among various species of *Crassostrea* oysters (Banks et al. 1993, O'Foighil et al. 1995, Boudry et al. 1998, Klinbunga et al. 2000, Klinbunga et al. 2001, Klinbunga et al. 2003), determine the origin of introductions (O'Foighil et al. 1998), verify a species' presence in a geographic area (Hedgecock et al. 1999), characterize new species (Lam & Morton 2003, Boudry et al. 2003, Wang et al. 2004), and distinguish among hatchery and wild stocks (Zhang et al. 2005). Initial studies commonly used mitochondrial markers such as the cytochrome oxidase subunit I (COI; Folmer et al. 1994, O'Foighil et al. 1998), and 16S rRNA (Banks et al. 1993; O'Foighil et al. 1995) gene regions. More recently, studies have also used nuclear markers such as the first internal transcribed spacer (ITS-1; Hedgecock et al. 1999) and 28S (Boudry et al. 2003) regions of the nuclear rRNA gene family, or some combination of mitochondrial and nuclear markers (Boudry et al. 1998, Klinbunga et al. 2003, Lam & Morton 2003, Boudry et al. 2003, Wang et al. 2004, Zhang et al. 2005). The most commonly used techniques to reveal genetic variation in these markers are polymerase chain reaction/restriction fragment-length polymorphism (PCR/RFLP) analysis (Banks et al. 1993, O'Foighil et al. 1998, Klinbunga et al. 2003, Boudry et al. 1998, Hedgecock et al. 1999, Boudry et al. 2003, Zhang et al. 2005) and sequencing (Banks et al. 1993, O'Foighil et al. 1998, Boudry et al. 2003, Lam & Morton 2003, Wang et al. 2004). Traditionally PCR/RFLP analyses were used because of relatively smaller commitments of equipment, time, and money; although this is changing as more molecular laboratories develop multiplex PCR protocols and acquire high-throughput sequencers.

Typically, previous species identification studies have differentiated two or three species of *Crassostrea* oysters (but see Wang & Guo 2008). Based on past studies, it would require two

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mitochondrial markers (COI and 16S) and a combination of PCR/RFLP and sequencing analyses to differentiate six of the nine *Crassostrea* species listed in Table 1. To our knowledge *C. madrasensis* and *C. gryphoides* have not been included in any previous studies.

Briefly stated, the objectives of this study were to develop a species identification key based on PCR/RFLP analyses of multiple molecular genetic markers for Indo-Pacific *Crassostrea* oysters, which are potentially sympatric with *C. ariakensis* over some or all of its range. Specifically, objectives were to: (1) develop a species identification key for as many Indo-Pacific *Crassostrea* species as possible, (2) base the key on the internal transcribed spacer (ITS-1) region of the nuclear rRNA gene as well as the cytochrome oxidase subunit I (COI) mitochondrial gene region, to provide for an internal check as well as to make the key useful in hybridization studies and for identification of hybrids in the wild, (3) Construct the key based on PCR/RFLP analyses and agarose gel electrophoresis protocols for ease of use, and (4) make the protocols and key readily available to researchers, managers, and government and other entities requiring positive identification of oyster species for purposes of restoration, management, and invasive species control.

MATERIALS AND METHODS

Virtual Restriction Enzyme Digestion (VRED) Analysis

Sample sequences of the nine species used in the VRED analysis were taken from the phylogenetic analysis of Reece et al. (2008), (Table 1). In that study, samples were collected from throughout the Indo-Pacific, provisionally assigned to species based on morphology and collection location, and a subset of each species sample subjected to phylogenetic analyses of both ITS-1 and COI gene region sequences. Additional sequences for some species downloaded from GENBANK were also included in the analysis (Reece et al. 2008). Those sample collections that formed monophyletic species groups with high bootstrap support on the resulting phylogenetic trees were used in the VRED analysis to develop the PCR/RFLP-based identification key. The eastern United States oyster species *Crassostrea virginica* was included in the present study in light of the proposed introduction of *C. ariakensis* into the Chesapeake Bay.

