Polymerase Chain Reaction–Restriction Fragment Length Polymorphism Analysis of a 16S rRNA Gene Fragment for Authentication of Four Clam Species

ALICIA FERNÁNDEZ, TERESA GARCÍA,* ISABEL GONZÁLEZ, LUIS ASENSIO, MIGUEL ÁNGEL RODRÍGUEZ, PABLO E. HERNÁNDEZ, AND ROSARIO MARTÍN

Departamento de Nutrición y Bromatología III, Facultad de Veterinaria, Universidad Complutense, 28040 Madrid, Spain

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ABSTRACT

Specific identification of four clam species, Ruditapes decussatus (grooved carpet shell), Venerupis pullastra (pullet carpet shell), Ruditapes philippinarum (Japanese carpet shell), and Venerupis rhomboides (yellow carpet shell), was achieved by polymerase chain reaction–restriction fragment length polymorphism analysis of a fragment of the mitochondrial 16S rRNA gene. Amplification of DNA isolated from the foot muscle produced fragments of 511 bp for V. pullastra, 523 bp for R. decussatus, 545 bp for R. philippinarum, and 502 bp for V. rhomboides. The restriction profiles obtained by agarose gel electrophoresis when amplicons were digested with endonucleases BsmAI and BsrI allowed unequivocal identification of the four clam species. This approach would be less costly, simpler, and quicker than conventional sequencing of polymerase chain reaction products followed by detailed comparison of individual sequences, especially when large numbers of samples need to be analyzed.

Accurate identification of clam species offered for sale is important for ethical, economic, and legal reasons (7). The four most popular clam species in the Spanish market are Venerupis pullastra (pullet carpet shell), Ruditapes decussatus (grooved carpet shell), Ruditapes philippinarum (Japanese carpet shell), and Venerupis rhomboides (yellow carpet shell). However, these clam species differ in price, with R. decussatus being the most expensive (approximately U.S. $13.30/kg), followed by V. pullastra (U.S. $10.10/kg), V. rhomboides (U.S. $6.40/kg), and R. philippinarum (U.S. $4.4/kg). It is difficult to detect the substitution of a cheaper clam species for a more expensive one when the shell has been removed. Thus, there is a need for analytical methods for species identification to ensure that consumers are receiving authentic fishery and aquaculture products and to enforce the labeling regulation.

Protein analysis techniques for fish and shellfish species differentiation include electrophoretic (6), chromatographic (11), and immunological techniques (5). However, DNA analysis offers several advantages over protein analysis (2). Because DNA is more thermostable than many proteins, analyses using nucleic acids are less liable to be affected by processing. Furthermore, DNA is present in all of the cells of an organism; thus, the same information can be obtained from any tissue used as a sample.

Most of the PCR-based methods for mollusk species identification have focused on phylogenetic and population studies and have been based on the amplification of conserved mitochondrial DNA genes followed by sequencing of the amplified fragments (4), restriction digests of mitochondrial DNA (3), polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) (13), or random amplified polymorphic DNA (1).

The objective of this work was to use the PCR amplification of selected regions of the mitochondrial 16S rRNA gene followed by restriction site analysis of amplified DNA fragments to differentiate the most common clam species found on the market for human consumption, R. decussatus, V. pullastra, R. philippinarum, and V. rhomboides.

MATERIALS AND METHODS

Sample collection and DNA extraction. R. decussatus, V. pullastra, R. philippinarum, and V. rhomboides were collected from Mercamadrid (Madrid Fish Central Market) and from different local markets in Madrid, Spain, over several months. Trained veterinarians morphologically identified each specimen. Fifteen individuals (including fresh and frozen samples) of each clam species were analyzed. Total DNA was extracted from 0.2 g of clam foot muscle with the Wizard Magnetic DNA Purification System for Food (Promega, Madison, Wis.) following the manufacturer’s instructions.

