JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Development of a Method for the Genetic Identification of Mussel Species Belonging to *Mytilus*, *Perna*, *Aulacomya*, and Other Genera

FRANCISCO J. SANTACLARA,* MONTSERRAT ESPIÑEIRA, ANA G. CABADO, ARRATE ALDASORO, NEREA GONZALEZ-LAVÍN, AND JUAN M. VIEITES

Area of Molecular Biology and Biotechnology, ANFACO-CECOPESCA, Vigo, 36310 Pontevedra, Spain

Legislation regarding the labeling of processed products is an important issue in the protection of consumer rights. This labeling is especially important in products that cannot be identified on the basis of their morphological characters, because these are removed from the animal in the transformation process. The goal of this study was the identification of mussel species using Polymerase Chain Reaction−Restriction Fragment Length Polymorphism (PCR-RFLP) and Forensically Informative Nucleotide Sequencing (FINS) methodologies. The molecular marker selected was *18S rDNA* (nuclear small-subunit rDNA gene), which allows identification at the genus level and at the species level in some cases. The genera included in this study were *Mytilus*, *Perna*, *Aulacomya*, *Semimytilus*, *Brachidontes, Choromytilus*, and *Perumytilus*. Different markers were used for genetic identification at the species level. To identify the species included in the genus *Perna* and *Choromytilus*, a fragment of *ITS 1* (Internal Transcribed Spacer 1) was amplified by multiplex PCR and digested with restrictases. The species of *Mytilus* were identified by length polymorphism and RFLP of the polyphenolic adhesive protein gene. This methodology was validated with products manufactured in the authors' pilot plant and applied to commercial samples. Therefore, this sequential method can be completely or partially used to determine the mussel genus or species present in any food product.

KEYWORDS: mussels; genetic identification; *Mytilus*; *Perna*; *Choromytilus*; *Aulacomya*; PCR-RFLP; FINS; multiplex PCR

INTRODUCTION

Mussels are a heterogeneous group that includes a high number of genera and each of them, a great number of species. These genera are *Mytilus*, the most common species in prepared food products, *Perna*, *Aulacomya*, *Semimytilus*, *Perumytilus*, *Choromytilus*, and *Brachidontes*.

Nowadays mussel importations in Europe from Third World countries show an increasing tendency, especially *Mytilus* and *Perna*. These bivalves are commercialized mostly without shells, and this fact prevents morphological identification. Therefore, this taxonomic group could be improperly labeled in the absence of shells as well as being misidentified, due to lack of analytical methods. All of these factors point out the necessity of developing methods based on nonmorphological techniques to guarantee the fulfillment of labeling laws of every state for manufactured mussels. For instance, different countries take the European Decision concerning labeling [Regulation (CE) 104/2000] as a model and publish their own laws to give correct information to consumers concerning seafood products.

* Author to whom correspondence should be addressed [telephone (34) 986 469 301; fax (34) 986 469 269; e-mail fransanta@anfaco.es].

A high number of genetic studies have dealt with mussels, due to their ability to generate hybrid individuals when two species are in contact (*1*−*5*), their exclusive manner of mitochondrial DNA inheritance (*6*), and their population structure and the distribution pattern of species (*7*−*9*) among others. There have been many findings in the field of genetic identification (*10*−*13*). Kenchington et al. (*14*) analyzed the nuclear small-subunit rRNA gene sequence to clarify the phylogeny and the status of the species that composed the *Mytilus edulis* complex defined by Gosling et al. (*15*) (*Mytilus edulis*, *Mytilus galloprovincialis*, and *Mytilus trossulus*). They found that neither phylogeny nor the species status could be resolved from that work.

Inoue et al. (*10*) developed one method based on the variable length of amplified PCR products, which was uniform in each species and different interspecifically. These authors found sizes of 126, 168, and 180 base pairs (bp) for *M. galloprovincialis*, *M. trossulus*, and *M. edulis*, respectively. Wood et al. (*16*) studied the method proposed by Inoue et al. to establish the genetic identification of larvae. These authors carried out crossings of different species in controlled conditions and analyzed the offspring using the methodology proposed by Inoue

10.1021/jf061400u CCC: $33.50
Published on Web 10/07/2006

Table 1. Samples Included in This Work

species	code	no. of samples	individuals per sample
Mytilus edulis	Me	18	10
Mytilus trossulus	Mt	5	10
Mytilus galloprovincialis	Mg	29	10
Mytilus chilensis	Mc	8	10
Mytilus californianus	Mcal	3	10
Perna perna	Pp	3	10
Perna canaliculus	Pc	3	10
Perna viridis	Pv	3	10
Chormomytilus chorus	Cch	3	5
Choromytilus meridionalis	Cm	3	5
Aulacomya ater	Aa	3	10
Brachidontes rodrigueiz	Brd	2	10
Brachidontes granulatus	Bgr	2	5
Perumytilus purpuratus	Ppu	2	10
Semimytilus algosus	Sa	3	10

et al. (*10*). Toro et al. (*17*) studied the taxonomic status of the Chilean blue mussel, *Mytilus chilensis*, using morphologic, mitochondrial, and nuclear DNA markers. Results of this work show evidence of genetic similarity among *M. chilensis*, *M. galloprovincialis*, and *M. edulis*. On the basis of these results a change in the current taxonomic status of *Mytilus chilensis* to *Mytilus edulis chilensis* was suggested. The same author developed a method for the genetic identification of four mussel species from the Chilean coast (*Mytilus chilensis*, *Choromytilus chorus*, *Aulacomya ater*, and *Perumytilus purpuratus*) using *ITS* regions, located between *18S* and *28S nuclear rDNA*, previously used by Health et al. (*3*) and points out that this technique could be very useful in planktonic surveys of bivalve larvae (*13*).

Rego et al. developed a Random Amplified Polymorphism DNA technique (RAPD) for the identification of mussel species (*12*). This technique allows the identification of *M. galloprovincialis* from other mussel species, because one pair of primers described in this work generates a 555 bp band. *Perna canaliculus* can also be identified with another pair of primers, which produce a specific pattern band ranging from 700 to 800 bp.

Recently, Bendezu et al. (*18*) have developed a method based on *16S* and *18S rDNA*, which allows the differentiation between *M. edulis*, *M. galloprovincialis*, and their hybrids and *Pecten maximus*. These authors did not find differences among mussel species with the molecular markers studied.

Among all of these works, only one of them can be applied to canned mussels because the sterilization process induces fragmentation of DNA into little pieces of about 200 bp (*19*). For this reason, molecular methods applied to commercial products subjected to thermal treatment should employ fragments around this size (*20*, *21*). This complex scenario creates the necessity of developing a unique method to identify these species and including all of the preparation formats.

In the present work, several DNA-based methods have been developed, which applied sequentially allow the genetic identification of mussel species in fresh, frozen, canned, or any other preparation.

MATERIALS AND METHODS

Sample Collection and Storage. Samples used in this work are displayed in **Table 1**. Position and location are represented in **Figure 1**. Samples were sent to our laboratory from their natural zones of distribution, frozen or immersed in absolute ethanol. Some scientists supplied us with their authenticated mussel lines, and other samples were taken from the natural environment. Samples were labeled after arriving at the laboratory and were preserved frozen at −80 °C for subsequent treatments.

DNA Extraction. DNA of samples was extracted from 30 mg of tissue as described by Roger and Bendich (*22*) with slight modifications. In the case of samples used in the validation method and market study, the oil of samples was removed with a solution of methanol/chloroform/water (2:1:0.8), and DNA was extracted from 300 mg of tissue.

Extracted DNA was visualized in agarose gels at 1% in TBE buffer with 10 mg/mL of ethidium bromide. The quality and quantity of the obtained DNA were measured with a spectrophotometer (Eppendorf Biophotometer), and it was optimal in all cases.

