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.

he market for oysters has experienced a considerable development during the last decades thanks to **L** 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

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 MT1 sequences are (5'-CGTCCGATCACCGAAGTTAA) MT2 and (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 MgCl₂, 1 µmol/L deoxyribonucleotide triphosphate (dNTP), I µmol/L of each primer, 2 U Tag 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 μ L) were mixed with 2 μ L gel loading solution (40% sacarose, 10 mMethylenediaminetetraacetic 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-Na₂ 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 pGEMTM-T Easy Vector System II (Promega, Alcobendas, Spain). The plasmid purification was performed by using the NucleoSpinTM 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 in Hence, primers each case. primers ED1 (5'-GACTTGCCATTTTAGAGGGTCT) and ED2 (5'-TGTTTAATTGGTGATAACGATGA) were specifically for O. edulis. These primers are located in the nontranscribed spacer (NTS) of 5S rDNA gene. A fragment of 300 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 μ mol/L of each primer, and the conditions were identical to those mentioned above in the PCR Amplification of the 5S rDNA Gene section.

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

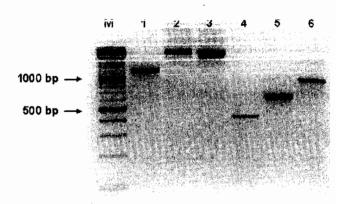


Figure 1. Electrophoretic analysis of the 5S rDNA products obtained from 4 oyster species. Lane M: 100 bp DNA ladder. Lanes 1–3: PCR products obtained with primers MT1 and MT2 in *C. angulata/C. gigas* (Lane 1), *O. edulis* (Lane 2), and *O. stentina* (Lane 3). Lanes 4–6: multiplex-PCR products obtained 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.

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).

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e angulata	_			22202		
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2000						
	11	ca	5.0	166	110	120
O stentina			GGGNAANGAG			
o edulis			ATGCTCACAT			
C angulata						
C gigas						
	130		150			
o stentina	ANANGGAATA	TTGACCGCCG	GNGGGAAAGA	GAGTACCAA-	ACCCCCNTGA	CCTGTGACCC
o edulis	GCGTGAAATG	TTTG	GAGAAAATGT	ACAAATCATC	ACCAGCGATA	CCTCAGACTC
C angulata			A			
C gigas			A	AGCCCCTGAC	ATCTCACCTC	TGACCAAAAA
	190		210		230	240
o_stentina			TGAGCGGAGG			
O_edulis			TGAACAGTCG			
C_angulata			CCATGTAGGG			
C_gigas	TCAGTTTAAT	ATCGTAGTTC	CCATGTAGGG	GAACGTATCA	GATATTAAGC	TGATAAGAAC
	250	260	270	290	290	300
Ostentina			TTGGAATAAA			
o_edulis			TCAGAGCCAT			
C_angulata			AGCCAAAAGG AGCCAAAAGG			
C_gigas	AGATACTACA	CTITGATCIT	AGCCRAARGG	CCGAGAAGCG	ATGCCCATAC	GUGGGCATGG
	310	320	330	340	. 350	360
o stentina			GGAAATGACA			
O_scencina	AGITITALA	CACACACATEG		ST1 →	CCATOCOGG	ATTCAT INCO
O edulis	A CONCRETE TOTAL	GMCG	TCAATCAACA		_CTYPECCEATTC	GG
C angulata			TCATTTTCAA			
C_gigas			TCATTTICAA			
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	370	380	390	400	410	420
O stentina	CCTATATAAT	GTTGCAATAT	ATTTAAATTAA	AATAAAAAAA	AAAAAAAA	ATGGAAATGA
O edulis	CATTTATTAT	ATCACAGTAT	ACAATAA	AACACAATTA	AAAATTATAG	TCCTAAACTT
C angulata	AAATTCGCAT	CAATGAGCGA	GTCAGGGTGA	TTGTACGATT	TITTTCCGAA	TAGATGTAAG
C_gigas	AAATTCGCAT	CAATGAGCGA	GTCAGGGTGA	TIGTACGATI	TITTTCCGAA	TAGATATAAG
	430	440	450	460	470	480
O_stentina			AAAACACATA			
O_edulis			ATTATTTACA			
Cangulata			ATTATTGTAA			
C_gigas	TIGITITAGGG	TATTIGCAAA	ATTATTGTAA	ACTTAXAART	AACAAAGGGG	TGAATTATCA
	40.0				520	540
O stentina	490	500	510	520	530	
O_stentina O edulis	CLOTTATTAAT	ACGCACACAC	GCCCCTTATA GCCCCTCATA	GTCCGCCTCC	CCGTCTCACC	CAMPETTECA
	CATTA	Oxeconstant a con-	TAGCAAAGTT	CTARAGETTEE	AUTHORITATE	TITTE COCCCC
C_angulata			INGCARAGIT	GIAGGIACGT	ATTACCTACG	7771000000
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C_gigas	ATCGCTATGA	10CTTTAATG	TAGCAAAGTT	GIAAATACGT	A TRUCTACE	TTTTGCGCGC

