FOOD COMPOSITION AND ADDITIVES

Species Identification of *Crassostrea* and *Ostrea* Oysters by Polymerase Chain Reaction Amplification of the 5S rRNA Gene

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A specific multiplex polymerase chain reaction (PCR) was developed for the identification of *Crassostrea angulata, C. gigas*, *Ostrea edulis,* and *O. stentina* oyster species. Universal primers were used for the amplification of complete repetition units of 5S rDNA in each of the 4 species. The alignment of the obtained sequences was the basis for the specific design of species-specific primers (ED1, ED2, ST1, ST2, CR1, and CR2) located in the nontranscribed spacer regions. The different sizes of the species-specific amplicons, separated by agarose gel electrophoresis, allowed identification of *Crassostrea* and *Ostrea* species. A multiplex PCR with a set of the 6 designed primers showed that they did not interfere with each other and bound specifically to the DNA target. This genetic marker can be very useful for traceability of the species, application in the management of oyster cultures, and conservation of the genetic resources of the species.

The market for oysters has experienced a considerable development during the last decades thanks to aquaculture. In Europe alone, the production in 2002 was 258 359 metric tons, and the export figures for fresh and chilled oysters reached 10 118 metric tons with a value of U.S. $28 443 000 [The Food and Agriculture Organization of the United Nations (FAO), 2002]. All oysters that are commercialized in Europe belong to either the *Crassostrea* or *Ostrea* genus, with *O. edulis, C. gigas,* and *C. angulata* being the main commercialized species. *O. edulis* is a flat oyster and *angulata* and *gigas* are cupped oysters, known as the Portuguese and Asian oysters, respectively. *C. angulata* is an oyster species which has its main natural beds at river estuaries in the southwest of the Iberian Peninsula, although its Asian origin has been reported recently (1, 2). *C. gigas* was introduced into Europe as a substitute for *C. angulata*, which was depleted as a result of an attack of an irodoviral infection from 1967 to 1972. Due to the variability in form and size of the shell, it is difficult to clearly differentiate some of these oysters species on the basis of their morphology.

In fact, classifications of *C. angulata* and *C. gigas* have been discussed widely (2–4) because the morphological similarities, the existence of hybrids between them, and the absence of genetic markers suggest that they might belong to the the same species (5). Furthermore, *O. stentina*, well known as a dwarf oyster, and *O. edulis* (flat oyster) are morphologically similar during the first stages of development, and only the adults can be distinguished by their size, the *O. stentina* species being the smallest. This misleading morphology creates an economic problem when cultures of flat oyster of high economic value are contaminated with dwarf oysters, causing an important loss of production (6). Moreover, *O. stentina*, *C. angulata,* and *C. gigas* are morphologically similar and, apart from the size of the adult individuals, the only differences are in the absence of the abductor muscle print in *O. stentina* and in the presence of denticles in the lower part of the umbo of valves of this species. Nevertheless, none of these characteristics is sufficiently definitive for an unequivocal distinction between these species.

Allozymes have been used mainly in the identification of oysters species, to date (2, 7, 8). However, the analysis of proteins has a number of disadvantages when compared with deoxyribonucleic acid (DNA) markers, such as the smaller variability, which in many cases prevents the identification of species, populations, and individuals (9, 10). Moreover, enzymatic activity and lack of reliability caused by degradation during the processing of products or to the absence of enzymes in the tissues to be analyzed may also result in difficulty of identification.

The use of the nucleic-acid-based analytical method can overcome these difficulties because DNA is a very stable and long-lived biological molecule that is present in all tissues of all organisms. Moreover, the introduction of polymerase chain reaction (PCR) has simplified earlier molecular methods that were complicated and time-consuming. PCR amplification of

Received June 10, 2005. Accepted by SG July 25, 2005.
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species-specific fragments has also been useful in fish for traceability (11, 12) and species differentiation (13, 14).

In oysters, the methods of species identification based on analysis of DNA are scarce. Restriction fragment length polymorphism (RFLP) of amplified fragments by PCR has enabled the differentiation of *C. angulata* and *C. gigas* (15). Other methods have been based on sequentiation of the 16S rRNA mitochondrial gene. Using this methodology, *C. virginica*, *C. gigas,* and *C. ariakensis* have been identified (16) and a distinction has been made between the *sikamea* and *gigas* species (17). In addition, *O. stentina* has been differentiated from *O. edulis* (18), and sequencing of a family of satellite DNA has enabled a distinction to be made between *O. edulis* and *O. stentina* (19).

