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Development of a Method for the Genetic Identification of Commercial Bivalve Species Based on Mitochondrial 18S rRNA Sequences

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In this study a genetic methodology based on the amplification of an 18S rRNA fragment by PCR and phylogenetic analysis of the obtained DNA sequences was developed. This technique allows the genetic identification of more than 50 bivalve species in fresh, frozen, precooked and canned products. The developed method was applied to 30 commercial samples to check their labeling, showing that 12 samples were incorrectly labeled (40%). Therefore, the proposed methodology is appropriate to study questions related to the correct labeling and traceability of commercial products and the control of imported bivalves and fisheries in order to guarantee the protection of consumers' rights and verify the transparency of the extractive and transforming industries.

KEYWORDS: Bivalves; genetic identification; FINS; clams; cockles; oysters; scallops; jackknife clams; mussels

INTRODUCTION

Bivalves belong to the phylum Mollusca, a group of organisms that includes a great diversity of species (among others, clams, scallops, mussels, and oysters), many of them cultured around the world. The worldwide bivalve production increased during the past 50 years, from 0.9 million t in 1950 to 14 million t in the year 2000, accounting for 26% of the world fishing production (FAO). This increase in bivalve production was due to the good acceptance of these products in the international market; natural populations had declined as a consequence of the massive exploitation of natural resources. These facts gave rise to the development of intensive culture systems for several bivalve species, which have an important presence in the current international market.

In many cases, clams and other bivalves are marketed alive, so that consumers can identify the different species on the basis of the morphological characteristics, chiefly the shell morphology. However, this task becomes impossible when the shells are absent, as, for instance, in processed products that include only the meat. In this context, commercial fraud is relatively easy, because it is not possible to carry out the assignation of these products to a particular species using morphological traits. Consumers and the fishing industry are concerned about this question, and they demand the correct labeling of seafood products and transparency in the international trade. In this sense, different regulations were developed with the aim of standardizing the labeling rules. The compulsory labeling of fishery and aquaculture products is established at regional, national, and international levels. International labeling standards applicable to food products are set down by the *Codex Alimentarius Food Labeling Committee*. It is worth pointing out that the labeling of fish products must show the common and scientific name, because even species belonging to different genera can be designated by the same commercial denomination.

All of the previous arguments justify the need to develop analytical methods to determine the authenticity of raw materials used in commercial products for which the morphological identification is not possible. In this field, molecular biology techniques allow the establishment of the unequivocal identity of commercialized species. In the past, many genetic methods were proposed for the identification of some bivalve species (*1*−*11*), but all of them have great disadvantages. The great majority of these works include a low number of species. Fernández et al. (*5*−*7*) carried out the genetic identification of three species of clams using three genetic markers. By means of the methodology developed by Fernández-Tajes (*8*) can be differentiated five species of jackknifes, and the work of Saavedra (*9*) is focused on the study of only two species of scallops. Other works include a higher number of species, but are centered in the group of mussels (*11*). Moreover, only the last work can be applied to highly processed products. For these reasons, a genetic methodology based on the amplification of an 18S rRNA fragment by PCR and subsequent phylogenetic analysis was developed. Unlike previous works, this tool allows the genetic identification of bivalves marketed around the world, independent of the treatment applied during the elaboration

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Table 1. Samples Included in This Work and Location of Collection[a]