PCR and Sequencing. Amplifications of DNA carried out in this work were performed in a thermocycling Uno II (Biometra) with puRE Taq Ready-to-Go PCR Beads (Amersham Biosciences) in a final volume of 25 μL, containing 2 mM $MgCl_2$, 2 μM of each primer, and 100 ng of DNA for raw mussels. In the case of processed mussels that underwent a thermal treatment, the DNA amount was between 1 and 2 μg. Primers used for PCR are shown in **Table 2**. PCR conditions were the following: a preheating step of 95 °C for 5 min, 35 cycles of 95 °C for 30 s, melting temperature (T_m) (**Table 2**) for 30 s, 72 °C for 30 s, followed by a final extension step of 72 °C for 5 min.

Correct PCR amplification was evaluated in agarose gels at 2% in TBE buffer, with 10 mg/mL of ethidium bromide for band detection. The size of amplified fragments was estimated from the molecular marker *pGEM* (Promega) or 50 bp ladder (Amersham Biosciences).

All PCR products obtained were purified before sequencing reaction with a GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences) following the supplier's protocol. Later, PCR products were directly sequenced in both directions with primers used for PCR amplification (**Table 2**). The sequencing process was performed in an ABI Prism 310 genetic analyzer using a BigDye Terminator Cycle Sequencing Ready Reaction Kit according to the manufacturer's recommendations (Applied Biosystems).

All sequences were analyzed with DNA Sequencing Analysis Software version 3.4.1 (Applied Biosystems) and corrected with CHROMAS version 1.45 (*23*).

a. Development of a PCR-RFLP and FINS Methodology Using Nuclear Small-Subunit rDNA Gene. a.1. Genetic Identification Using PCR-RFLP of an 18S rDNA Fragment. DNA was amplified with primer set *Myt18S* (**Table 2**). Sequences were obtained as indicated previously, and the alignments were performed with BioEdit version 7.0.0 *(24)*. Sequences from the National Center for Biotechnology Information (NCBI) with accession numbers AY527062, L78854, L33448, X59118, L24489 (*M. edulis*); L33453 to L33455, L24490 (*M. trossulus*); L33451, L33452 (*M. galloprovincialis*); and L33449 (*Mytilus californianus*) were included in the alignment together with ones obtained in this research and used for obtaining haplotypes with DnaSP 4.0 (*25*). From these haplotypes were designed the restriction maps with Webcutter 2.0 (*26*) and obtained variable positions using MEGA version 3.0 (*27*). A new region including variable positions between species that coincides with points of cut for several enzymes was selected. Next, a new primer set (*MusRFLP*) was designed for amplification of this DNA fragment. This PCR product was purified before enzymatic digestion with a GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences) to eliminate primer−dimer and primer excess. Digestions with the selected enzymes were developed separately, using 4 units of each enzyme and 1 μg of DNA. Incubations were carried out overnight in a final volume of 20 μL, at the specific temperature recommended for each enzyme.

Digested PCR products were electrophoresed in agarose gels at 3% in TBE buffer with ethidium bromide (10 mg/mL) for 120 min at 70 V (agarose LM, Pronadisa). Gels were observed in an image analyzer Gel Doc XR (Bio-Rad) under ultraviolet light. Size fragments were estimated from a ladder of 50 bp (Amersham Biosciences).

a.2. Genetic Identification Using FINS Technique. Primers *MusRFLP F* and *MusFINS R* were used to amplify and sequence the PCR product, as described previously. Once sequences were obtained, these were aligned with BioEdit version 7.0.0 (*24*), and phylogenetic analysis was carried out with MEGA 3 *(27)*. The algorithm selected for genetic distance calculation was Tamura−Nei, and the method to construct

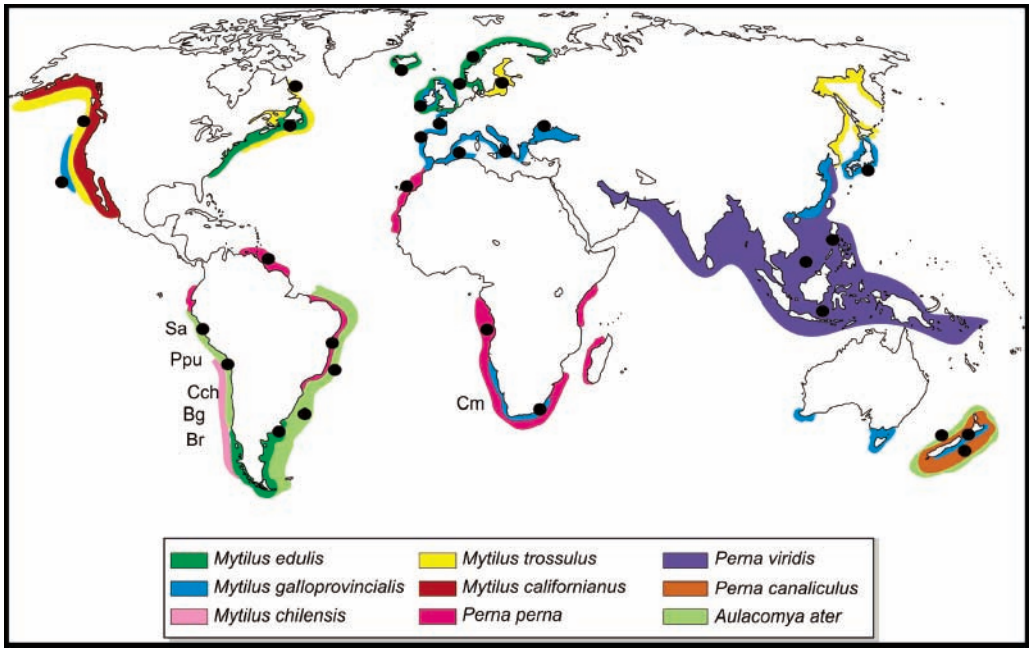

Figure 1. Distribution areas of mussel species studied and areas where samples were taken (points). Positions of samples of *S. algosus* (Sa), *Brachidontes granulatus* (Bg), *Brachidontes rodrigueiz* (Br), *Choromytilus chorus* (Cch), *Choromytilus meridionalis (*Cm), and *Perumytilus purpuratus* (Ppu) are indicated on the map.

Table 2. Description of Primers Used in This Work

DNA region	name		sequence (5′-3′)	LEN[a] (bp)	T_m[b] (°C)	fragment size (bp)	described
rDNA 18S	*Myt18S*	F	CAACCTGGTTGATCCTGCCAGT	22	56	919–924	in this work
		R	CACCTCTAACACCGTAATACGA	22			
	MusRFLP	F	CGAGGCCCCGTAATTGGAATGA	22	63	230–237	in this work
		R	TCAGTCAAGAGCACCAAGGGC	21			
	MusRFLP	F	CGAGGCCCCGTAATTGGAATGA	22	58	167–169	in this work
	MusFINS	R	AAACCGGGAGGTAGGTCAGG	20			
ITS 1	*Xela ITS1*	1	AAGTAAAAGTCGTAACAAGGTTTCCGTAGG	30	67	383–394	Pérez et al. (*28*)
	OnmylTS1	2	CAAGCCGAGTGATCCACCGC	20			
	Pspp	F	AYAAACGGAGGTTACGGTTTC	21	62	171–207	in this work
		R	GTGCGAGGCMRAWRWGGAAAA	21			
	CHORO	F	AGGATCATTACCGCAATACGAT	22	62	100	in this work
		R	AAACGACGKAGGACTTTGCRT	21			
adhesive protein gene	*Me*	15	CCAGTATACAAACCTGTGAAGA	22	63	126–180	Inoue et al. (*10*)
		16	TGTTGTCTTAATAGGTTTGTAAGA	24			

[a] Length of the primer. [b] Melting temperature.

the phylogenetic tree was neighbor-joining. The reliability of the constructed tree was evaluated by means of a Bootstrap test with 2000 replications.

b. Identification of Species Belonging to Perna, Choromytilus, and Mytilus Genera. b.1. Development of a Multiplex PCR-RFLP Methodology for Genetic Identification of Perna and Choromytilus Species Using ITS 1. Species included in *Perna* and *Choromytilus* genera were studied (**Table 1**). An *ITS 1* fragment was amplified with primers described by Pérez et al. (**Table 2**) (*28*). To obtain DNA sequences, PCR products were cloned into pGEM-T Easy Vector System II (Promega), following the supplier's recommendations. These were analyzed, as in the case of *18S rDNA gene*, by obtaining haplotypes and restriction maps. Two internal regions, which include variable positions between species that coincide with points of cut for restriction enzymes, were selected to design two new primer sets, called *Pspp* and *CHORO*. These primer sets were calibrated in a multiplex PCR, to determine if the analyzed individual belongs to the *Perna* or *Choromytilus* genus, according to the PCR product size. The only parameter adjusted was melting temperature, whereas the remaining conditions were the same as described above.