Oyster ITS-1 sequences for 122 clones from 67 individuals representing 9 different species (4–15 individuals per species) used in the phylogenetic analysis of Reece et al. (2008) were aligned using the CLUSTALW option (Thompson et al. 1994) in the MacVector 8.1.2 Sequencing Analysis Package (MacVector, Inc., Cary, NC, USA). Aligned sequences were subjected to virtual digestion using the restriction enzyme analysis option in MacVector to determine the smallest number of restriction enzymes required to discriminate the maximum number of species. Criteria used in determining the usefulness of each restriction enzyme included maximizing the number of distinguished species, minimizing intraspecific variation, and production of fragment patterns that could be visualized with simple agarose gel electrophoresis and ethidium bromide (EtBr) staining. Potentially useful restriction endonucleases identified by the virtual digestion analyses were then tested in a PCR/RFLP agarose gel analysis of the ITS-1 marker in multiple individuals of each species.

A second marker based on the COI mitochondrial gene region was developed to provide a means of verifying results from the first marker and to make it possible to investigate cases of interspecific hybridization. COI sequences (Reece et al. 2008) for 70 clones from 69 individuals representing the nine species (2–14 individuals per species) were subjected to the same virtual restriction enzyme digestion analyses outlined earlier.

Tissue Samples

Sample sizes, sampling location, and collector information for each species used in the PCR/RFLP agarose gel analysis are given in Table 1. Samples consisted of mantle or adductor muscle tissue preserved either in DMSO storage buffer (25 mM EDTA, 20% DMSO, saturated NaCl), or 95% ethanol and

TABLE 1.

Samples used to construct a molecular genetic key for distinguishing among various oyster species based on PCR/RFLP analysis. Samples from collections also used in the phylogenetic analyses of Reece et al. (2008, Table 1) are indicated with an *.

Species	Sample Location	Collection Date	Sample Code	N	Source
Crassostrea virginica	Horn Point Hatchery	02/2006	CviHP	120	R. Newell
C. belcheri	Pulau Aman near Penang, Malaysia	10/2000	CarMal*	24	I. Kornfeld
C. iredalei	Chonburi, Thailand	02/2001	CiCB*	2	S. Klinbunga
	Phangnga, Thailand	02/2001	CiPN *	2	S. Klinbunga
C. madrasensis	India	06/2000	CmInd*	25	F. Obeirn
C. gryphoides	India	06/2000	CgrInd*	27	F. Obeirn
C. gigas	Ariake Bay, Japan	05/2004	Cgi	60	C. Langdon
C. sikamea	Nantong, Jiangsu Province, China	05/2006	Csi	38	K. Reece
C. ariakensis	Itoki R., Japan	07/1999	CarIR*	20	E. Francis
	Yellow River, Shandong Province, China	06/1999	CarYR*	20	H. Que
	Kahwa River, South Korea		CarKR	20	H. An
C. hongkongensis[a]	Dafeng R., Guangxi Province, China	05/1999	CarDR*	20	H. Que
	Yamen R., Guangdong Province, China	05/1999	CarZ*	20	H. Que
	Souchang R., Guangdong Province, China	05/1999	CarYJ*	20	H. Que

[a]Historically misidentified as *C. gigas* and *C. ariakensis* (Lam & Morton 2003, Boudry et al. 2003, Wang et al. 2004, Zhang et al. 2005).

stored at room temperature. Genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen Inc., Santa Clara, CA, USA) according to the manufacturer's instructions. In the final step samples were eluted with 200 µL of AE elution buffer and stored at –20°C until needed.

PCR Amplifications

PCR amplifications of the COI and ITS-1 gene regions were carried out using the primers of Folmer et al. (1994) and Hedgecock et al. (1999), respectively. Amplifications consisted of 25 µL reactions containing 15.375 µL sterile dH_2O, 2.5 µL × 10 PCR buffer, 0.75 µL 50 mM $MgCl_2$, 0.5 µL 10 mM each dNTPs, 0.25 µL 10 µM forward and reverse primers, 0.125 µL *Taq* I polymerase (0.20 U total), and 0.25 µL DNA (approximately 5ng DNA total). Samples were first denatured for 3 min at 95°C, followed by 30 cycles of PCR amplification performed under the following conditions: 1 min at 95°C, 2 min at 52°C, and 2 min at 72°C, with a 5 min final extension at 72°C.