PCR amplification. The PCR amplification reactions were carried out in a total volume of 50 μl with a Progene Thermal Cycler (Techne Ltd., Cambridge, UK). The 16S rRNA mitochondrial gene was PCR amplified with universal oligonucleotide primers 16SAR (5’-dCGCCTGTGTATCAAAAACAT-3’) and 16SBR (5’-dCCGCTGACTACATACGATCAGT-3’) (10) under

* Author for correspondence. Tel: 34-913943747; Fax: 34-913943743; E-mail: tgarcia@vet.ucm.es.
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FIGURE 1. DNA sequences of the 16S rRNA PCR products from V. pullaster, R. decussatus, V. rhomboides, and R. philippinarum aligned with the M. edulis sequence (accession number U22864). Restriction sites for BsmAI (5' [GTCTC(N)14]3 or 3' [CAGAG(N)5]5) and BsrI (5' [ACTGG(N)5]3 or 3' [TGAC(N)5]5) are shadowed. Nucleotides in italic bold type indicate the sequences of primers 16SAR and 16SBR, used for PCR amplification, and are not necessarily identical to the original sequences present in the genome of the four clam species.

the following reaction conditions: a denaturation step of 94°C for 3 min, followed by 30 cycles of 20 s at 94°C for denaturation, 20 s at 50°C for annealing, and 45 s at 72°C for extension, with the final extension step being extended by 5 min.

PCR products (10 μl) were mixed with 2 μl of gel loading solution (Sigma Chemical Co., St. Louis, Mo.) and loaded in a 1.5% low-electroendosmosis D1 agarose gel (Hispanlab S.A., Torrejón de Ardoz, Spain) containing 1 mg of ethidium bromide per ml in Tris acetate buffer (0.04 M Tris acetate, 0.001 M EDTA [pH 8.0]). Electrophoretic separation was performed at 14 V/cm for 45 min. The resulting DNA fragments were visualized by UV transillumination and analyzed with the Geldoc 1000 UV Fluorescent Gel Documentation System-PC (Bio-Rad, Hercules, Calif.).

Cleanup and sequencing of the PCR products. The PCR products (120 μl) were purified with the QIAquick Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Sequences were determined at the DNA Sequencing Center (Facultad de Farmacia, Universidad Complutense de Madrid, Madrid, Spain) with an ABI PRISM Model 377 DNA Sequencer (Perkin-Elmer Applied Biosystems Division, Foster City, Calif.). The Wisconsin Package (version 9.0, Genetics Computer Group, Madison, Wis.) was used for sequence analysis and restriction map comparison. The sequences were deposited in the EMBL database under accession numbers AJ417845 (V. pullaster), AJ417846 (R. decussatus), AJ417847 (R. philippinarum), and AJ417848 (V. rhomboides).

Restriction site analysis of PCR products. Digestions of PCR products with BsmAI and BsrI (Roche, Basel, Switzerland) were performed in separate tubes in a total volume of 20 μl. Each restriction reaction contained 100 ng of amplified DNA, 5 μl of the selected enzyme, and 2 μl of 10X digestion buffer, as recommended by the manufacturer. BsmAI digestions were incubated at 55°C for 16 h, and BsrI digestions were incubated at 65°C for 16 h. Good results were obtained with purified PCR products and without previous purification of the amplified DNA fragments. The resulting fragments were separated by electrophoresis in a 3% high-resolution MS8 agarose gel (Hispanlab) for 1 h at 13 V/cm. The sizes of the resulting DNA fragments were estimated by comparison with a commercial 1 kilobase plus DNA ladder (Gibco BRL, Life Technologies Inc., Rockville, Md.).

RESULTS AND DISCUSSION

Application of DNA techniques in molecular studies of mollusks is seriously hampered by difficulties in extracting high-molecular-weight DNA free from PCR inhibitors, because mollusks secrete mucopolysaccharides and polyphenolic proteins, which interfere with the enzymatic processing of nucleic acids (8). However, the Wizard Magnetic DNA Purification System for Food (Promega) proved to extract high-molecular-weight DNA that was suitable for genetic analysis from clam samples in <2 h, with A260/A280 ratios of 1.7 to 2.0.