Next, PCR products obtained in multiplex PCR were cleaned, digested with the selected restriction enzymes, and electrophoresed as reported in previous cases.

b.2. Development of a PCR and Length Polymorphism for Genetic Identification of Mytilus Species Using Adhesive Protein Gene. Species belonging to the *Mytilus* genus included in **Table 1** have been studied in this paper following the method described by Inoue et al. for the genetic identification of mussels (*10*), based on a length polymorphism among the three species of the *M. edulis* complex. PCR products were obtained as indicated by the authors (*10*), and two techniques have been used to evaluate this polymorphism: agarose gels and fragment analysis. Agarose gel studies were carried out in conditions identical to those used for RFLP of *18S rDNA* and *ITS 1*, and for fragment analysis, one primer of these pairs was labeled with the fluorochrome 6-FAM to enable the determination of size fragments using an ABI 310 Genetic Analyzer and the software GeneScan Analysis, version 3.1.2 (Applied Biosystems). Fragment size was estimated from GeneScan 400 HD (ROX DYE) Standard (Applied Biosystems).

Mussel species belonging to the *Mytilus* genus were genotyped in this way.

b.3. Identification of M. galloprovincialis and M. chilensis. To identify these two species, nucleotidic sequences of the fragment

Table 3. Information of All Samples and Methods Included in This Paper That Have Been Applied to Genetic Identification of Mussels

				individuals analyzed							
				18S			ITS 1		adhesive protein gene		
species	code	no. of samples	individuals per sample	seq[a] 924 pb	FINS	PCR-RFLP	seq[a]	PCR-RFLP	seq[a]	LP[b]	PCR-RFLP
Mytilus edulis	Me	18	10	2	3	10			2	180	
Mytilus trossulus	Mt	5	10	2	3	10			2	80	
Mytilus galloprovincialis	Mg	29	10	2	5	10			11	290	50
Mytilus chilensis	Mc	8	10	3	4	10			11	50	50
Mytilus californianus	Mcal	3	10	2	3	10					
Perna perna	Pp	3	10	4	4	10	4	10			
Perna canaliculus	Pc	3	10	3	3	10	4	10			
Perna viridis	Pv	3	10	5	5	10	2	10			
Choromytilus chorus	Cch	3	5	3	2	5	2	5			
Choromytilus meridionalis	Cm	3	5	2	3	5	2	5			
Aulacomya ater	Aa	3	10	2	5	10					
Brachidontes rodrigueiz	Br	2	10	2	3	5					
Brachidontes granulatus	Bg	2	5	2	2	5					
Perumytilus purpuratus	Ppu	2	10	2	2	5					
Semimytilus algosus	Sa	3	10	2	3	10					

[a] Seq, sequenced individuals. [b] LP, length polymorphism.

described by Inoue et al. (*10*) were obtained. A representative number of individuals per sample and species were sequenced (**Table 3**). From these sequences, a method based on RFLP was developed as previously. This RFLP methodology was applied to a high number of individuals per species (**Table 3**). The restriction profile was visualized in agarose gels, in the same conditions as the previous ones.

Methodological Validation and Market Study. The aim of this process was to evaluate the correct performance of the methodologies herein proposed. Authenticated samples were used to manufacture cans of product with different covering sauces in our pilot plant. These preserved products, including canned and frozen samples, were analyzed with the methods developed.

The methodological strategy described previously was also applied to 30 commercial samples from canned or frozen products acquired in shops and stores from Pontevedra, Spain, to check the correct labeling of the analyzed products.

RESULTS AND DISCUSSION

The present work includes a group of methods that must be used sequentially, allowing the genetic identification of mussel species in any commercial format. The combination of several molecular markers has been necessary due to the great variability in the genera that compose mussels.

In the present work a methodology that consists of two steps was developed to perform genetic identification. Several alternatives are given in each step depending on the availability of equipment in each laboratory (**Figure 3**).

a. Development of PCR-RFLP and FINS Methodology Using Nuclear Small-Subunit rRNA Gene. *a.1. Development of PCR-RFLP Technique.* The importance of this first methodological step is based on the assignment at genus level. Therefore, one can apply the next steps with total reliability. When this first step is not carried out, the subsequent methodology was not useful and led to an incorrect identification.

In a previous paper, Kenchington et al. (*14*) used *18S rDNA* to study the relationship among different taxa belonging to *Mytilus*. The main characteristic of this marker is its high degree of conservation. These authors found two different nucleotidic positions in three *Mytilus* species from a total of 1825 bp evaluated. In the present paper we sequenced a small fragment inside this one, limited by primer set *Myt18S* (**Table 2**). The sequences obtained had sizes between 919 and 924 bp (accession numbers DQ640506−DQ640543). This nucleotidic fragment showed a high degree of conservation in all studied species, and this feature makes this marker very suitable for the genetic identification of species, because interspecific variability is enough for genetic identification and intraspecific variability detected was very low and never affected the target recognized by the restriction enzymes.

From these sequences, the primer set *MusRFLP* was designed, which amplified a PCR product of 230−237 bp (**Table 2**) that includes those variable positions pointed out by Kenchington et al. (*14*) and those described in the present work.

The identification of *Mytilus* spp., *M. californianus*, *Perna* spp., *Choromytilus* spp., and *Aulacomya ater* together with one group composed by species of minor commercial importance can be carried out by using three endonucleases, *Bsa*HI, *Cac*8I (New England Biolabs), and *Afa*I (Amersham Biosciences) (**Table 4** and **Figure 4**). Some species of this last group (*Brachidontes* spp., *Perumytilus purpuratus*, and *Semimytilus algosus*) can be identified with *Msp*I and *Nla*III enzymes (New England Biolabs) (**Table 5** and **Figure 5**). These restriction enzymes will be used only in a few cases because the three previous ones are able to determine the more important commercial species (**Figure 3**).

a.2. Alternative to PCR-RFLP: FINS Technique. The FINS technique was described by Bartlett and Davidson (*29*), who carried out genetic identification using phylogenetic analysis. Phylogenetic trees can be constructed from a distance matrix, which is calculated using the sequence of an undetermined or unknown species and reference sequences belonging to known species. Results of this analysis can be displayed by a phylogenetic tree, where sequences of the same species are grouped into clades. This methodology was applied previously to the genetic identification of fishes with total success (*20, 21, 30*−*32*).

The DNA fragment amplified with *MusRFLP* F and R primers contains a poli timine (T) zone with intraindividual length polymorphism in some *Mytilus* species, which prevents correct sequencing. Therefore, a new primer called *MusFINS* R was designed to be used together with *MusRFLP* F. This new amplified region does not contain the poli T zone. The size of this new PCR product was between 167 and 169 bp according to the species and allowed direct sequencing in all cases (accession numbers DQ640544−DQ640555) (**Table 2** and **Figure 2**).