Sizes of the undigested ITS-1 and COI PCR products for each species were compared by electrophoresis on 1% agorose gels in × 1 TBE for approximately 20 min. at 100 V. Gels were stained in a 0.5 µg/mL EtBr bath and visualized using a UV transilluminator and AlphaImager 5.5 software (Alpha Innotech Co., San Leandro, CA, USA).

RFLP Analysis

Amplified ITS-1 and COI products were digested with diagnostic restriction enzymes identified in the VRED analysis following manufacturer protocols (New England Biolabs, Inc). The digested products were separated by electrophoresis on 3% (1:1 agarose: low melt agarose; Fisher Scientific) gels in 1X TBE. Gels were run at 100 V for approximately 90 min. and visualized as described earlier.

RESULTS

ITS-1 Marker

Among the nine species surveyed, sequence data (Reece et al. 2008) revealed a size range of 519–610 bp (including primer sites) for the PCR-amplified, undigested ITS-1 marker (Table 2). Preliminary tests using sequences from multiple individuals of six (*Crassostrea virginica*, *C. belcheri*, *C. gigas*, *C. sikamea*, *C. ariakensis*, and *C. hongkongensis*) of the nine species listed in Table 1 were performed using the virtual restriction enzyme analysis option in MacVector. Results indicated that only two (*Hae* III and *Mnl* I) restriction enzymes produced fragment patterns that differed among at least 4 of the six species. Because of the large number of small fragments generated for all six of the species by *Mnl* I, this enzyme was excluded from further analysis, because differences in the interspecific patterns would have been difficult to resolve using agarose gel electrophoresis.

Virtual digestions were then performed on sequences from multiple individuals of all nine species using *Hae* III. Individual species exhibited one or two restriction fragment patterns, and five of the nine species exhibited patterns that were not shared by any other species. Species pairs that shared at least one

TABLE 2.

Alleles and haplotypes, band sizes, and total sizes of amplified fragments for ITS-1 and COI genetic markers subjected to restriction fragment-length polymorphism (RFLP) analysis in nine species of *Crassotrea* oysters. Band sizes and total sizes of amplified fragments are based on consensus sequences (with primer sites included) for each species. Because of the difficulty in distinguishing bands <40 bp some bands listed will not appear in the agarose gel images (Figs. 1 and 2). Band sizes are listed based on order of restriction sites. The heading n_a is the number of times a particular allele was seen in the ITS-1 analysis, n_h is the number of times a particular haplotype was seen in the COI analysis. *Denotes the most frequently seen allele/haplotype for each species and marker. **Denotes alleles found during the gel electrophoresis screening process that have not yet been sequenced. [1, 2, 3] indicate band patterns from species pairs that are difficult to distinguish by agarose gel electrophoresis, requiring a second restriction enzyme for species determination.

