Identification of the Clam Species *Ruditapes decussatus* (Grooved Carpet Shell), *Venerupis pullastra* (Pullet Carpet Shell), and *Ruditapes philippinarum* (Japanese Carpet Shell) by PCR-RFLP

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PCR-RFLP analysis has been applied to the identification of three clam species: *Ruditapes decussatus* (grooved carpet shell), *Venerupis pullastra* (pullet carpet shell), and *Ruditapes philippinarum* (Japanese carpet shell). PCR amplification was carried out using a set of primers designed from the DNA nucleotide sequences reported for α-actins from humans and various animals. Restriction endonuclease analysis based on sequence data of the PCR products of each clam species revealed the presence of species-specific polymorphic sites for *Mae*III and *Rsa*I endonucleases. Electrophoretic analysis of the amplicons digested with *Mae*III and *Rsa*I produced species-specific profiles that allowed the genetic identification of the three clam species.

Keywords: *Species identification; PCR-RFLP; α-actin gene; clam species; Ruditapes decussatus; Venerupis pullastra; Ruditapes philippinarum*

INTRODUCTION

The clam species *Ruditapes decussatus* (grooved carpet shell), *Venerupis pullastra* (pullet carpet shell), and *Ruditapes philippinarum* (Japanese carpet shell) are among the most common clam species found in the market for human consumption. Because of its delicate taste and meat texture, *R. decussatus* is the clam species preferred by consumers and also the most expensive. Its value on the market can reach 4-fold the price of any other clam species.

When the clams are sold alive, they can be identified by morphological criteria, such as siphon shape and location as well as shell characteristics (shape, color, and striations). However, due to their difference in price, the possibility exists for fraudulent substitution of clam species, especially if the shell is removed and the clams are sold mixed with other ingredients, usually frozen. Nonmorphological methods for clam species identification are then necessary to enforce labeling regulations in the authentication of bivalves.

In contrast to the numerous analytical methods that have been developed for meat (García et al., 1994; Matsunaga et al., 1999), milk (Anguita et al., 1995; Dennis, 1996), or fish (Carrera et al., 1999a,1999b; Rehbein et al., 1999) species identification, molluscs have received little attention so far.

Allozyme electrophoresis has been used for the identification of mussel species (Sanjuan et al., 1996) to identify their geographical distribution. However, for species identification, it would be preferable to analyze DNA rather than allozymes. Analysis of DNA variation is rapidly becoming the method of choice because of its greater potential sensitivity, the vast number of loci to be tapped, and the higher probability of variation being neutral. Regions of the genome evolve at rates varying from those suitable for the identification of individuals (Jeffreys et al., 1985) to those suitable for resolving deep phylogenetic branches (Olsen, 1987).

Genetic methods have been purposed for the identification of some mollusc species. Borgo et al. (1996) developed a PCR-RFLP technique for the identification of three snail species based on the amplification of mitochondrial 16S rRNA and 12S rRNA genes using universal primers followed by restriction site analysis of the PCR products. Heath et al. (1995) employed PCR-based nuclear markers for identifying alien blue mussel (*Mytilus* spp.) genotypes on the west coast of Canada. Martínez-Lage et al. (1995) described the differences among *M. edulis*, *M. galloprovincialis*, and *M. trossulus* on the basis of chromosomal markers.

Actins are highly conserved contractile proteins and are usually encoded by multiple genes in multicellular eukariotes (Shah et al., 1983). Because actin genes are very conserved at the amino acid level, they have been used to study codon bias and phylogenetic relationships across large phylogenetic distances (Fang and Brandhorst, 1994; He and Haymer, 1995). Although actin genes have been studied in a variety of organisms, there are few genetic studies at the molecular level in bivalves. In this work, we have studied the potential use of PCR-RFLP, using a set of primers designed from the DNA nucleotide sequences reported for α-actins from humans and various animals, for the identification of three clam species (*R. decussatus*, *V. pullastra*, and *R. philippinarum*).