The objective of the present work was to develop a straightforward and reliable method of identification, by means of the amplification by multiplex PCR of fragments of 5S rDNA, of 4 oyster species of the *Ostrea* and *Crassostrea* genus that are normally found in the market: *O. edulis*, *O. stentina*, *C. angulata,* and *C. gigas*. The method should be applicable to the characterization of stock breeders used in oyster culture in order to obtain their correct description, and to the conservation of marine genetic resources.

Experimental

Origin of Samples and Method of DNA Extraction

Ten individuals of each of species *C. angulata*, *C. gigas*, *O. stentina*, and *O. edulis* were studied. The samples of *O. edulis* were obtained from local markets in Cádiz (Spain) and those of *O. stentina* from a natural population at Puerto de Santa María (Cádiz, Spain) as specified by Pascual (20). The samples of *C. angulata* were obtained from a natural population in the estuary of the Rio Guadalquivir in Sanlúcar de Barrameda (Cádiz, Spain) and the samples of *C. gigas* were provided by Amalthea S.L. (Cádiz, Spain). Samples (50–80 mg) of total DNA were extracted from pieces of mantle by means of the NucleoSpin© Tissue kit (Macherey-Nagel, Hoerdt, France) according to the instructions of the manufacturer. Because of the morphological similarity among the analyzed species, the genus *Ostrea* was identified according to the method given by Pascual (20). In the case of the *Crassostrea* samples, the analysis confirming that they belong to the species *angulata* was performed by an RFLP of the PCR-amplified cytochrome oxidase C subunit, following the protocol described in Boudry et al. (15).

PCR Amplification of the 5S rDNA Gene

For the amplifications of 5S rDNA, a pair of contiguous primers of *Mytilus galloprovincialis* with opposed orientation located in positions 13–32 and 36–56 of the 5S coding region whose sequences are MT1 (5′-CGTCCGATCACCGAAGTTAA) and MT2 (5′-ACCGGTGTTTTCAACGTGAT) were used (according to a personal communication from Martinez-Lage, University of La Coruña). The amplification reactions were performed in a total volume of 50 µL. The reaction mixture contained 4 µL template DNA, 3 µM $MgCl_2$, 1 µmol/L deoxyribonucleotide triphosphate (dNTP), 1 µmol/L of each primer, 2 U *Taq* polymerase (Roche Molecular Biochemicals, Sant Cugat del Vallés, Spain) and the appropriate buffer for the polymerase. DNA was amplified in a GeneAmp PCR System 2700 (Applied Biosystems, Madrid, Spain). The cycles of amplification were 94°C/5 min, followed by 35 cycles of 94°C/45 s, 59°C/45 s, 72°C/1 min, and then followed by an extension of 72°C/10 min.

The PCR products (10 µL) were mixed with 2 µL gel loading solution (40% sacarose, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.25% bromophenol blue) and electrophoresed in a 2% agarose gel, containing 0.5 µg/mL ethidium bromide in Tris–boric buffer (0.89 M Tris, 0.02 M EDTA–Na_2 salt and 0.89 M boric acid) for 1.5 h at 70 V. The resulting DNA fragments were viewed by ultraviolet (UV) transillumination and analyzed using Geldoc 1000 UV fluorescent gel system and Molecular Analyst software (Bio-Rad Laboratories, Alcobendas, Spain). PCR products were cleaned with the High Pure PCR Product Purification Kit (Roche Molecular Biochemicals) and cloned into pGEM™-T Easy Vector System II (Promega, Alcobendas, Spain). The plasmid purification was performed by using the NucleoSpin™ Plasmid Quick Pure (Macherey-Nagel). DNA sequencing was performed with fluorescence-labeled terminators (BigDye Terminator v 3,1 Cycle Sequencing Kit; Applied Biosystems) for an automated sequencer (Model ABI Prism 373XL Stretch; Applied Biosystems). Sequencing curves were analyzed with the Chromas 2.0 program and the sequences obtained were aligned using the ClustalX 1.81 program (21).

Purification, Cloning, and Sequentiation of PCR Products

The PCR products obtained with primers MT1 and MT2 were purified with High Pure PCR Product Purification Kit (Roche Molecular Biochemicals). Later, they were cloned into pGEM-T Easy Vector System II (Promega) following the manufacturer's instructions, and transformed into *Escherichia coli* JM109 high efficiency cells. Then they were plaqued onto plates of medium (Luria-broth; LB)-ampicillin with isopropyl-beta-D-thiogalactopyranoside (IPTG) and the 5-bromo-4-chloro-3-indolyl-beta-D-galactoside (X-GAL). Extraction and purification of plasmids of the recombinant clones were performed using the NucleoSpin Plasmid Quick Pure (Macherey-Nagel). DNA sequencing was performed with fluorescence-labeled terminators as mentioned above.