Species	Allele	n_a	Band Sizes (bp)	Total (bp)	Species	Haplotype	n_{hh}	Band Sizes (bp)	Total (bp)
			ITS-1					COI	
Hae III					*Dde I*				
C. virginica	A*	178	111, 86, 62, 180, 80	519	*C. virginica*	A	120	284, 47, 97, 272	700
	B	62	197, 62, 180, 80		*C. belcheri*	B	24	115, 169, 416	700
C. belcheri	C	48	169, 166, 132, 150	579	*C. madrasensis*	C[3]	25	115, 7, 578	700
C. madrasensis	D[1]	50	178, 67, 132, 133, 100	610	*C. iredalei*	C[3]	4	115, 7, 578	700
C. iredalei	D[1]	8	178, 67, 132, 133, 100	610	*C. gryphoides*	D	27	417, 283	700
C. gryphoides	E	54	181, 188, 106, 43, 90	608	*C. gigas*	E[4]	60	115, 302, 283	700
C. gigas	F	120	153, 53, 242, 97	545	*C. sikamea*	F*	36	700	700
C. sikamea	G[2]	76	169, 44, 128, 109, 99	549		G**	2	275,425	
C. ariakensis	H	120	161, 55, 58, 65, 124, 96	559	*C. ariakensis*	E[4]	60	115, 302, 283	700
C. hongkongensis	I* [2]	119	171, 50, 125, 126, 103	575	*C. hongkongensis*	H	60	50, 65, 585	700
	J**	1	221, 125, 126, 103						700
Hinf I					*Hae III*				
C. sikamea	A	75	411, 138	549	*C. madrasensis*	A	25	583, 117	700
					C. iredalei	B	2	432, 151, 117	700
					C. gigas	C	60	502, 198	700
C. hongkongensis	B*	118	182, 229, 164	575	*C. ariakensis*	D	60	700	700
	C**	2	411, 164						

restriction pattern included *C. madrasensis*/*C. iredalei* and *C. sikamea*/*C. hongkongensis*. After reviewing the virtual digestions previously performed we found that the restriction enzyme *Hinf* I produced unique fragment patterns that distinguished *C. sikamea* from *C. hongkongensis*; to date no enzymes have been found that can differentiate between *C. madrasensis* and *C. iredalei* based on the ITS-1 region sequences.

To verify the results of the virtual digestions, 4–120 individuals (Table 1) of each species were subjected to RFLP analysis using *Hae* III and *Hinf* I according to protocols outlined in the materials and methods section. For *Hae* III, individual species still exhibited one or two restriction fragment patterns (Table 2, Fig. 1A). For *Hinf* I, fragment patterns for *C. madrasensis* and *C. iredalei* remained indistinguishable; however, the restriction enzyme did differentiate *C. sikamea* and *C. hongkongensis* (Table 2, Fig. 1B).

COI Marker

Sequence data (Reece et al. 2008) revealed a size of 700 bp (including primer sites) for the PCR-amplified, undigested COI marker (Table 2) in all nine species. Preliminary virtual digestions of COI sequences were preformed on the six species listed earlier for the ITS-1 marker analysis. Four (*Dde* I, *Mnl* I, *Mse* I, *Tsp*R I) restriction enzymes resulted in fragment patterns that differed among at least four of the six species. Virtual digestions were then performed on sequences from multiple individuals of all nine species using these four enzymes. Because of the large number of small fragments generated for many of the nine species by *Mnl* I and *Mse* I, these enzymes were not considered further. Of the two remaining enzymes, *Dde* I uniquely distinguished the greatest number of species, and was therefore assessed by agarose gel electrophoresis to verify its usefulness for the genetic identification key.

Using the same samples as the ITS-1 analysis above, individuals of each of the nine species were subjected to RFLP analysis using *Dde* I. Species exhibited one or two restriction fragment patterns, and five of the species were clearly differentiated based on exhibited patterns (Table 2). Species pairs that shared at least one restriction pattern included *C. madrasensis*/*C. iredalei* and *C. gigas*/*C. ariakensis*. Review of the virtual digestions showed that the restriction enzyme *Hae* III would distinguish *C. madrasensis* from *C. iredalei* and *C. gigas* from *C. ariakensis*. This was confirmed by gel electrophoresis; thus all nine species could be distinguished based on the COI marker using two restriction enzymes (Fig. 2).