The mitochondrial gene for 16S ribosomal RNA has proved to be a powerful tool in phylogenetic studies and has provided information on the systematics of terrestrial and marine vertebrates (9) and invertebrates (12). In this work, universal primers 16SAR and 16SBR (10) were used to amplify the mitochondrial 16S rRNA gene from four
clam species of interest. PCR products from at least two individuals of each species were sequenced with primers 16SAR and 16SBR, and the analysis of these sequences indicated that the lengths of the PCR products were 511 bp for V. pullastra, 523 bp for R. decussatus, 545 bp for R. philippinarum, and 502 bp for V. rhomboides. These lengths are similar to that of the amplified fragment of the mitochondrial 16S RNA gene from the Mytilus edulis species complex (527 nucleotides (12)).

To confirm that the amplified products were from the target 16S rRNA regions, PCR sequences from V. pullastra, R. decussatus, R. philippinarum, and V. rhomboides were aligned and compared with the M. edulis 16S rRNA gene sequence (U22864) and those of the clam species analyzed in this work. Moreover, percentages of identity between the 16S rRNA sequence of R. decussatus and the rest of clam species were 80.58% for V. pullastra, 78.86% for R. philippinarum, and 79.11% for V. rhomboides (Fig. 1). A detailed comparison of the restriction maps of the PCR products obtained in this study allowed the identification of at least two restriction endonucleases (BsmAI and BsrI) that could be used to reliably differentiate the four clam species analyzed (Fig. 1). According to sequence analysis, there are three restriction sites for BsmAI in the sequences of V. pullastra, R. decussatus, and V. rhomboides that should cleave the PCR products from these species into four fragments of 302, 111, 52, and 46 bp in V. pullastra; 304, 157, 41, and 21 bp in R. decussatus; and 293, 158, 30, and 21 bp in V. rhomboides. However, there are two restriction sites for this enzyme in the sequences of R. philippinarum that should produce three fragments of 386, 113, and 46 bp. On the other hand, there is only one restriction site for BsrI in the PCR products from R. philippinarum that should yield two fragments of 494 and 51 bp, and there are two restriction sites for V. rhomboides that should yield three fragments of 328, 126, and 48 bp. Nevertheless, this enzyme does not cleave PCR products from V. pullastra or R. decussatus.

Digestion of the clam PCR products with endonucleases BsmAI and BsrI showed that band sizes obtained by electrophoresis in 3% MS8 agarose gel were in agreement with the expected sizes of the restriction fragments inferred from sequence analysis (Fig. 2). Intraspecific polymorphism in the PCR-RFLP pattern of the 16S rRNA gene was not observed when 15 individuals of each of the four clam species were analyzed. It should be noted that the resolution of the agarose gel did not allow visualization of the smallest fragments (<30 bp) that resulted from digestions. Despite the fact that agarose gels cannot fully separate DNA fragments that differ in length by a few nucleotides, PCR-RFLP patterns obtained with endonuclease BsmAI showed enough differences to distinguish the four clam species. Moreover, digestion with endonuclease BsrI improved the differentiation between R. decussatus and V. rhomboides, for which BsmAI RFLP patterns were similar.

The results obtained in this study demonstrate that PCR-RFLP of the 16S rRNA mitochondrial gene is a powerful technique for the genetic differentiation of the clam species R. decussatus, V. pullastra, R. philippinarum, and V. rhomboides. This approach would be less costly than conventional sequencing of PCR products followed by detailed comparison of individual sequences, especially when large numbers of samples are to be analyzed.

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FIGURE 2. Electrophoretic analysis (3% MS8 agarose) showing the restriction profiles of the 16S rRNA PCR products obtained from the clam species V. pullastra (1, 5, 9), R. decussatus (2, 6, 10), R. philippinarum (3, 7, 11), and V. rhomboides (4, 8, 12). Lanes 1 to 4, no digestion; lanes 5 to 8, digestion with BsmAI; lanes 9 to 12, digestion with BsrI. M = molecular weight marker, 1 kilobase plus DNA ladder.