order	family	scientific name	common name	samples	location
Veneroida	Veneridae	*Tapes pullastra*	pullet carpet shell	10	ESP, PRT, FRA, UK, ITA
		Tapes decussata	grooved carpet shell	10	ESP, PRT, CHN, UK, ITA
		Tapes rhomboides	banded carpet shell	9	ESP, FRA, UK, ITA
		Paphia undulata	baby clam	2	CHN, JPN
		Venerupis philippinarum	Japanese carpet shell	5	ESP, JPN, IRL, CHN
		Venerupis variegata	variegated carpet shell	5	KOR, JPN,
		Venus verrucosa	warty venus	6	ESP, FRA, ITA, USA, GRE, MOR
		Venus antiqua	Chilean littleneck	2	CHI
		Dosinia exoleta	mature dosinia	5	ESP, POR, UK, GRE
		Tivela mactroides	trigonal tivela	2	PER, BRA
		Donax trunculus	wedge shell	2	ESP
		Donax vittatus	no common name	2	BEL
		Chamelea gallina	striped venus	4	ESP, ITA
		Austrovenus stutchburyi	New Zealand cockle	1	NZL
		Protothaca thaca	taca clam	3	CHI
		Callista chione	hard clam	2	ESP
	Mesodesmatidae	*Mesodesma donacium*	surf clam	2	CHI
		Paphies subtriangulata	New Zealand tuatua	2	NZL
		Paphies australis	New Zealand pipi	2	NZL
	Cardiidae	*Cerastoderma edule*	common cockle	4	ESP, POR, MOR
		Cerastoderma glaucum	olive green cockle	2	ITA, ESP
	Solecurtidae	*Tagelus dombeii*	tongue duckbill tagelus	5	CHI, PER
		Tagelus californianus	California tagelus	AM774536	
		Tagelus peruanus	no common name	5	PER, BRA
	Mactridae	*Spisula solida*	solid surf clam	7	ESP, POR, BEL, UK, MOR
		Spisula elliptica	no common name	5	BEL, FRA, UK
		Mulinia lateralis	dwarf surfclam	L11268	
	Pharidae	*Ensis ensis*	razor shell	7	ESP, FRA, CRO, UK, IRL
		Ensis arcuatus	razor clam	6	ESP, UK, BEL, IRL
		Ensis macha	macha jackknife clam	3	CHI, PER, ARG
		Ensis directus	atlantic jackknife	6	USA, UK, FRA, CAN
		Ensis americanus	American jackknife	2	NER, GER
		Ensis siliqua	sword razor shell	4	ESP, MAR
	Solenidae	*Solen marginatus*	European razor clam	5	ESP, POR, BEL, UK
	Tellinidae	*Macoma balthica*	Baltic macoma	2	BEL
	Corbiculidae	*Corbicula fluminea*	Asian clam	2	ESP
	Petricolidae	*Petricola pholadiformis*	no common name	1	BEL
Ostreoida	Ostreidae	*Ostrea edulis*	common oyster	8	ESP, FRA, ITA, UK, IRL
		Ostrea chilensis	Chilean oyster	6	NZL, CHI, ARG
		Crassostrea gigas	giant Pacific oyster	5	ESP, POR
		Crassostrea virginica	American cupped oyster	3	USA, CAN
		Crassostrea angulata	Portuguese oyster	5	ESP, POR
	Pectinidae	*Placopecten magellanicus*	sea scallop	2	CAN
		Patinopecten yessoensis	Japanese scallop	2	JPN
		Aequipecten opercularis	queen scallop	2	CHI
		Pecten maximus	great Atlantic scallop	7	ESP, NOR, FRA, UK, IRL
		Pecten jacobaeus	great Mediterranean scallop	6	GRE, ITA, CRO, ESP, FRA
		Argopecten purpuratus	Peruvian scallop	3	PER
		Chlamys varia	variegated scallop	2	ESP, IRL
		Chlamys islandica	Iceland scallop	2	NOR
Arcoida	Arcidae	*Anadara granosa*	blood cockle	2	MAS
		Anadara antiquata	ark shell	2	VIE
	Glycymerididae	*Glycymeris glycymeris*	dog cockle	2	BEL

[a] Location abbreviations: ARG, Argentina; BEL, Belgium; BRA, Brazil; CAN, Canada; CHI, Chile; CHN, China; CRO, Croatia; ESP, Spain; FRA, France; GER, Germany; GRE, Greece; IRL, Ireland; ITA, Italy; JPN, Japan; KOR, Korea; MOR, Morocco; NED, Netherlands; NOR, Norway; NZL, New Zealand; PER, Peru; PRT, Portugal; UK, United Kingdom; USA, United States; VIE, Vietnam. Only one of the possible common names for each species is given.

process. The developed tool could be of great help in controlling the correct labeling of these products, improving the protection of consumers' rights and avoiding unfair competition between fishing industry operators.