FINS alternative allowed identification of *Mytilus califor-*

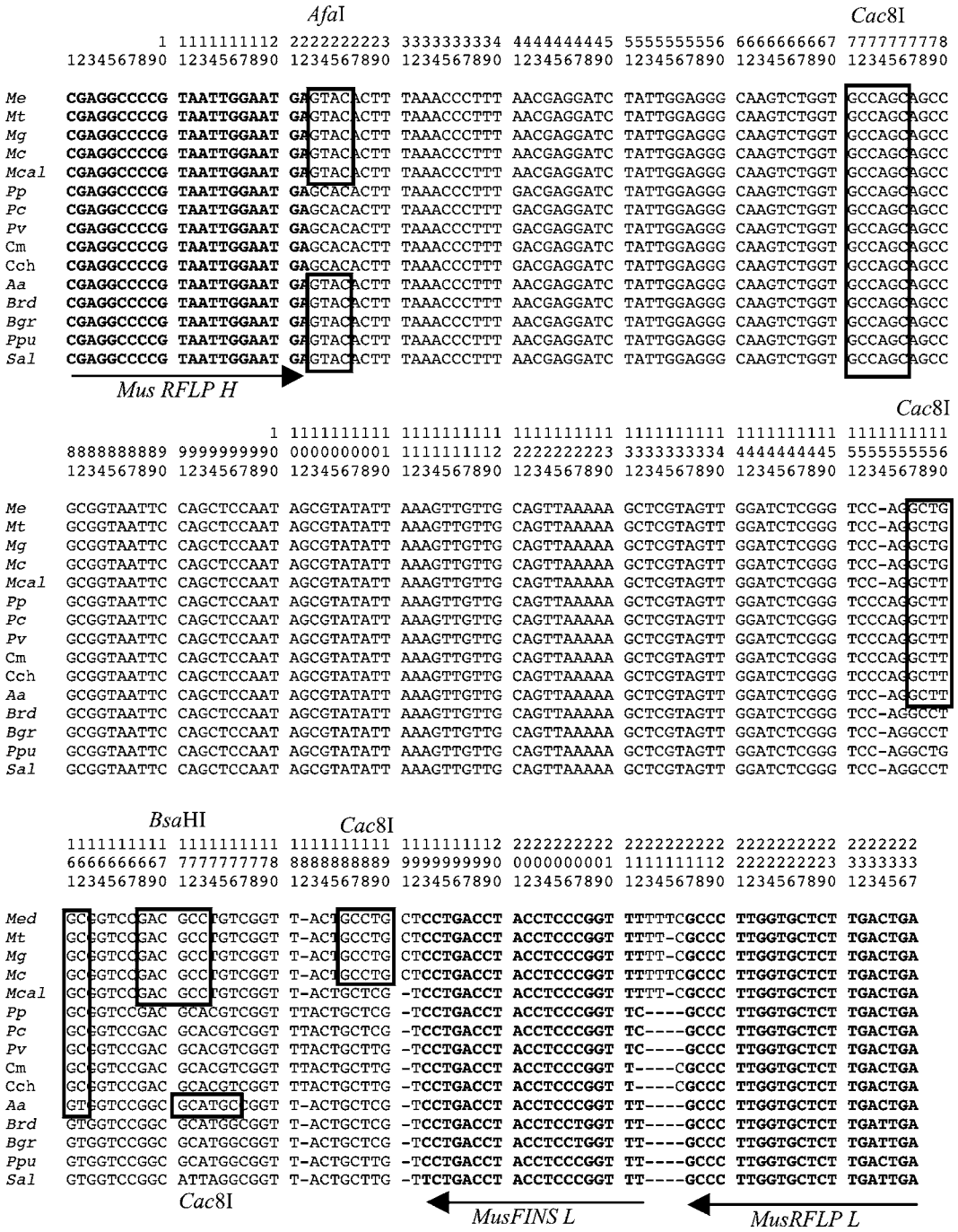

Figure 2. Sequence of 230–237 bp fragment of the studied species and target of restriction enzymes. Arrows show the positions of the primer sets used to amplify this region.

nianus, *Aulacomya ater*, *Perumytilus purpuratus*, *Perna viridis*, and *Semimytilus algosus* at the level of species and the following groups: *Mytilus* spp. (group 1), *Perna perna* and *Perna canaliculus* (group 2), *Choromytilus* spp. (group 3), and *Brachidontes* spp. (group 4) (**Figure 6**). Bootstrap values are shown over crucial branches and reflect the reliability of the species assignation.

Group 4 consists of two species of *Brachidontes* genus (*B. granulatus* and *B. rodrigueiz*). These species are rarely used for the elaboration of commercial products. Therefore, they were not studied in more depth, and we were able to carry out identification at only the genus level.

b. Identification of Species Belonging to *Perna*, *Choromytilus*, and *Mytilus* Genera. It is important to point out that the methodological second step is necessary to identify species that belong to groups 1−3 (*Mytilus* spp., *Perna* spp., and *Choromytilus* spp.) when PCR-RFLP or FINS is used (**Figures 4** and **6**).

Due to the characteristics of *18S rDNA*, this marker does not allow the identification of some species included in the genera *Perna*, *Choromytilus*, and *Mytilus*, because it is highly conserved and these species are genetically very close. Therefore, other genomic regions characterized by a higher rate of change than nuclear small-subunit rRNA gene were selected. These regions were *ITS 1*, which allowed us to identify the species of *Perna* and *Choromytilus* genera, and the adhesive protein gene described by Inoue et al. (*10*), which allowed us to identify all of the *Mytilus* species studied.

Development of these methods was very similar to that of the previous ones based on nuclear small subunit rDNA gene and is explained hereafter.