DISCUSSION

Using published PCR primers for two molecular genetic markers we have developed an identification key for nine species of oysters, including eight Indo-Pacific *Crassostrea* species. Utilization of sequence information for both the ITS-1 and COI markers available from the phylogenetic study of Reece et al. (2008) greatly facilitated optimization of the key by allowing us to perform virtual restriction endonuclease digestion (VRED) analyses, which saved time by eliminating much of the laboratory-intense trial-and-error usually associated with identifying informative restriction endonucleases. Because the key is based on two independent markers, species identifications can be verified internally, and because a nuclear and a mitochondrial locus are used, instances of interspecific hybridization can be identified and the maternal and paternal species assigned. The key has been used to verify the production

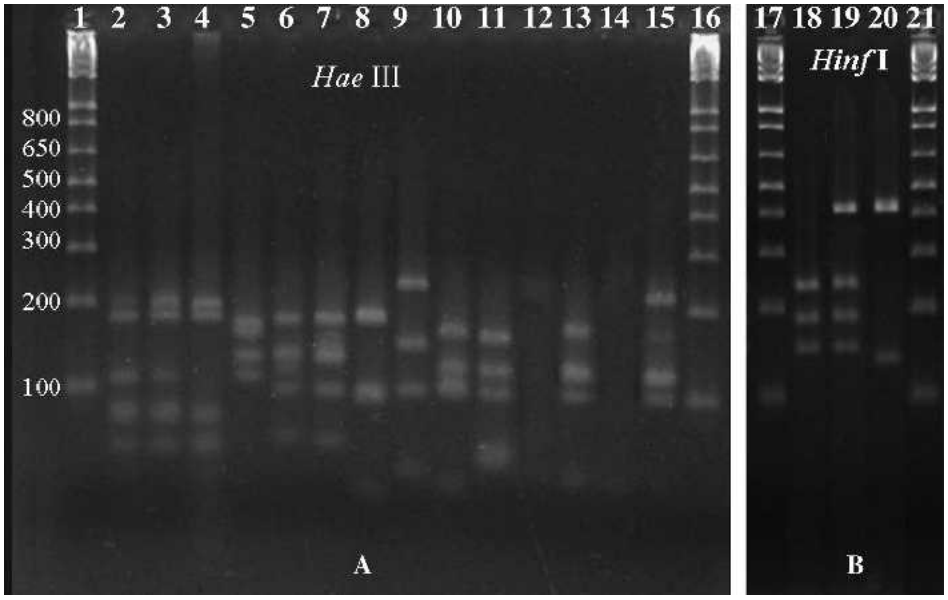

Figure 1. Polymerase chain reaction (PCR) amplified ITS-1 nDNA gene region for nine species of Pacific oysters digested with two restriction endonucleases: (A) *Hae* III; Lanes 1 and 16 are 1 Kb + size standards; 2 = *C. virginica* AA homozygote; 3 = *C. virginica* AB heterozygote; 4 = *C. virginica* BB homozygote; 5 = *C. belcheri* CC; 6 = *C. madrasensis* DD; 7 = *C. iredalei* DD; 8 = *C. gryphoides* EE; 9 = *C. gigas* FF; 10 = *C. sikamea* GG; 11 = *C. ariakensis* HH; 12 = blank; 13 = *C. hongkongensis* II; 14 = blank; 15 = *C. hongkongensis* IJ*, and (B) *Hinf* I; Lanes 17 and 21 are 1 Kb + size standards, 18 = *C. hongkongensis* BB; 19 = *C. hongkongensis* BC; 20 = C. *sikamea* AA. * In most cases second alleles were rare (see Table 2) and homozygotes for these alleles were not found.