Design of Specific Primers for O. edulis, O. stentina, C. angulata, and C. gigas

The sequences obtained from the PCR products with primers MT1 and MT2 were used for the design of specific primers in each case. Hence, primers ED1 (5′-GACTTGCCATTTTAGAGGGTCT) and ED2 (5′-TGTTTAATTGGTGATAACGATGA) were designed specifically for *O. edulis*. These primers are located in the nontranscribed spacer (NTS) of 5S rDNA gene. A fragment of

800 base pairs (bp) was amplified in a flat oyster and no fragments were amplified in the 3 other analyzed species. The sequence of 5S from *O. stentina* was used in order to design 2 specific primers for this species: ST1 (5′-CGGGAAATGACAGCAGAAAT) and ST2 (5′-TGCAACAGTAATGGAGATGACA), which are located in the spacer of 5S rDNA gene and produce a fragment of 596 bp. No amplification took place in the other 3 analyzed species. Finally, the sequence of 5S rDNA of species *C. angulata* and *C. gigas* was used for designing 2 primers, CR1 (5′-CAGTCGCTATGATGCTTTAATGT) and CR2 (5′-GAAAGATGAAAAGTGGGGAGAA), which amplify in both species a fragment of 400 bp and do not amplify any product in *O. edulis* or *O. stentina*. Primers were designed with the aid of the Primer3 program (22) and the sequences were analyzed with the Bioedit 5.0.9 program (23).

PCR Amplification of Specific Fragments of 5S rDNA Gene

Primers ED1, ED2, ST1, ST2, CR1, and CR2 were used to amplify specific fragments of *O. edulis*, *O. stentina,* and the 2 species of *Crassostrea*: *C. angulata* and *C. gigas* (Figure 1). The PCR-amplified products obtained were directly sequenced without cloning. They determined the 5S rDNA sequence corresponding to the heterologous primers MT1 and MT2 and the sequence present between them in the coding region (Figure 2).

A set of these 6 primers was used to make a multiplex PCR. Amplification was performed by using 1 μmol/L of each primer, and the conditions were identical to those mentioned above in the *PCR Amplification of the 5S rDNA Gene* section.

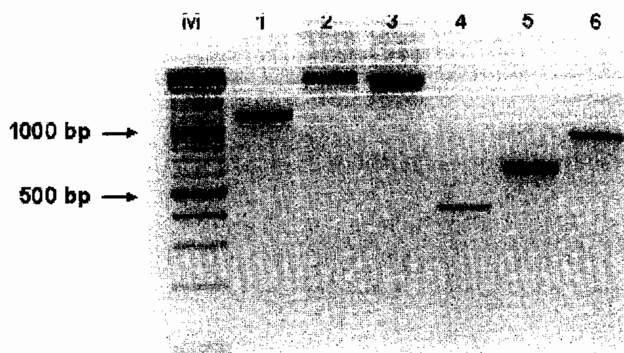

Figure 1. Electrophoretic analysis of the 5S rDNA products obtained from 4 oyster species. Lane M: 100 bp DNA ladder. Lanes 1–3: PCR products obtained with primers MT1 and MT2 in *C. angulata/C. gigas* (Lane 1), *O. edulis* (Lane 2), and *O. stentina* (Lane 3). Lanes 4–6: multiplex-PCR products obtained with primers CR1–CR2, ED1–ED2, ST1–ST2, amplified from *C. angulata/C. gigas* (Lane 4), *O. stentina* (Lane 5), and *O. edulis* (Lane 6). This picture is a reverse image of the ethidium bromide-stained gel.

Results and Discussion

There is a growing need to develop techniques that allow the correct identification and traceability of fish and seafood products in order to satisfy the requirements of current legislation (13). The DNA-based markers have been shown to be very useful for this purpose, mainly because the generalized use of amplified products by PCR has simplified the technology.

We have chosen the 5S ribosomal ribonucleic acid (RNA) as a marker for the genetic identification of related species of oysters because its structure converts it into a species-specific gene in the higher eukaryotes (24). The 5S rDNA gene forms a multigene family of tandem arrays, whose unit of repetition is composed of one conserved coding region of 120 bp and an NTS variable in both length and sequence (25). This structure presents many advantages in the identification of species, because the design of primers located in the NTS allows species-specific amplifications by PCR.