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O stentina		GCSTTTCGAG				ANCATOGAT
O_cdulis		GCGTGTCGFC				
C_angulata		CTOSOGGTAT				
P 91988	AGRICOVIACION	CTCGCGGTAT	ATATACAATC	GLINY GLLW.	Shahil Biring	ACTGATOAAA
	610	628	630	640	C50	660
C_stentine		$\widehat{G}_{i}(\widehat{G})=(\widehat{G}_{i}(\widehat{G}),\widehat{G}_{i}(\widehat{G}))=(\widehat{G}_{i}(\widehat{G}),\widehat{G}_{i}(\widehat{G}),\widehat{G}_{i}(\widehat{G}))$				
o elulia		CANCAGACTA				
C angulata		ACATCARATC				
C gigas	ACTUATAÇÃO	ACATCAAATC	COUGAGTTTG	AATTACCOTC	AATCAATGTT	TTGAACTAAT
	670	690	690	700	710	720
0 stentina	AGTAATATAA	CTACTTTTAA	TACATCCCTT	CACTGGTCTA	TTCACAAAAT	AAACACATGG
0 edulis	AGAGTAATAA	C-GCCTTACA	CCCAACTCTT	CACTGATGTA	GTCGCAAATT	AAACAACTAT
C angulata	ACTTACCAAC	TRECTGREET	GAAATCACTA	ATATCTATTT	TACTIGITIC	TAACTCTTAG
C gigas	ACTTACCAAC	TTCCTGTCCT	GAAATCACTA	ATATCTATTT	TACTIGITIE	TAACTCTTAG
	730	740	750	760	770	780
O stentina	TGRATGTCTA	OGGCCATATC	ACGTTGRAR-	CACCGGTTCT	CGTCCGATCA	CCGAAGTTAX
o edulis		CCCCCATATC				
C angulata	TYGTTGTCTA	CGGCCATATC	ACCTTGARAG	CACCGGTTCT	CGTCCGATCA	CCGAAGTTAA
C gigas	TTGTTGTCTA	CACCCATATC	ACGTTGAAAG	CACCGGTTCT	COTCCGATCA	CCGAAGTTAA
		+	2	Primer MT1 →		
	790	800	810	820	830	840
O stentina	GCARCGTCGA	CCTTCGTTTAG	TACTTGGATG	GGTGACCGCC	TGGGAATACC	ARCTGTCCTA
O_stentina O_edulis		CCTTGGTTAG				
o edulis	CCARCGTCGA		TACTIGGATE	GGTGACCGCC	TEGGRATACC	AGGTGTCCTA
O_edulis C_angulata	GCAACGTCGA GCAACGTAGA	CCCTGGTTAG	TACTIGGATE TACTIGGATE	GGTGACCGCC	TGGGAATACC TGGGAATACC	AGGTGTCGTA AGGTGTCGTA
o edulis	GCAACGTCGA GCAACGTAGA	GCCTGGTTAG GCTTGGTTAG	TACTIGGATE TACTIGGATE	GGTGACCGCC	TGGGAATACC TGGGAATACC	AGGTGTCGTA AGGTGTCGTA
O_edulis C_angulata	GCAACGTCGA GCAACGTAGA	GCCTGGTTAG GCTTGGTTAG	TACTIGGATE TACTIGGATE	GGTGACCGCC	TGGGAATACC TGGGAATACC	AGGTGTCGTA AGGTGTCGTA
O_edulis C_angulata	GCAACGTCGA GCAACGTAGA GCAACGTAGA 850	CCTGGTTAG CCTTGGTTAG CCTTGGTTAG	TACTIGGATG TACTIGGATG TACTIGGATG 070	GGTGACCGCC GGTGACCGCC GGTGACCGCC	TEGGRATACC TEGGRATACC TEGGRATACC	AGGTGTCGTA AGGTGTCGTA AGGTGTCGTA 900