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MATERIALS AND METHODS

Sample Collection. *R. decussatus, V. pullastra*, and *R. philippinarum* were collected from different markets in Madrid (Spain) over several months. Trained veterinarians morphologically identified all specimens. Fifteen fresh and frozen individuals of each clam species were analyzed in this work.

DNA Extraction. Four different DNA extraction methods have been assayed to obtain high molecular weight DNA suitable for PCR amplification. Extraction protocols essentially followed those used by De Salle et al. (1995), Borgo et al. (1996), Jones et al. (1993), and Bagshaw (1991) and are briefly described bellow.

Method 1 (De Salle et al., 1995). Muscle tissue (0.1 g) was homogenized with 500 μL of TSM buffer (0.2 M Tris, 0.1 M EDTA, and 1% SDS), containing $^1/_{50}$ volume of protease K (10 mg/mL), and incubated overnight at 55 °C with vigorous shaking. After the lysis step, $^1/_{10}$ volume of 5 M potassium acetate was added to the lysate, mixed by inverting the tubes, and incubated on ice for 30 min. The tubes were centrifuged at 8000*g* for 10 min, and the supernatant was transferred to a clean tube. DNA was extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol in a 25:24:1 ratio and once with chloroform. The DNA was then precipitated twice with ethanol. The pellet was allowed to dry at 20 °C and resuspended in 100 μL of sterile distilled water.

Method 2 (Borgo et al., 1996). Muscle tissue (0.1 g) was homogenized in 600 μL of TEK buffer (100 mM Tris-HCl, 20 mM EDTA, and 1.5% KCl, pH 7.8) containing proteinase K (100 μg/mL) and was incubated for 4 h at 55 °C. Mitochondria were lysed in 2 volumes of alkaline SDS (0.2 M NaOH and 1% SDS) for 10 min on ice. One-half volume of 5 M potassium acetate buffer, pH 4.8, was then added, and the lysate was kept on ice 10 min. The tubes were spun, and the supernatant was extracted twice with phenol and once with chloroform/isoamyl alcohol (24:1). DNA was precipitated by adding 1 volume of 2-propanol and 0.1 volume of 5 M sodium acetate, stored overnight at −20 °C, and centrifuged at 18000*g*. The DNA was washed with 70% ethanol, dried, and resuspended in 30 μL of sterile water.

Method 3 (Jones et al., 1993). Twenty grams of muscle tissue was homogenized with 180 mL of PBS buffer (136 mM NaCl, 1.4 mM KH_2PO_4, 8.09 mM $Na_2HPO_4 \cdot 12\ H_2O$, and 2.6 mM KCl, pH 7.2). One milliliter clam homogenate was washed twice with 1 mL of PBS, vortexed, and centrifuged at 12000*g*. The supernatant fluid was discarded, and the washed pellets were treated with 25 μL of 5.9 M guanidine thiocyanate (Sigma Chemical Co., St. Louis, MO), vortexed, and incubated at 60 °C for 90 min. The suspension was diluted to 0.3 M guanidine thiocyanate by adding 0.2 mL of InstaGene (Bio-Rad, Hercules, CA) and 0.26 mL of sterile distilled water to the samples, which were then mixed and boiled for 8 min in a water bath. The samples were cooled to room temperature, and sodium acetate was added to a final concentration of 0.3 M. Subsequently, the samples were centrifuged at 12000*g* for 10 min at 4 °C, and the supernatant fluids were transferred to new tubes and extracted twice with an equal volume of chloroform. The DNA was precipitated with 95% ethanol, sedimented by centrifugation, and resuspended in sterile distilled water.

Method 4 (Bagshaw, 1991). Muscle tissue (0.5 g) was homogenized with 5 mL of DNA extraction buffer (50 mM Tris, pH 8; 0.1 M NaCl, 0.1 M EDTA, and 0.5% SDS) and 15 μL of proteinase K (20 mg/mL) (Roche, Basel, Swizerland) for 6 h at 55 °C or until complete lysis of samples. After incubation, 5 mL of phenol equilibrated to pH 8 was added to the lysate, and tubes were incubated overnight at 20 °C with gentle rocking. The samples were then centrifuged at 1500*g* for 10 min, and the aqueous phase was carefully transferred to a fresh tube. The DNA was extracted with phenol/chloroform (1:1) for at least 1 h and precipitated with ethanol. Pellets were washed with 2 mL of 70% ethanol for 1 h, dried, and dissolved in 2 mL of TE buffer (10 mM Tris, pH 8, and 1 mM EDTA).