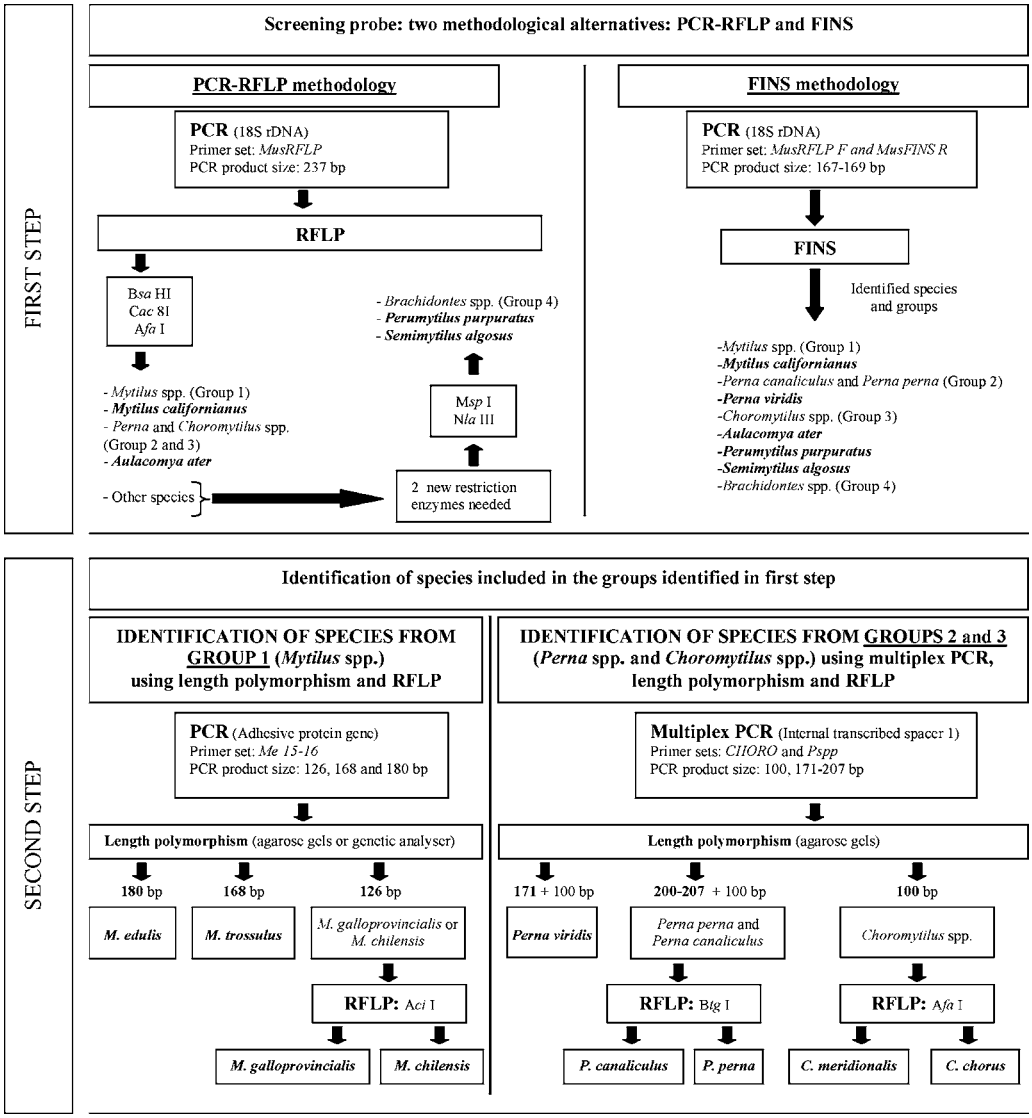

Figure 3. Diagram of methodology used in the genetic identification of mussel species.

Table 4. Haplotypes Generated after Digestion with Restriction Enzymes

	*Bsa*HI		*Afa*I		*Cac*8I			
species	size fragment	H[a]	size fragment	H[a]	size fragment	H[a]	CH[b]	identified species
Mytilus edulis	169, 68	A	24, 213	A	73, 85, 28, 51	A	AAA	*Mytilus* spp.
Mytilus galloprovincialis	169, 68	A	24, 213	A	73, 85, 28, 51	A	AAA	
Mytilus trossulus	169, 68	A	24, 213	A	73, 85, 28, 51	A	AAA	
Mytilus chilensis	169, 68	A	24, 213	A	73, 85, 28, 51	A	AAA	
Mytilus californianus	169, 68	A	24, 213	A	73, 85, 79	B	AAB	*Mytilus californianus*
Perna perna	237	B	237	B	73, 86, 78	B	BBB	*Perna* and *Choromytilus* spp.
Perna canaliculus	237	B	237	B	73, 86, 78	B	BBB	
Perna viridis	237	B	237	B	73, 86, 78	B	BBB	
Choromytilus meridionalis	237	B	237	B	73, 86, 78	B	BBB	
Choromytilus chorus	237	B	237	B	73, 86, 78	B	BBB	
Aulacomya ater	237	B	24, 213	A	73, 99, 65	C	BAC	*Aulacomya ater*
other species	237	B	24, 213	A	73, 164	D	BAD	other species

[a] H, haplotype. [b] CH, combined haplotype.

b.1. Development of a Multiplex PCR-RFLP Methodology for Genetic Identification of Perna and Choromytilus Species Using ITS 1 Region. ITS 1 is located between the *18S rDNA* (nuclear small-subunit rRNA gene) and *5.8S rDNA* genes. This region has been used previously in the genetic identification of species (*28*), giving satisfactory results, in part, due to the characteristic mode of evolution, concerted evolution (*33*), and the multicopy nature of this gene (*34*).

Nucleotidic sequences for several clones and individuals per species were obtained using the primers described by Pérez et al. (*28*) (**Tables 2** and **3**) (accession numbers DQ640556−DQ640585). Direct sequencing has not been possible, due to the intraindividual length polymorphism of *ITS 1*. Therefore, PCR products have been cloned into the pGEM-T Easy Vector System II (Promega).

These sequences allowed us to select internal regions for

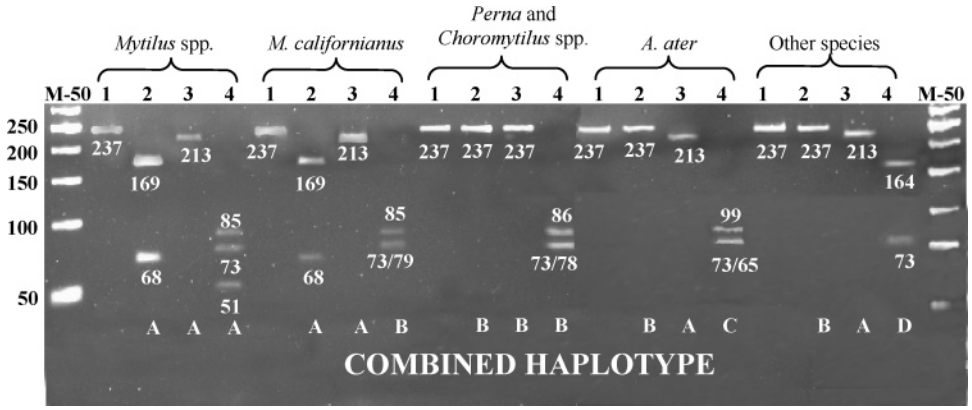

Figure 4. RFLP analysis of PCR products obtained by PCR with *MusRFLP* primer set. Lanes: 1, PCR products; 2, PCR products after digestion with *Bsa*HI; 3, PCR products after digestion with *Afa*I; 4, PCR products after digestion with *Cac*8I; M-50, molecular size marker (50 bp DNA ladder, Amersham Biosciences).

Table 5. Haplotypes Generated after Digestion with Restriction Enzymes in the Case of Species with Minor Commercial Importance

	*Msp*I		*Nla*III			
species	size fragment	H[a]	size fragment	H[a]	CH[b]	identified species
Brachidontes rodrigueiz	237	A	174, 63	A	AA	*Brachidontes* spp.
Brachidontes granulatus	237	A	174, 63	A	AA	
Perumytilus purpuratus	203, 34	B	174, 63	A	BA	*Perumytilus purpuratus*
Semimytilus algosus	203, 34	B	237	B	BB	*Semimytilus algosus*

[a] H, haplotype. [b] CH, combined haplotype.

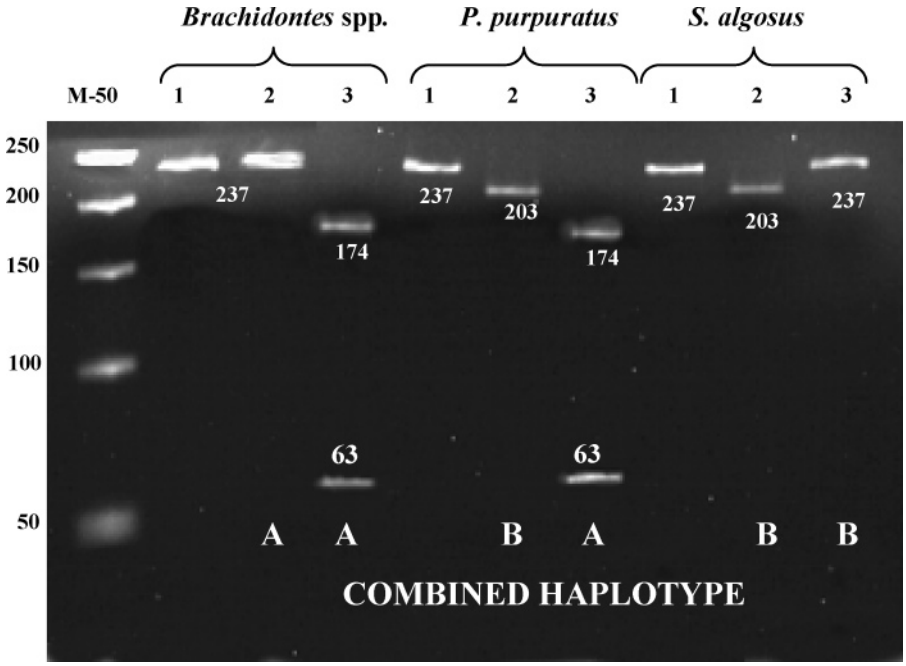

Figure 5. RFLP analysis of PCR products belonging to species with minor commercial importance. Lanes: M-50, molecular size marker (50 bp DNA ladder, Amersham Biosciences); 1, PCR products; 2, PCR products after digestion with *Msp*I; 3, PCR products after digestion with *Nla*III.

designing two new primer sets: *Pspp* and *CHORO*. These primer sets were selected to generate different PCR product sizes in *Perna* and *Choromytilus* species.

