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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. ireadiei 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 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 46° 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. ireadiei, 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>
<thead>
<tr>
<th>Species</th>
<th>Sample Location</th>
<th>Collection Date</th>
<th>Sample Code</th>
<th>N</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Crassostrea virginica</em></td>
<td>Horn Point Hatchery</td>
<td>02/2006</td>
<td>CviHP</td>
<td>120</td>
<td>R. Newell</td>
</tr>
<tr>
<td><em>C. belcheri</em></td>
<td>Puluau Aman near Penang, Malaysia</td>
<td>10/2000</td>
<td>CarMal*</td>
<td>24</td>
<td>I. Kornfeld</td>
</tr>
<tr>
<td><em>C. iredalei</em></td>
<td>Chonburi, Thailand</td>
<td>02/2001</td>
<td>CiCB*</td>
<td>2</td>
<td>S. Klinbunga</td>
</tr>
<tr>
<td><em>C. madrasensis</em></td>
<td>Phangnga, Thailand</td>
<td>02/2001</td>
<td>CiPN*</td>
<td>2</td>
<td>S. Klinbunga</td>
</tr>
<tr>
<td><em>C. gryphoides</em></td>
<td>India</td>
<td>06/2000</td>
<td>CmInd*</td>
<td>25</td>
<td>F. Obeirn</td>
</tr>
<tr>
<td><em>C. gigas</em></td>
<td>Ariake Bay, Japan</td>
<td>06/2000</td>
<td>CgInd*</td>
<td>27</td>
<td>F. Obeirn</td>
</tr>
<tr>
<td><em>C. sikamea</em></td>
<td>Nantong, Jiangsu Province, China</td>
<td>05/2004</td>
<td>Cgi</td>
<td>60</td>
<td>C. Langdon</td>
</tr>
<tr>
<td><em>C. ariakensis</em></td>
<td>Itoki R., Japan</td>
<td>05/2006</td>
<td>Csii</td>
<td>38</td>
<td>K. Reece</td>
</tr>
<tr>
<td><em>C. hongkongensis</em></td>
<td>Yellow River, Shandong Province, China</td>
<td>06/1999</td>
<td>CarYR*</td>
<td>20</td>
<td>H. Que</td>
</tr>
<tr>
<td></td>
<td>Kahwa River, South Korea</td>
<td>06/1999</td>
<td>CarKR</td>
<td>20</td>
<td>H. An</td>
</tr>
<tr>
<td><em>C. hongkongensis</em></td>
<td>Dafeng R., Guangxi Province, China</td>
<td>05/1999</td>
<td>CarDR*</td>
<td>20</td>
<td>H. Que</td>
</tr>
<tr>
<td></td>
<td>Yamen R., Guangdong Province, China</td>
<td>05/1999</td>
<td>CarZ*</td>
<td>20</td>
<td>H. Que</td>
</tr>
<tr>
<td></td>
<td>Souchang R., Guangdong Province, China</td>
<td>05/1999</td>
<td>CarYJ*</td>
<td>20</td>
<td>H. Que</td>
</tr>
</tbody>
</table>