NaCl was added to the DNA solution to bring the final concentration to 0.7 M, and Tris, pH 8, was also added to a final concentration of 0.1 M. One-ninth volume of a solution containing 10% cetyltrimethylammonium bromide and 0.7 M NaCl was then added and mixed gently. Finally, DNA was extracted twice with chloroform, precipitated with ethanol, and washed in 70% ethanol. The dried pellet was dissolved in 150−300 μL of sterile distilled water.

PCR Amplification. The PCR amplification reactions were performed in a total volume of 50 μL. Each reaction mixture contained 2000−3000 ng of template DNA, 2 mM $MgCl_2$, 100 pmol of each primer, 200 μM each dNTP, and 2 units of DNA polymerase (Biotools, Madrid, Spain) in a reaction buffer containing 75 mM Tris-HCl, pH 9.0, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, and 0.001% BSA.

The forward primer, act2 (5′-dTACCT(GC)ATGAAGATC-CTCACTGAG-3′), and the reverse primer, act4 (5′-dTCCT-GCTTGGTGATCCACATCTG-3′), used for amplification were designed by Watabe et al. (1995) and were based on sequences for α-actin from humans and various animals such as chick, rat, and mouse.

The Polymerase Chain Reaction was carried out in a Progene thermal cycler (Techne Ltd., Cambridge, U.K.), programmed to perform a denaturation step of 93 °C for 3 min, followed by 35 cycles consisting of 1 min at 93 °C for denaturation, 1.5 min at 55 °C for annealing, and 2 min at 72 °C for extension. The last extension step was 5 min longer.

PCR products (10 μL) were mixed with 2 μL of gel loading solution (Sigma) and loaded in a 1.5% D1 agarose gel (Hispanlab S.A., Alcobendas, Spain) containing 1 μg/mL ethidium bromide in Tris−acetate buffer (0.04 M Tris−acetate and 0.001 M EDTA, pH 8.0). Electrophoretic separation was performed at 100 V for 45 min. The resulting DNA fragments were visualized by UV transillumination and analyzed using a Geldoc 1000 UV fluorescent gel documentation system PC (Bio-Rad).

Cleanup and Sequencing of the PCR Products. The PCR product (120 μL) was loaded in a 1.5% LM-2 agarose gel (Hispanlab) containing 1 μg/mL ethidium bromide in Tris−acetate buffer and electrophoresed at 90 V for 70 min. The DNA of the band was excised under UV light and purified using the QIAquick Gel Extraction kit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions.

Purified PCR products were sequenced at the Centro de Investigaciones Biológicas (Consejo Superior de Investigaciones Científicas, Madrid, Spain), in a ABI PRISM model 377 DNA sequencer (Perkin-Elmer/Applied Biosystems Division, Foster City, CA), using primers act2 and act4 with the dRhodamine Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer). Wisconsin Package version 9.0 (Genetics Computer Group, Madison, WI) was used for sequence analysis.

Restriction Site Analysis of the PCR Products. PCR products were gel purified using the QIAquick Gel Extraction kit protocol (QIAGEN), following the manufacturer's instructions. However, good results were also obtained without previous purification of the amplified DNA fragments.

Restriction maps of the actin PCR products from *R. decussatus*, *V. pullastra*, and *R. philippinarum* were compared using the Wisconsin Package version 9.0. From the detailed comparison of the sequences, *Mae*III and *Rsa*I endonucleases (Roche) were selected as suitable candidates for identification of the clam species. Digestions with *Mae*III were performed in a total volume of 20 μL containing 100−200 ng of amplified DNA, 2 units of enzyme, and 10 μL of 2× digestion buffer recomended by the manufacturer and were incubated at 55 °C for 16 h. Digestions with *Rsa*I were also performed in a total volume of 20 μL containing 100−200 ng of DNA, 10 units of enzyme *Rsa*I, and 2 μL of 10× buffer provided by the manufacturer and incubated for 16 h at 37 °C.