These two primer sets were calibrated in a multiplex PCR to determine if the analyzed individual belongs to the *Perna* or *Choromytilus* genus according to the PCR product size. Melting temperature was adjusted to 62 °C (**Table 2**).

The first primer set generated PCR products between 171 and 207 bp with *Perna* species. Neither species of *Choromytilus* nor any other species included in this study showed amplification with this primer set, so it is specific for *Perna* spp. One length polymorphism between *Perna viridis* (171 bp) and the group composed by *Perna canaliculus* and *Perna perna* was observed, which amplified one fragment between 200 and 207 bp. Therefore, *P. viridis* can be easily identified in agarose gels because this species shows a fragment size smaller than that of *P. canaliculus* and *P. perna* (**Table 6** and **Figure 7**).

The second primer set designed, *CHORO* (**Table 2**), amplified a PCR product of 100 bp in *Perna* and *Choromytilus* species (**Table 6** and **Figure 7**).

When the PCR product size is not characteristic of any mentioned species, RFLP must be carried out, allowing the

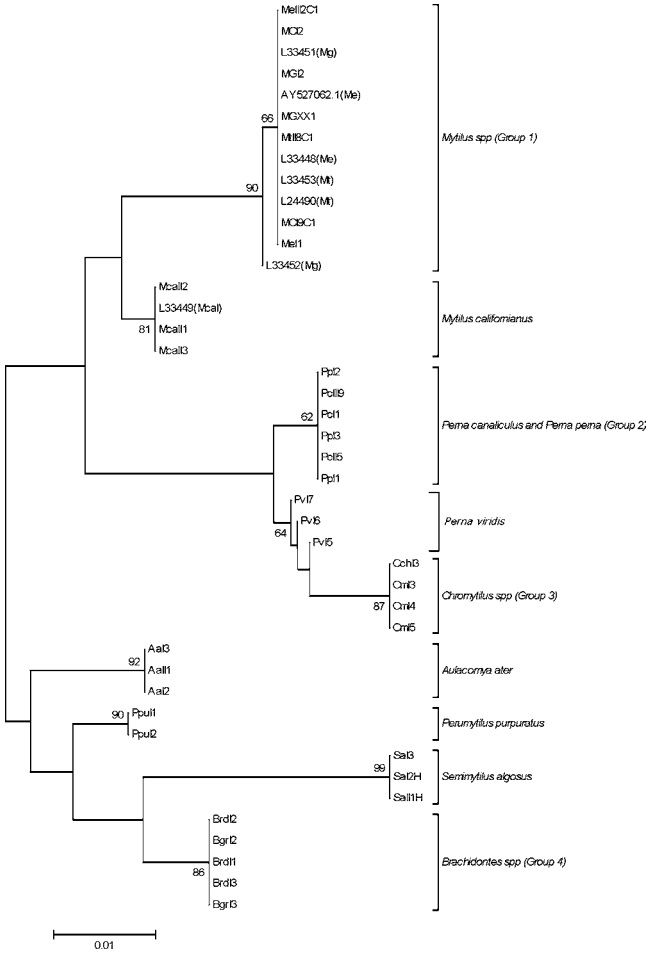

Figure 6. Neighbor-joining tree of genetic relationships among studied mussel species, carried out from the alignment of 167–169 bp sequences. Sequences with codes AY527062.1, L24490, L33448, L33449, L33451, L33452, and L33453 were obtained from the NCBI database.

Table 6. PCR Products Obtained by Multiplex PCR in *Perna* and *Choromytilus* Species and Restriction Haplotypes Generated after Digestion with *Btg*I and *Afa*I

species	PCR product size	restriction enzyme	size fragment	RH[a]
Perna viridis	171, 100	*Btg*I	171, 100	A
Perna canaliculus	207, 100		207, 100	B
Perna perna	200, 100		98, 102, 100	C
Choromytilus chorus	100	*Afa*I	24, 21, 28, 26	D
Choromytilus meridionalis	100		24, 50, 26	E

[a] RH, restriction haplotype.

genetic identification of these species in all cases (**Figure 3**). The restriction enzyme *Btg*I was selected to differentiate *P. perna* and *P. canaliculus*, because it induces one cut in *P. perna* and it does not cut in *P. canaliculus*. This cut profile allows differentiation between these two species (**Table 6** and **Figure 8**). The species of *Choromytilus* can be differentiated using the endonuclease *Afa*I, because C*horomytilus meridionalis* shows a characteristic 50 bp fragment in RFLP analysis, whereas *Choromytilus chorus* shows small fragments of 21−28 bp and the absence of the 50 bp band (**Table 6** and **Figure 8**).

b.2. Development of a PCR and Length Polymorphism for the Genetic Identification of Mytilus Species Using Adhesive Protein Gene. A series of markers usually employed for the genetic identification of marine species have been studied: *5S rDNA* gene (*35*), *ITS 1* and *ITS 2* (*3*, *13*, *28*), *NTS* (nontranscribed spacer) (*36*); however, the interspecific variability detected in *Mytilus* species was not appropriate to develop an identification method (data not shown). Therefore, the method proposed by Inoue et al. (*10*) was selected, because PCR product sizes are small enough to allow application in canned seafood and showed correct functioning when it was applied in previous works (*16*). Other methods have been used for this task (*3*, *12*, *13*, *17*), but elevated PCR product sizes prevent their application in canned products.

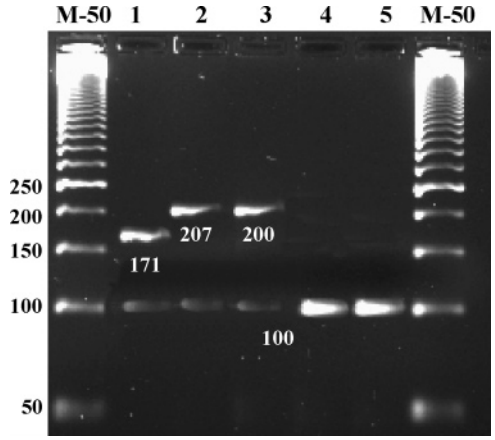

Figure 7. PCR product profiles obtained in the multiplex PCR, with DNA template of *Perna* and *Chromytilus* species. Lanes: M-50, molecular size marker (50 bp DNA ladder, Amersham Biosciences); 1, *Perna viridis*; 2, *Perna perna*; 3, *Perna canaliculus*; 4, *Choromytilus chorus*; 5, *Choromytilus meridionalis*.

Table 7. PCR Products Obtained in *Mytilus* Species and Restriction Profile Generated after Digestion with *Aci*I in *M. galloprovincialis* and *M. chilensis*

species	PCR product size	*Aci*I size fragment	RH[a]
Mytilus edulis	180		
Mytilus trossulus	168		
Mytilus galloprovincialis	126	57, 69	A
Mytilus chilensis	126	126	B

[a] RH, restriction haplotype.

The method developed by Inoue et al. was applied to *Mytilus* individuals included in this study (**Table 3**). Agarose gels and fragment analysis allowed identification of the three species and some of their hybrids (**Figures 9** and **10**), as the authors described (*10*).