In this work, MT1 and MT2 primers that correspond to 2 sequences of the 5S coding region of *Mytilus* spp. have allowed us to amplify whole units of 5S rDNA of 4 species of oysters: *O. edulis, O. stentina, C. angulata,* and *C. gigas*. Sizes of amplified products were about 2000 bp in *Ostrea* and about 1100 bp in *Crassostrea* (Figure 1). The size of amplification products was estimated to be the same in 10 individuals of each species. Although sizes of amplified fragments with primers MT1 and MT2 allow us to differentiate between *Crassostrea* and *Ostrea*, an unknown species of oyster with an NTS of similar size could be misidentified as pertaining to *O. edulis*, *O. stentina*, *C. angulata,* or *C. gigas* if its NTS fragments were similar in length. Hence, in order to identify specifically the oyster species studied, we decided to design specific primers for each species. We purified, cloned, and sequenced amplification products corresponding to 2 different individuals of each species.

The complete 5S rDNA gene of *C. angulata* and *C. gigas*, and the flanking regions of the coding region of 5S rDNA of 2 individuals of *O. edulis* and *O. stentina* were sequenced. When the sequences were aligned, a low similarity between the spacer regions of 4 species was shown to exist, which was very useful for our purposes (Figure 2). Moreover, when considering the genus *Crassostrea*, the aligning of the sequences of the *angulata* and *gigas* species rendered a close identity between them. The close identity between sequences shows a genetic differentiation between both taxa that was not greater than that which exists between reproductively isolated populations within other species. Moreover, the sequencing of NTS in each species did not render species-specific polymorphisms [sequences are available in the European Molecular Biology Laboratory (EMBL) database under the accession No. AY765359 and AY765364 for *C. angulata* and *C. gigas,* respectively]. They seemed to be the same species (3, 4, 8). The conclusion seems to add one more controversy over the classification of these 2 species. Nevertheless, more recently the study of the mitochondrial DNA confirmed the original classification of both species like *C. angulata* and *C. gigas* (1, 15).

Figure 2. 5S rDNA sequences of 4 oyster species. The coding gene region is in bold. The sequences of the specific primers from each species are underlined (CR1, CR2, ST1, ST2, ED1, ED2). MT1 and MT2 were the primers used for the amplification of complete repetition units. The complete NTS of the *C. angulata* and *C. gigas* are shown, but in the case of *O. stentina* and *O. edulis*, only the flanking regions of 5S rDNA coding necessary for the designing of the primers are shown.

In order to unequivocally identify them, we designed 3 pairs of primers that were species-specific to *O. stentina*, *O. edulis*, and of *C. angulata/C. gigas*. To confirm the effectiveness of the designed primers, they were tested separately. Primers ED1 and ED2, located in the NTS of *O. edulis*, flanking the 5S rDNA coding region, amplified an 818 bp product, whereas none of the products were amplified in *O. stentina*, *C. angulata*, and *C. gigas*. Also, ST1 and ST2 primers amplified a 576 bp fragment only in *O. stentina*, whereas no amplification was observed in the other analyzed oysters. Finally, primers CR1 and CR2 amplified a 400 bp fragment in *C. angulata* and a 402 bp fragment in *C. gigas*, and no amplification was produced *O. edulis* and *O. stentina*.

Our purpose was to find a straightforward and reliable method of identification of the described species, and to check that no disturbance occurred among these pairs of primers during the amplification reaction. We also wanted to show that the primers specific to each DNA species annealed on their target sequences. In order to do this, we used a multiplex PCR with a set of 6 primers (ED1, ED2, ST1, ST2, CR1, and CR2) in the same reaction. The results obtained (Figure 1) showed that primers did not interfere with each other and that they bound specifically to the DNA target. The amplified fragments showed the same length as they did when the primers were used separately.

The results presented in this work show that, by using a multiplex PCR of specific fragments of 5S rDNA, different species of oysters can be identified in a quick and efficient way. Designed primers (ED1, ED2, ST1, ST2, CR1, and CR2) allowed us to identify the species (*O. stentina, O. edulis, C. angulata/C. gigas*) not only by the size of the amplified product, but also by the presence of specific targets of each species. Due to the speed, sensitivity, and ease of the described method, as well as the possibility of performing it in nonspecialized laboratories, this method offers the advantage of traceability of the species as well as application in the management of oyster culture.

Acknowledgments

We would like to thank A. Martínez-Lage (University of La Coruña) for the primer sequences and Amalthea S.L. (Cádiz, Spain) for providing the samples of *C. gigas*. This work has been supported by projects INTERREG IIIA OPAM from the European Economic Community (EEC) and CVI219 from the Junta de Andalucía (Spain).

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