The resulting fragments were separated by electrophoresis in a 3% MS8 agarose gel (Hispanlab) for 1 h at 90 V. The sizes of the resulting DNA fragments were estimated by comparison with a commercial 1 kb plus DNA ladder (Gibco BRL, Life Technologies Inc., Rockville, MD).

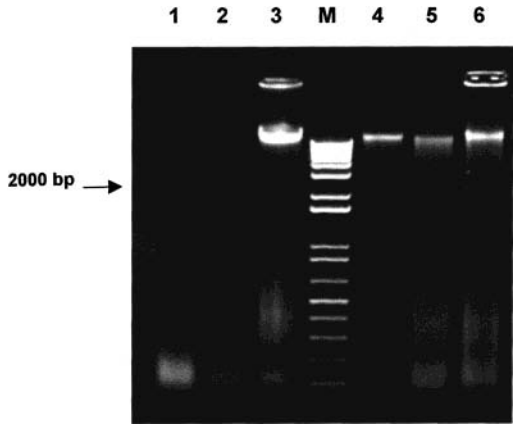

Figure 1. Electrophoretic analysis of the DNAs from clams following the methods described by (1) Jones et al. (1993), (2) Borgo et al. (1996), (3) DeSalle et al. (1995), and (4−6) Baghsaw (1991). Clam species used for DNA extraction were *R. philippinarum* (1−3 and 6), *R. decussatus* (4), and *V. pullastra* (5). M = molecular weight marker 1 kb plus DNA ladder.

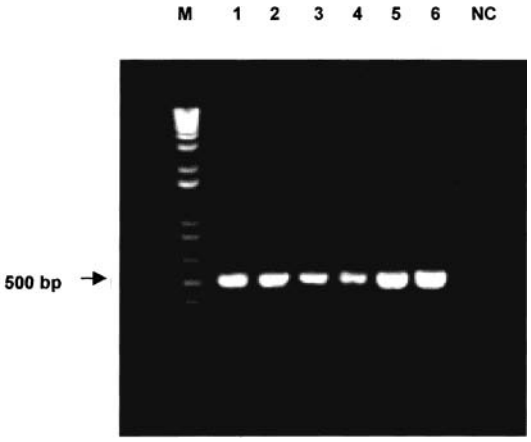

Figure 2. Electrophoretic analysis of the actin PCR products obtained from two individuals of the clam species *R. decussatus* (1 and 2), *V. pullastra* (3 and 4), and *R. philippinarum* (5 and 6). M = molecular weight marker 1 kb plus DNA ladder; NC = negative control.

RESULTS AND DISCUSSION

Genetic differentiation of the clam species *R. decussatus*, *V. pullastra*, and *R. philippinarum* has been achieved by PCR amplification of a DNA fragment using primers based on the nucleotide sequences of several α-actin genes, followed by analysis of the polymorphism in the fragments generated by appropriate restriction endonucleases (PCR-RFLP).

A previous step in the development of genetic markers is to obtain DNA from the clam samples that are suitable for PCR amplification. The method described by DeSalle et al. (1995) was first tested, because it had been used to obtain high-quality DNA from fish samples (Carrera et al., 1999b; Céspedes et al., 1999a). When applied to clams, high molecular weight DNA (Figure 1, lane 3) with an absorbance ratio A_{260}/A_{280} of 1.8−2.0 was obtained. However, it was not possible to amplify any fragment from these DNAs using universal primers for several mitochondrial (Bartlett and Davidson, 1991; Céspedes et al., 2000) and nuclear genes (Pendas et al., 1995; Watabe et al., 1995). The lack of amplification could be produced by the extraction of unidentified inhibitor compounds from the mollusc tissues together with the DNA (Wilson, 1997). Thus, several extraction methods intended for DNA amplification from molluscs were tested. The method described by Borgo et al. (1996) for snail meat DNA extraction produced fragments smaller than 500 bp and having absorbance ratios A_{260}/A_{280} of 1.6−1.7 and was unsuitable for PCR amplification (Figure 1, lane 2). The method described by Jones et al. (1993) also yielded degraded DNA unsuitable for amplification (Figure 1, lane 1). Finally, using a modification of the protocol described by Bagshaw (1991), DNA was obtained with little degradation (Figure 1, lanes 4−6) having absorbance ratios A_{260}/A_{280} of 1.8−2.0 and was suitable for PCR amplification. Probably, the overnight extraction with phenol is essential to eliminate inhibitors present in the clam tissues, which are otherwise difficult to separate from the DNA.