The method reported by Inoue et al. (*10*) includes the species of the *M. edulis* complex, *M. edulis* with a PCR product size of 180 bp, *M. trossulus* of 168 bp, and *M. galloprovincialis* of 126 bp. However, it is possible that other species that do not belong to the *Mytilus* genus have a length polymorphism identical to that of one of the *Mytilus* species. In the present study, length polymorphism of polyphenolic adhesive protein gene has been verified in *Mytilus* and other species. It has been observed that species which do not belong to *Mytilus* yield the same length polymorphism as *Mytilus* species. For instance, in *P. viridis*, we amplified a 126 bp fragment. This could give rise to misidentifications if a previous step based on *18S rDNA* gene is obviated, which fits perfectly at genus level, and it would indicate if a second step is necessary.

Moreover, the method proposed by Inoue et al. (*10*) shows a lack of specificity because *M. galloprovincialis* and *M. chilensis*

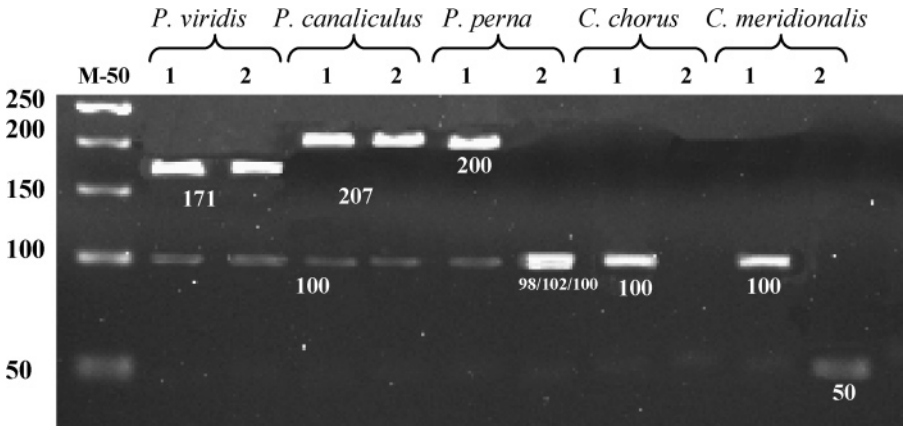

Figure 8. RFLP analysis of PCR products obtained by PCR with *Pspp* and *CHORO* primer sets. Lanes: M-50, molecular size marker (50 bp DNA ladder, Amersham Biosciences); 1, PCR products; 2, PCR products after digestion with *Btg*I (*Perna* spp.) and *Afa*I (*Choromytilus* spp.).

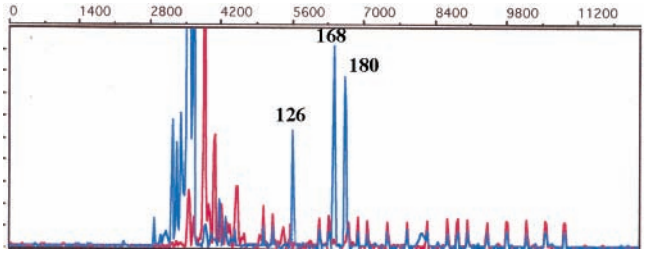

Figure 9. Electropherogram obtained in an ABI 310 Genetic Analyzer (Applied Biosystems) where 126, 168, and 180 bp PCR fragments have been loaded. Fluorescence of these PCR products is shown in blue, because one primer of this set was labeled with 6-FAM. In red is shown the standard GeneScan 400 HD (ROX DYE) (Applied Biosystems), which is used to determine the PCR product size in the ABI 310 Genetic Analyzer.

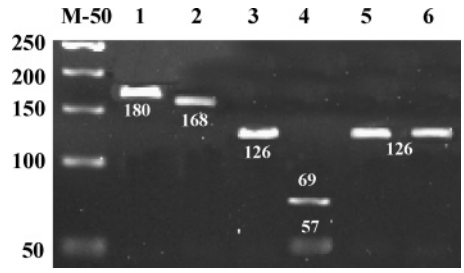

Figure 10. Length polymorphism and restriction profiles obtained with *Mytilus* spp. Lanes: M-50, molecular size marker (50 bp DNA ladder, Amersham Biosciences); 1, PCR product of *M. edulis*; 2, PCR product of *M. trossulus*; 3, PCR product of *M. galloprovincialis*; 4, RFLP of *M. galloprovincialis* with *Aci*I enzyme; 5, PCR product of *M. chilensis*; 6, RFLP of *M. chilensis* with *Aci*I enzyme.

are not differentiated; both presented identical length polymorphism (126 bp fragment) (**Figure 10**). In that study *M. chilensis* was not analyzed (*10*).

b.3. Identification of M. galloprovincialis and M. chilensis. To study differentiation of *M. galloprovincialis* and *M. chilensis*, PCR products of 126 bp from both species were sequenced (accession numbers DQ640586−DQ640610). These sequences allowed development of a RFLP methodology, which was applied to 50 individuals of each of these two species (**Table 3**).

The restriction enzyme *Aci*I was selected because it includes one interspecific variable position in its target sequence. Therefore, the restrictase has diagnostic value, and it produces a cut in the amplicon of *M. galloprovincialis*, generating two fragments of 57 and 69 bp size. In contrast, the enzyme does not cut in *M. chilensis* (**Table 7** and **Figure 10**).

c. Methodological Validation and Market Study. Genetic identification of the products manufactured in our pilot plant from authenticated individuals was correct because analyzed samples were always identified as expected. Commercial samples were analyzed following the diagram shown in **Figure 3**. All of the analyzed samples were identified as some species of those included in this work by PCR-RFLP, FINS, and length polymorphism methodology. All of these methodological approximations showed concordant results and allowed us to verify the species included in the analyzed products and their correct labeling.

Altogether, this paper describes DNA-based methods that applied sequentially allow the genetic identification of mussel species in fresh, frozen, canned, or any other preparation. Lack of other methods for this task makes this methodology the unique alternative to genetic key, which can be applied independently on the transformation process. Therefore, this method can be useful in studies of traceability and correct labeling.

ACKNOWLEDGMENT

We thank P. Pringent, B. McDonald, J. Sanchez, R. Luengo, M. Pfaff, C. Ruiz, M. L. Zettler, Q. Jo, H. Furuya, E. C. Amar, R. D. Sepulveda, D. Torring, V. Pienaar, P. J. Seguel, B. Gudbjornsdottir, J. Deboer, G. Moeser, L. M. Chi-Chang, A. Staniszewski, B. Becker, G. Smith, K. Lehtonen, K. Park, H. Waite, and others for the collection of samples.

LITERATURE CITED

(1) Daguin, C.; Bonhomme, F.; Borsa, P. The zone of sympatry and hybridization of *Mytilus edulis* and *M. galloprovincialis*, as described by intron length polymorphism at locus mac-1. *Heredity* **2001**, *86*, 342−354.

(2) Heath, D. D.; Hatcher, D. R.; Hilbish, T. J. Ecological interation between sympatric Mytilus species on the west coast Canada investigated using PCR markers. *Mol. Ecol.* **1996**, *5*, 443−447.

(3) Heath, D. D.; Rawson, P. D.; Hilbish, T. J. PCR-based nuclear markers identify alien blue mussel (*Mytilus* spp.) genotypes on the west coast of Canada. *Can. J. Fish. Aquat. Sci.* **1995**, *52* (12), 2621−2627.

(4) Inoue, K.; Odo, S.; Noda, T.; Nakao, S.; Takeyama, S.; Yamaha, E.; Yamazaki, F.; Harayama, S. A possible hybrid zone in the *Mytilus edulis* complex in Japan revealed by PCR markers. *Mar. Biol.* **1997**, *128* (1), 91−95.