O_edulis C_angulata C_gigas	GCAACGTAGA GCAACGTAGA GCAACGTAGA 850 GACTTTTTCT	GCCTGGTTNG GCTTGGTTNG GCTTGGTTNG 860	TACTIGATE TACTIGATE TACTIGATE 070 TGTTTTACCT	GGTGACCGCC GGTGACCGCC GGTGACCGCC 880 TCAGACATTT	TEGGRATACC TEGGRATACC TEGGRATACC 890 TACATTATTT	AGGTGTCGTA AGGTGTCGTA AGGTGTCGTA 900 TCTTGTCATC
O edulis C angulata C gigas O stentina O edulis	GCAACGTAGA GCAACGTAGA GCAACGTAGA 850 GACTTTTTCT GACTTTTTCA	GCCTGGTTAG GCTTGGTTAG GCTTGGTTAG 960 TTTTCCATTT CTTTT	TACTIGGATG TACTIGGATG TACTIGGATG 070 TGTTTTACCT TTTTCTTCCT	GOTGACCECC GOTGACCECC GOTGACCECC 980 TCAGACATTT	TEGGRATACC TEGGRATACC TEGGRATACC 890 TACATTATTT CACATTATTT	AGGTGTCGTA AGGTGTCGTA AGGTGTCGTA 900 TCTTGTCATC TCTTTTTATT
O edulis C_angulata C_gigas O stentina O edulis C_angulata	GCAACGTCGA GCAACGTAGA GCAACGTAGA 850 GACTTTTTCCA GACTTTTTCCA	GCCTGGTTAG GCTTGGTTAG GCTTGGTTAG 960 TTTTCCATFT CTTTT CCCCTCTCTT	TACTIGGATG TACTIGGATG TACTIGGATG 070 TGTTTTACCT TTTTCTTCCT TCTCCCACTT	GGTGACCGCC GGTGACCGCC GGTGACCGCC 980 TCAGACATTTATTT TTCACCTTTC	TEGGRATACC TEGGRATACC TEGGRATACC 890 TACATTATTT CACATTATTT TTGACAGTAA	AGGTGTCGTA AGGTGTCGTA AGGTGTCGTA 900 TCTTGTCATC TCTTTTTATT CATTTTATT
O edulis C angulata C gigas O stentina O edulis	GCAACGTCGA GCAACGTAGA GCAACGTAGA 850 GACTTTTTCCA GACTTTTTCCA	GCCTGGTTAG GCTTGGTTAG GCTTGGTTAG 960 TTTTCCATTT CTTTT	TACTIGGATE TACTIGGATE 010 TGTTTTACCT TTTTCTCCT TCTCCCACTT TCTCCCACTT	GOTGACCECC GOTGACCECC GOTGACCECC 980 TCAGACATTTATTT TTCACCTTTC TTCATCTTTC	TEGGRATACC TEGGRATACC TEGGRATACC 890 TACATTATTT CACATTATTT TTGACAGTAA	AGGTGTCGTA AGGTGTCGTA AGGTGTCGTA 900 TCTTGTCATC TCTTTTTATT CATTTTATT
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o_edulis C_angulata c_gigas O_stentina o_edulis C_angulata C_gigas	GCANCGTAGA GCANCGTAGA GCANCGTAGA 850 GACTTTTTCT GACTTTTTCC GACTTTTTCC 910	GCTGGTTAG GCTTGGTTAG GCTTGGTTAG 960 TTTTCCATTT CCTTTT CCCTCTCTT CCCCTCTCTT 920	TACTIGATE TACTIGATE 070 TGTTTTACCT TTTCTCCACTT TCTCCCACTT	GOTGACCEC GOTGACCEC GOTGACCECC 880 TCAGACATTT ATTT TTCACCTTTC TTCATCTTTC Mer CR2 940	TEGGRATACE TEGGRATACE 890 TACATTATTT CACATTATTT TTGACAGTAA TTGACAGTAA 950	AGGIGICCETA AGGIGICCETA AGGIGICCETA 900 TCTTGTCATC TCTTTTTATT CATTTTATTT CATTTTATTT CATTTTATTT 950
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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.