Actins constitute a multigene family of highly conserved proteins found in all eucaryotic cells (Zakut et al., 1982). They are globular proteins that polymerize into filaments for most of their biological functions, such as muscle contraction, cell motility, and cellular structure (Pollard and Cooper, 1986). Nucleotide sequences of actin genes are available in the databanks for several species, such as humans (Taylor et al., 1988), mouse (Hu et al., 1986), rat (Zakut et al., 1982), chick (Fornwald et al., 1982), carp and goldfish (Watabe et al., 1995), and sea scallop (Patwary et al., 1996). As they are highly conserved, Watabe et al. (1995) used published sequences of α-actin genes from various animals to design a set of primers (act2 and act4) that amplify part of the α-actin cDNA from carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*). This set of primers encompasses 521 nt, which cover 173 amino acids near the C terminus.

Primers act2 and act 4 consistently amplified a fragment of 520 bp from genomic DNA of the clam species *R. decussatus*, *V. pullastra*, and *R. philippinarum* (Figure 2).

PCR products from at least two individuals of each species were sequenced in both directions to confirm that the amplified products were the target actin genes. Sequences were aligned and compared using Wisconsin Package, GCG, version 9.0 (Figure 3). For the 477 bp sequenced, excluding primers, *R. decussatus* PCR products differed at 16 nucleotides from *V. pullastra*, whereas 14 positions were different from those of *R. philippinarum*. Once PCR products from reference samples have been sequenced, several approaches can be followed for specific identification of unknown samples. Polymorphic sites with diagnostic value can be used for the identification of closely related species (Unseld et al., 1995). Also, phylogenetic analysis using parsimonia criteria (Bartlett et al., 1992) and genetic distance measurement with phylogenetic tree construction (Quinteiro et al., 1998) have demonstrated their value to identify closely related species. However, all such methods require sequencing of the PCR products from each unknown sample, which is costly and time-consuming. An alternative approach to sequencing is the analysis of PCR fragments with endonucleases that detect interspecific restriction fragment length polymorphisms (Meyer et al., 1995; Ram et al., 1996; Carrera et al., 1999a).

A detailed comparison of the restriction maps of the PCR products obtained in this work allowed identification of at least two restriction endonucleases (*Rsa*I and *Mae*III) that could be used to reliably differentiate the three clam species analyzed (Figure 3). According to