(5) Rawson, P. D.; Agrawal, V.; Hilbish, T. J. Hybridization between the blue mussels *Mytilus galloprovincialis* and *M. trossulus* along the Pacific coast of North America: evidence for limited introgression. *Mar. Biol.* **1999**, *134* (1), 201−211.
(6) Zouros, E. The exceptional mitochondrial DNA system of the mussel family Mytilidae. *Genes Genet. Syst.* **2000**, *75* (6), 313−318.
(7) Daguin, C.; Borsa, P. Genetic relationships of *Mytilus galloprovincialis* Lamarck populations worldwide: evidence from nuclear-DNA markers. The evolution and biology of the Bivalvia. *Geol. Soc.* **2000**, *177*, 389−397.
(8) Sarver, S. K.; Foltz, D. W. Genetic population-structure of a species complex of blue mussels (*Mytilus* spp.). *Mar. Biol.* **1993**, *117* (1), 105−112.
(9) Apte, S.; Gardner, J. P. A. Absence of population genetic differentiation in the New Zealand greenshell mussel *Perna canaliculus* (Gmelin 1791) as assessed by allozyme variation. *J. Exp. Mar. Biol. Ecol.* **2001**, *258* (2), 173−194.
(10) Inoue, K.; Waite, J. H.; Matsuoka, M.; Odo, S.; Harayama, S. Interspecific variations in adhesive protein sequences of *Mytilus edulis*, *M. galloprovincialis*, and *M. trossulus*. *Biol. Bull.* **1995**, *189* (3), 370−375.
(11) Ohresser, M.; Borsa, P.; Delsert, C. Intron-length polymorphism at the actin gene locus mac-1: a genetic marker for population studies in the marine mussels *Mytilus galloprovincialis* Lmk and *M. edulis* L. *Mol. Mar. Biol. Biotechnol.* **1997**, *6* (2), 123−130.
(12) Rego, I.; Martinez, A.; Gonzalez-Tizon, A.; Vieites, J.; Leira, F.; Mendez, J. PCR technique for identification of mussel species. *J. Agric. Food Chem.* **2002**, *50*, 1780−1784.
(13) Toro, J. E. Molecular identification of four species of mussels from southern Chile by PCR-based nuclear markers: the potential use in studies involving planktonic surveys. *J. Shellfish Res.* **1998**, *17* (4), 1203−1205.
(14) Kenchington, E.; Landry, D.; Bird, C. J. Comparison of taxa of the mussel *Mytilus* (Bivalvia) by analysis of the nuclear small-subunit rRNA gene sequence. *Can. J. Fish. Aquat. Sci.* **1995**, *52* (12), 2613−2620.
(15) Gosling, E. Systematics and geographic distribution of *Mytilus*. In *The Mussel Mytilus: Ecology, Physiology, Genetics and Culture*; Goling, E., Ed.; Elsevier Science: Amsterdam, The Netherlands, 1992; pp 1−20.
(16) Wood, A. R.; Beaumont, A. R.; Skibinski, D. O. F.; Turner, G. Analysis of a nuclear-DNA marker for species identification of adults and larvae in the *Mytilus edulis* complex. *J. Molluscan Stud.* **2003**, *69*, 61−66.
(17) Toro, J. E. PCR-based nuclear and mtDNA markers and shell morphology as an approach to study the taxonomic status of the Chilean blue mussel, *Mytilus chilensis* (Bivalvia). *Aquat. Living Resour.* **1998**, *11* (5), 347−353.
(18) Bendezu, I. F.; Slater, J. W.; Carney, B. F. Identification of *Mytilus* spp. and *Pecten maximus* in Irish waters by standard PCR of the *18S rDNA gene* and multiplex PCR of the *16S rDNA gene*. *Mar. Biotechnol.* **2005**, *7* (6), 687−696.
(19) Quinteiro, J.; Sotelo, C. G.; Rehbein, H.; Pryde, S. E.; Medina, I.; Perez-Martin, R. I.; Rey-Mendez, M.; Mackie, I. M. Use of mtDNA direct Polymerase Chain Reaction (PCR) sequencing and PCR-restriction fragment length polymorphism methodologies in species identification of canned tuna. *J. Agric. Food Chem.* **1998**, *46*, 1662−1669.
(20) Terol, J.; Mascarell, R.; Fernandez-Pedrosa, V.; Perez-Alonso, M. Statistical validation of the identification of tuna species: bootstrap analysis of mitochondrial DNA sequences. *J. Agric. Food Chem.* **2002**, *50*, 963−969.
(21) Jerome, M.; Lemaire, C.; Verrez-Bagnis, W.; Etienne, M. Direct sequencing method for species identification of canned sardine and sardine-type products. *J. Agric. Food Chem.* **2003**, *51*, 7326−7332.
(22) Roger, S. O.; Bendich, A. J. Extraction of DNA from plant tissues. *Plant Mol. Biol. Manual* **1988**, (A6), 1−10.
(23) McCarthy, C. Chromas version 1.45. School of Health science, Griffifth University, Gold Coast Campus, Queensland, Australia, 1996.
(24) Hall, T. A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **1999**, *No. 41*, 95−98.
(25) Rozas, J.; Sánchez-DelBarrio, J. C.; Messeguer, X.; Rozas, R. DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* **2003**, *19*, 2496−2497.
(26) Heiman, M. Webcutter 2.0. http://www.firstmarket/cutter/cut2.html, 1997.
(27) Kumar, S.; Tamura, K.; Nei, M. MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings Bioinf.* **2004**, *5*, 150−163.
(28) Pérez, M.; Cabado, A. G.; Vieites, J. M.; Presa, P. Experimental assessment of a new rDNA-based method for the identification of *Merluccius capensis* and *Merluccius paradoxus* in commercial products. *J. Aquat. Food Prod. Technol.* **2004**, *13*, 49−57.
(29) Bartlett, S. E.; Davidson, W. S. FINS (forensically informative nucleotide sequencing): a procedure for identifying the animal origin of biological specimens. *Biotechniques* **1992**, *12* (3), 408−411.
(30) Chapela, M. J.; Sotelo, C. G.; Calo-Mata, P.; Perez-Martin, R. I.; Rehbein, H.; Hold, G. L.; Quinteiro, J.; Rey-Mendez, M.; Rosa, C.; Santos, A. T. Identification of cephalopod species (Ommastrephidae and Loliginidae) in seafood products by forensically informative nucleotide sequencing (FINS). *J. Food Sci.* **2002**, *67* (5), 1672−1676.
(31) Chapela, M. J.; Sotelo, C. G.; Perez-Martin, R. I. Molecular identification of cephalopod species by FINS and PCR-RFLP of a cytochrome *b* gene fragment. *Eur. Food Res. Technol.* **2003**, *217* (6), 524−529.
(32) Sotelo, C. G.; Calo-Mata, P.; Chapela, M. J.; Perez-Martin, R. I.; Rehbein, H.; Hold, G. L.; Russell, V. J.; Pryde, S.; Quinteiro, J.; Izquierdo, M.; Rey-Mendez, M.; Rosa, C.; Santos, A. T. Identification of flatfish (Pleuronectiforme) species using DNA-based techniques. *J. Agric. Food Chem.* **2001**, *49*, 4562−4569.
(33) Cortadas, J.; Pavon, M. C. The organization of ribosomal genes in vertebrates. *MBO J.* **1982**, *1*, 1075−1080.
(34) Long, E. O.; Dawid, I. B. Repeated genes in eukaryotes. *Annu. Rev. Biochem.* **1980**, *49*, 727−764.
(35) Moran, P.; Garcia-Vazquez, E. Identification of highly prized commercial fish using a PCR-based methodology. *Biochem. Mol. Biol. Educ.* **2006**, *34* (2), 121−124.
(36) Aranishi, F. PCR-RFLP analysis of nuclear nontranscribed spacer for mackerel species identification. *J. Agric. Food Chem.* **2005**, *53*, 508−511.

Received for review May 18, 2006. Revised manuscript received August 4, 2006. Accepted August 8, 2006. This work was funded with Grant PGIDIT05RMA00801CT from Consellería de Innovación e Industria da Xunta de Galicia.

JF061400U