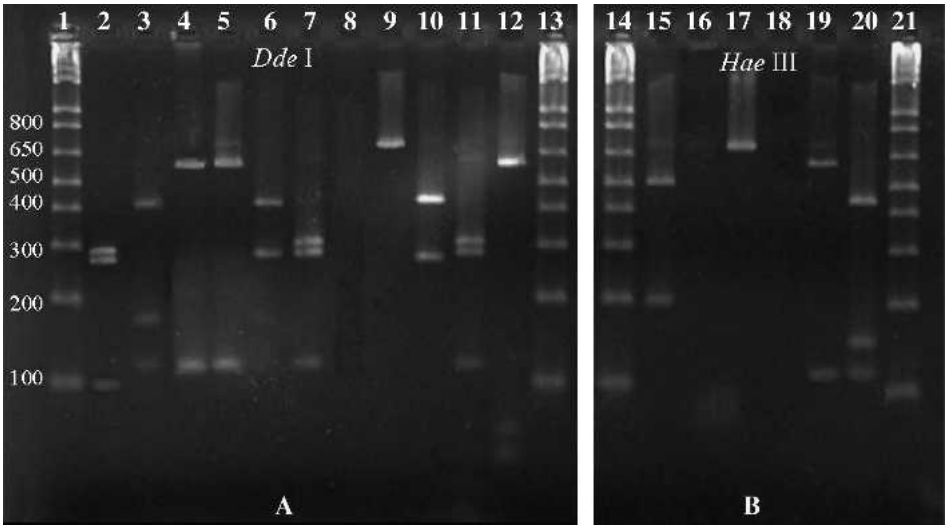

Figure 2. Polymerase chain reaction (PCR) amplified COI mtDNA gene region for nine species of Pacific oysters digested with two restriction endonucleases: (A) *Dde* I; Lanes 1 and 13 are 1 Kb + size standards; 2 = *C. virginica* haplotype A; 3 = *C. belcheri* B; 4 = *C. madrasensis C*; 5 = *C. iredalei* C; 6 = *C. gryphoides* D; 7 = *C. gigas* E; 8 = blank; 9 = *C. sikamea* F; 10 = *C. sikamea* G; 11 = *C. ariakensis* E; 12 = *C. hongkongensis* H, and (B) *Hae* III; Lanes 14 and 21 are 1 Kb + size standards, 15 = *C. gigas* C; 16 = blank; 17 = *C. ariakensis D*; 18 = blank; 19 = *C. madrasensis* A; 20 = *C. iredalei* B.

of F1 hybrids from a hatchery cross of *C. sikamea* and *C. gigas*, although the presence of extra heteroduplex bands (apparently resulting from reannealing of single strands from different species in the PCR process) added a layer of complexity to the key (Camara et al. 2008). Because the key is PCR-based, little sample tissue is required, making the key potentially useful for identification of spat and larvae. In addition, use of the key does not require expensive DNA sequencing equipment, and therefore should be useful to a wider variety of researchers with access to basic molecular biology laboratories.

The key is unable to distinguish *C. madrasensis* and *C. iredalei* based on the ITS-1 marker, although the species pair was resolved when using COI. This is reflected in their phylogenetic relationship derived from sequences of the same loci, in which *C. iredalei* forms a fairly well supported (75% bootstrap value) monophyletic group nested within *C. madresensis* based on ITS-1, but the two form distinct (100% bootstrap support) sister species in the COI analysis (Reece et al. 2008). Unfortunately, we were able to obtain only a limited number of *C. iredalei* samples. Initially we included in the present study a sample identified morphologically as *C. iredalei* from southern China. Preliminary phylogenetic analyses of COI and ITS-1 sequences indicated the sample was an unidentified species aligned loosely with the *Saccostrea commercialis* and *S. cucullata* outgroups (KSR, unpublished data). The PCR/RFLP analysis of the present study also differentiated this sample from other *C. iredalei* collected closer to the center of its geographic range (Angell 1986, Quayle & Newkirk 1989) in Thailand, based on the size of the undigested ITS-1 amplified region as well as the restriction patterns for both markers (JFC, unpublished data). For these reasons the sample was dropped from the study, though we continue attempts to positively identify it for future incorporation into the key. Until then, results regarding *C. iredalei* must be viewed as provisional. Additionally, the ITS-1 key was not able to distinguish between *C. sikamea* and *C. hongkongensis* using only *Hae* III. Though the underlying sequences (Reece et al. 2008) and the RFLP band patterns (Table 2) did differ, the difference was difficult to distinguish using agarose gel electrophoresis, requiring a second enzyme for positive identification and/or restriction analysis of COI with *Dde* I.