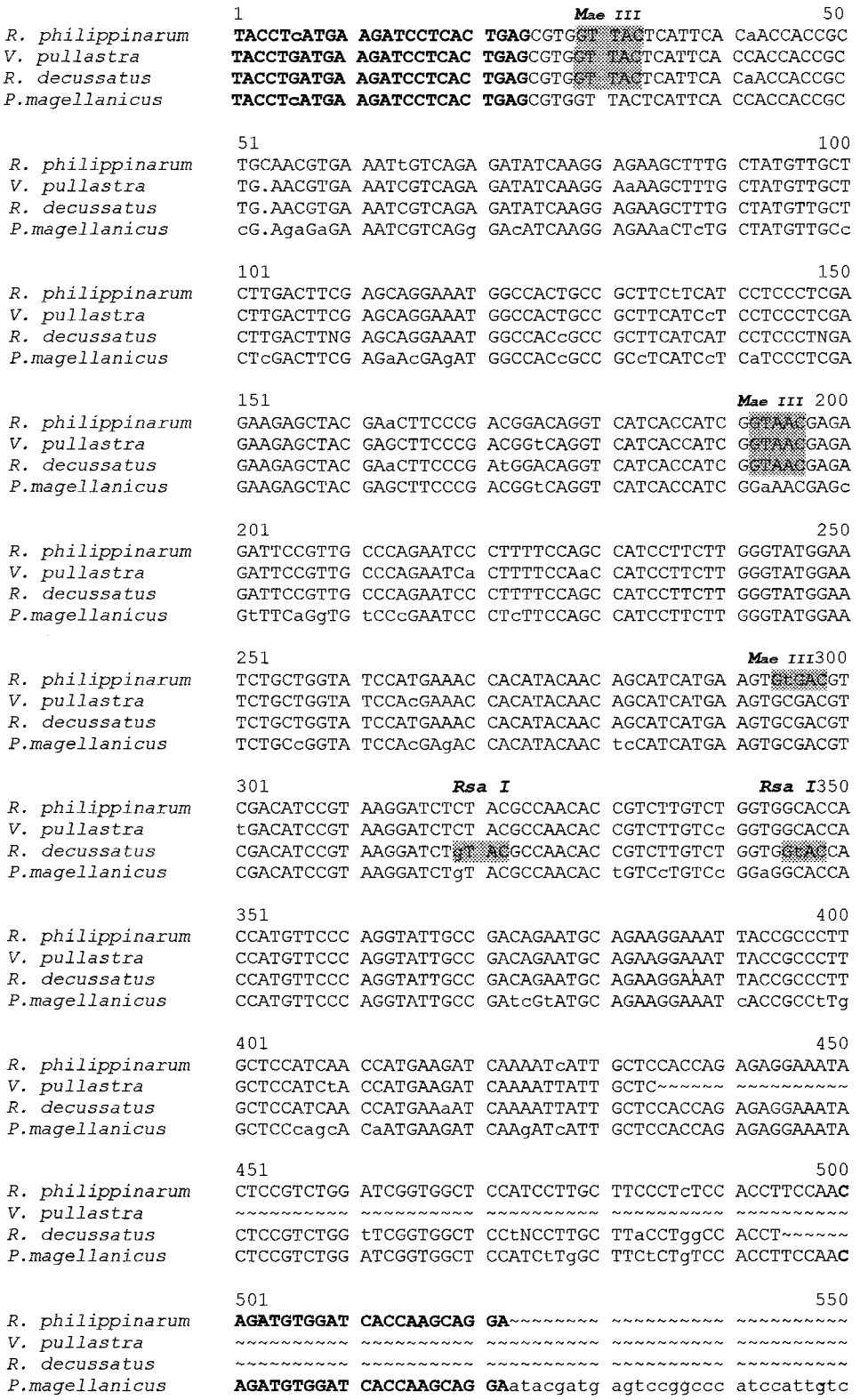

Figure 3. DNA sequences of the actin PCR products from *R. decussatus*, *V. pullastra*, and *R. philippinarum* aligned with the α-actin cDNA sequence of *P. magellanicus* obtained from GenBank (accession no. U55046). Restriction sites for *Rsa*I (GT↓AC) and *Mae*III (↓GTNAC) are shown with shading. Bold-type nucleotides indicate the position of primers act2 and act4 used for PCR amplification.

sequence analysis, there are two restriction sites for *Rsa*I in the sequences of *R. decussatus* that cleave the PCR products from this species into three fragments of 320, 170, and 30 bp. Nevertheless, this enzyme does not cleave PCR products from *V. pullastra* or *R. philippinarum*. On the other hand, there are two restriction sites for *Mae*III in *R. decussatus* and *V. pullastra* PCR products that would yield three fragments of 330, 160, and 30 bp. An extra restriction site for *Mae*III is present in the PCR products of *R. philippinarum*, thus yielding four fragments of 230, 160, 100, and 30 bp.