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References

(1) O'Foighil, D., Gaffney, P.M., Wilbur, A.E., & Hilbish, T.J. (1998) Mar. Biol. 131, 497–503

- 148
- (2) Buroker, N.E., Hershberger, W.K., & Chew, K.K. (1979) Mar. Biol. 54, 157–169
- (3) Mathers, N.F., Wilkins, P.N., & Walne, P.R. (1974) Biochem. Syst. Ecol. 2, 93–96
- (4) Mattiucci, S., & Villani, F. (1983) Parasitology 25, 21–27
- (5) Menzel, R.W. (1974) J. Fish. Res. Board Can. 31, 453-456
- (6) Saavedra, C. (1997) J. Shellfish Res. 16, 441-446
- (7) Buroker, N.E. (1982) J. Shellfish Res. 2, 157-163
- (8) Buroker, N.E., Hershberger, W.K., & Chew, K.K. (1979) Mar. Biol. 54, 171–184
- (9) Grant, W. (1984) Copeia 3, 357-364
- (10) Utter, F.M., Milner, G., Stahl, G., & Teel, D. (1989) Fish. Bull. U.S. 87, 239–264
- (11) Céspedes, A., García, T., Carrera, E., González, I., Fernández, A., Hernández, P.E., & Martín, R. (1999) J. Agric. Food Chem. 47, 1046–1050
- (12) Carrera, E., García, T., Céspedes, A., González, I., Fernández, A., Asensio, L.M., Hernández, P.E., & Martín, R. (2000) Int. J. Food Sci. Technol. 35, 401–406
- (13) Asensio, L., González, I., Fernández, A., Céspedes, A., Rodriguez, M.A., Hernández, P.E., García, T., & Martín, R. (2001) J. AOAC Int. 84, 777–781
- (14) Rego, I., Martínez, A., González-Tizón, A., Vieites, J., Leira, F., & Méndez, J. (2002) J. Agric. Food Chem. 50, 1780–1784

- (15) Boudry, P., Heurtebise, S., Collet, B., Conette, F., & Gérard, A. (1998) J. Exp. Mar. Biol. Ecol. 226, 279-291
- (16) O'Foighil, D., Gaffney, P.M., & Hilbish, T.J. (1995) J. Exp. Mar. Biol. Ecol. 192, 211–220
- (17) Banks, M.A., Hedgecock, D., & Waters, C. (1993) Mol. Mar. Biol. Biotechnol. 2, 129–136
- (18) Comesaña, A.S., Fossum, A., & Sanjuan, A. (2001) in I Congreso Internacional de Ciencia y Tecnología Marina, Pontevedra, Spain
- (19) López-Flores, I., de la Herrán, R., Garrido-Ramos, M.A., Boudry, P., Ruiz-Rejón, C., & Ruiz-Rejón, M. (2004) Gene 339, 181–188
- (20) Pascual, E. (1972) Invest. Pesq. 36, 287-310
- (21) Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., & Higgins, D.G. (1997) Nucleic Acids Res. 24, 4876–4882
- (22) Rozen, S., & Skaletsky, H.J. (2000) in Bioinformatics Methods and Protocols: Methods in Molecular Biology, S. Krawetz & S. Misener (Eds), Humana Press, Totowa, NJ, pp 365–386
- (23) Hall, T.A. (1999) Nucleic Acids Symp. Ser. 41, 95-98
- (24) Rodriguez, M.A., García, T., González, I., Asensio, L., Fernández, A., Lobo, E., Hernández, P.E., & Martín, R. (2001) J. Agric. Food Chem. 49, 2717–2721
- (25) Suzuki, H., Sakurai, S., & Matsuda, Y. (1996) Cytogenet. Cell Genet. 72, 1–4