Three Indo-Pacific *Crassostrea* species of interest (*C. nippona*, *C. angulata*, and *C. plicatula*) were not included in the key for a number of reasons. In the case of *C. nippona* and *C. angulata* we were unable to obtain samples, although sequences for both species are available from GenBank (http://www.ncbi.nih.gov/Genbank/index.html). Initial VRED analysis of *C. nippona* COI sequences indicates that the PCR-amplified product would be uncut by *Dde* I, making it easily distinguishable from all of the other *Crassostrea* species in the key except *C. sikamea* haplotype A. VRED analysis of ITS-1 sequences also obtained from GenBank indicates *C. nippona* cannot be distinguished from *C. hongkongensis* based on the two restriction endonucleases in our key, but several other endonucleases will differentiate between the two (JFC, unpublished data). Phylogenetic analyses of various Indo-Pacific *Crassostrea* species based on COI, 16S, and ITS-1 gene regions (Lam & Morton 2003, Reece et al. 2008) suggest *C. nippona* may be most closely related to *C. hongkongensis* and *C. ariakensis*.

Some phylogenetic studies based on COI sequences (O'Foighil et al. 1998, Lapeìgue et al. 2004, Reece et al. 2008) have suggested that *C. angulata* is distinct from but closely related to *C. gigas*, whereas another based on ITS-1 sequences (Reece et al. 2008) did not provide support for the two as distinct species. Boudry et al. (1998) differentiated Asian and transplanted European populations of *C. gigas* and *C. angulata* based on PCR/RFLP analysis of COI using four restriction endonucleases, although they did not test the two (*Dde* I and *Hae* III) used in the present study. Only COI sequences were available from GenBank for *C. angulata*, and VRED analysis of these sequences suggested that the two species may be distinguishable based on *Dde* I digestions (JFC, unpublished data). However, larger sample sizes incorporating existing intraspecific variation would be

necessary to confirm these preliminary VRED analyses for both *C. nippona* and *C. angulata*.

The phylogenetic status of *C. plicatula* is problematic. Using samples identified morphologically as *C. plicatula* from northern China, Yu et al. (2003) was unable to distinguish them from samples of *C. ariakensis* collected in the same general area based on a phylogenetic analysis of COI sequences, and speculated that they may have been a *C. ariakensis* ecotype adapted to higher salinity waters. Reece et al. (2008) incorporated Yu's *C. plicatula* and *C. ariakensis* sequences into their expanded COI phylogeny and found that they all formed part of a well supported (90% bootstrap value) clade with *C. sikamea* samples collected in Japan. Reece et al. (2008) included another sample of oysters morphologically identified as *C. plicatula* and collected in southern China in their COI and ITS-1 phylogenies. In the COI analysis individuals grouped with the *C. angulata*/*C. gigas* clade; in the ITS-1 analysis, individuals grouped with *C. gigas* (no samples collected as *C. angulata* were included in the ITS-1 analysis). Because there is no clear genetic evidence for *C. plicatula* as a distinct species to date and we could not verify the identification of our samples collected as *C. plicatula*, they were not included in the key. We hope to add this species as well as *C. nippona* and *C. angulata* in the future.

To date the molecular genetic identification key developed in this study has proven useful in a number of ways. It has been used to identify potentially mislabeled individuals in archived samples of oyster tissue collected over the past decade, as well as verify the morphologically-based species identifications accompanying newly arrived samples. On a recent collection trip to China, the key was used to quantify the species composition of samples collected in the field before they were brought back (live and preserved in ethanol) to the United States (JFC, unpublished data). In addition, the key has been used to investigate possible instances of reproductively viable *C. ariakensis* in the Chesapeake Bay, where sterile triploid *C. ariakensis* are currently being used in experimental deployments to ascertain the suitability of this species for large-scale introduction. Our laboratory has performed tests similar to these examples for other researchers, but our hope is that publication will allow any moderately well equipped molecular laboratory to use the key. To facilitate this, plans exist to establish a database linked to our laboratory web site (http://www.vims.edu/env/research/dna/) that will contain protocols, additional gel images, and periodic updates as new species, intraspecific variants, and hybrids are added to the key.

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