Results following digestion of the clam PCR products from the three species showed that band sizes obtained by electrophoresis in 3% MS8 agarose gel were in

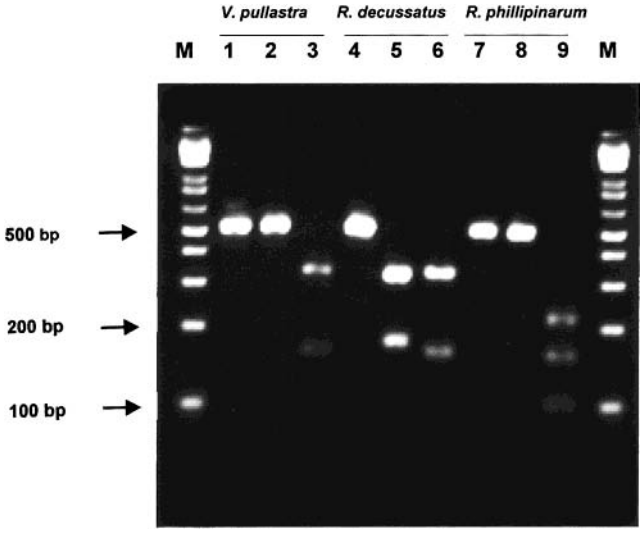

Figure 4. Electrophoretic analysis (3% MS8 agarose) showing the restriction profiles of the actin PCR products obtained from the clam species *V. pullastra* (1−3), *R. decussatus* (4−6), and *R. phillipinarum* (7−9). Lanes: 1, 4, and 7, undigested; 2, 5, and 8, digested with *Rsa*I; 3, 6, and 9, digested with *Mae*III. M = molecular weight marker 1 kb plus DNA ladder.

agreement with the expected sizes of the restriction fragments inferred from sequence analysis (Figure 4). It should be noted that resolution of agarose gel did not allow visualization of the smallest fragments (30 bp) that resulted from digestions. At least 15 individuals from each clam species were analyzed using PCR-RFLP of the actin gene, and results did not show intraspecific polymorphism.

The sequences of clam PCR products obtained in this work were compared to human (M20543), mouse (M12866), and chick (K02259) skeletal muscle actin genes and also to carp (D50028), goldfish (D50029), and sea scallop (U55046) skeletal actin cDNA sequences available in the Genbank/EMBL databank. As a result of comparison, it was found that sequences of clam PCR products resemble those of cDNA. Thus, they lack the three introns present in conserved positions within this fragment in human, rat, mouse, and chick α-actin genes (Taylor et al., 1988; Zakut et al., 1982; Hu et al., 1986; Fornwald et al., 1982).

The lack of introns in the clam PCR products is probably due to amplification of processed pseudogenes. Moreover, the insertion of a single nucleotide at position 54 of the *R. philippinarum* sequence produces a shift in the reading frame generating several stop codons, which are frequently found in pseudogenes (Proudfoot, 1980). Amplification of intronless pseudogenes when using primers designed for conserved exon sequences has been previously described. Lessa and Applebaum (1993) had some difficulty using exonic primers designed for insulin gene. Similarly, Slade et al. (1993) had to design primers with several bases inside the intron sequence to avoid amplification of intronless pseudogenes of histone H2AF and myoglobin genes. They concluded that processed pseudogenes seem to be the rule rather than the exception. These interfere with amplification of the intron-containing genes because, being substantially shorter, they amplify more efficiently. However, pseudogenes and noncoding DNA sequences such as nontranscribed intergenic spacers (NTSs) have been described as sequences for comparing closely related species due to their exhibiting rapid substitution and homogenization (Slade et al., 1993; Sajdak et al., 1998).

Our results suggest that PCR-RFLP of the actin pseudogene amplified in this work allows clear identification of the clam species *R. decussatus*, *V. pullastra*, and *R. philippinarum*. Interpretation of the restriction profiles can be performed visually, avoiding the need for tedious sequencing and sequence analysis methods. The PCR-RFLP method described herein can be useful for the identification of mislabeled clam species and also for shellfish industries as a tool to warrant the quality and identity of the clams offered for sale.

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