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 dH₂O, 2.5 μL × 10 PCR buffer, 0.75 μL 50 mM MgCl₂, 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 5 ng 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% agarose gels in 1X 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>
<thead>
<tr>
<th>Species</th>
<th>Allele</th>
<th>n&lt;sub&gt;a&lt;/sub&gt;</th>
<th>Band Sizes (bp)</th>
<th>Total (bp)</th>
<th>Species</th>
<th>Haplotype</th>
<th>n&lt;sub&gt;a&lt;/sub&gt;</th>
<th>Band Sizes (bp)</th>
<th>Total (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hae III</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>Dde I</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. virginica</em></td>
<td>A&lt;sup&gt;+&lt;/sup&gt;</td>
<td>178</td>
<td>111, 86, 62, 180, 80</td>
<td>519</td>
<td><em>C. virginica</em></td>
<td>A</td>
<td>120</td>
<td>284, 47, 97, 272</td>
<td>700</td>
</tr>
<tr>
<td><em>C. belcheri</em></td>
<td>B</td>
<td>62</td>
<td>197, 62, 180, 80</td>
<td>519</td>
<td><em>C. belcheri</em></td>
<td>B</td>
<td>24</td>
<td>115, 169, 416</td>
<td>700</td>
</tr>
<tr>
<td><em>C. madrasensis</em></td>
<td>D&lt;sup&gt;1&lt;/sup&gt;</td>
<td>50</td>
<td>178, 67, 132, 133, 100</td>
<td>610</td>
<td><em>C. madrasensis</em></td>
<td>C&lt;sup&gt;3&lt;/sup&gt;</td>
<td>25</td>
<td>115, 7, 578</td>
<td>700</td>
</tr>
<tr>
<td><em>C. iredalei</em></td>
<td>D&lt;sup&gt;1&lt;/sup&gt;</td>
<td>8</td>
<td>178, 67, 132, 133, 100</td>
<td>610</td>
<td><em>C. iredalei</em></td>
<td>C&lt;sup&gt;3&lt;/sup&gt;</td>
<td>4</td>
<td>115, 7, 578</td>
<td>700</td>
</tr>
<tr>
<td><em>C. griffithioides</em></td>
<td>E</td>
<td>54</td>
<td>181, 188, 106, 43, 90</td>
<td>608</td>
<td><em>C. griffithioides</em></td>
<td>D</td>
<td>27</td>
<td>417, 283</td>
<td>700</td>
</tr>
<tr>
<td><em>C. gigas</em></td>
<td>F</td>
<td>120</td>
<td>153, 53, 242, 97</td>
<td>545</td>
<td><em>C. gigas</em></td>
<td>E&lt;sup&gt;4&lt;/sup&gt;</td>
<td>60</td>
<td>115, 302, 283</td>
<td>700</td>
</tr>
<tr>
<td><em>C. sikamea</em></td>
<td>G&lt;sup&gt;2&lt;/sup&gt;</td>
<td>76</td>
<td>169, 44, 128, 109, 99</td>
<td>549</td>
<td><em>C. sikamea</em></td>
<td>F&lt;sup&gt;*&lt;/sup&gt;</td>
<td>36</td>
<td>417</td>
<td>700</td>
</tr>
<tr>
<td><em>C. ariakensis</em></td>
<td>H</td>
<td>120</td>
<td>161, 55, 58, 65, 124, 96</td>
<td>559</td>
<td><em>C. ariakensis</em></td>
<td>G&lt;sup&gt;**&lt;/sup&gt;</td>
<td>2</td>
<td>275, 425</td>
<td>700</td>
</tr>
<tr>
<td><em>C. hongkongensis</em></td>
<td>I&lt;sup&gt;2&lt;/sup&gt;</td>
<td>119</td>
<td>171, 50, 125, 126, 103</td>
<td>575</td>
<td><em>C. hongkongensis</em></td>
<td>E&lt;sup&gt;4&lt;/sup&gt;</td>
<td>60</td>
<td>115, 302, 283</td>
<td>700</td>
</tr>
<tr>
<td><em>Hin I</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><em>Hae III</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. sikamea</em></td>
<td>A</td>
<td>75</td>
<td>411, 138</td>
<td>549</td>
<td><em>C. madrasensis</em></td>
<td>A</td>
<td>25</td>
<td>583, 117</td>
<td>700</td>
</tr>
<tr>
<td><em>C. ariakensis</em></td>
<td>B&lt;sup&gt;+&lt;/sup&gt;</td>
<td>118</td>
<td>182, 229, 164</td>
<td>575</td>
<td><em>C. iredalei</em></td>
<td>B</td>
<td>2</td>
<td>432, 151, 117</td>
<td>700</td>
</tr>
<tr>
<td><em>C. hongkongensis</em></td>
<td>C&lt;sup&gt;**&lt;/sup&gt;</td>
<td>2</td>
<td>411, 164</td>
<td>575</td>
<td><em>C. gigas</em></td>
<td>C</td>
<td>60</td>
<td>502, 198</td>
<td>700</td>
</tr>
<tr>
<td><em>C. hongkongensis</em></td>
<td>D</td>
<td>60</td>
<td>700</td>
<td>700</td>
<td><em>C. ariakensis</em></td>
<td>D</td>
<td>60</td>
<td>700</td>
<td>700</td>
</tr>
</tbody>
</table>

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 *Crassostrea* 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<sub>a</sub> is the number of times a particular allele was seen in the ITS-1 analysis, n<sub>a</sub> 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.
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 *HinI* 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 *HinI* 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 *HinI* 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 performed on the six species listed earlier for the ITS-1 marker analysis. Four (*Dde I*, *Mnl I*, *Msp I*, *TspI*) 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 *Msp 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
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 identifying 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. madrasensis 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, Lapeigue 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

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; 4 = C. madrasensis; 5 = C. iredalei; 6 = C. gryphoides; 7 = C. gigas; 8 = blank; 9 = C. sikamea; 10 = C. sikamea; 11 = C. ariakensis; 12 = C. hongkongensis H; and (B) Hae III; Lanes 14 and 21 are 1 Kb + size standards, 15 = C. gigas; 16 = blank; 17 = C. ariakensis D; 18 = blank; 19 = C. madrasensis A; 20 = C. iredalei B.
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 north-
ern 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 col-
clected 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 accom-
panying 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 cur-
rently 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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