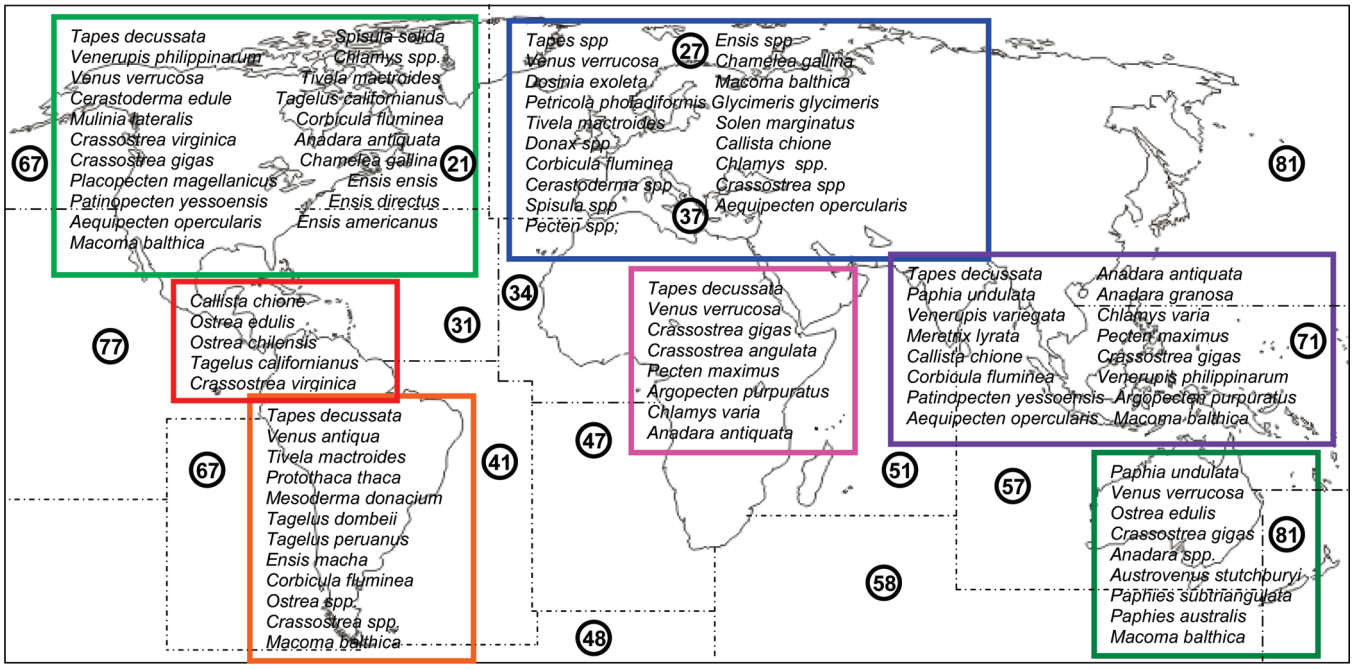

Figure 1. Map showing the distribution areas of bivalve species included in this study.

Table 2. Primers Used in This Work

name	sequence 5′−3′	T_a (°C)	size (bp)	described
Myt18S F	CAA CCT GGT TGA TCC TGC CAG T	50	912–964	Santaclara et al. (*11*)
Myt18S R	CAC CTC TAA CAC CGT AAT ACG A			
DtCed18S	CAC CTC TCS CGC CGC ART ACG T	60		in this work
BIVALVE 1F	TCT AGA GCT AAT ACA TGC			
BIVALVE 1R	ATA GGK CAG ACA YTT GAA AG	56	162–196	in this work
BIVALVE 2Fmod	AAA TTA GAG TGY TCA AAG CAG GC	56	148–151	in this work
DtCed18S	CAC CTC TCS CGC CGC ART ACG T			

MATERIALS AND METHODS

Sample Collection. A total of 53 species of adult bivalves were collected from different locations around the world (**Table 1**; **Figure 1**) and sent, frozen or immersed in absolute ethanol, to the Molecular Biology and Biotechnology laboratory of CECOPESCA (Spanish National Centre of Fish Processing Technology). Some samples were provided from universities and research centers located around the world. Once received, the samples were labeled and preserved at −80 °C. When possible, the specimens were identified on the basis of external morphological characteristics according to several bibliographic references (*12, 13*). Commercial samples were provided by import industries or obtained in local markets.

DNA Extraction. Genomic DNA was extracted from 30−50 mg of mantle tissue using the NucleoSpin Tissue kit (Macherey-Nagel) following the manufacturer's instructions. The obtained DNA was diluted in 100 μL of 1× Tris-EDTA (TE) buffer (Sigma). In the case of manufactured products used in the methodological validation and commercial samples, the DNA was extracted from a piece of 200 mg of tissue using the CTAB method previously described (*14*). The DNA was purified by means of the Nucleospin Extract II kit (Machery-Nagel) following the supplier's protocol.

The concentration and purity of DNA were determined by measuring the absorbance at 260 nm and the 260/280 and 234/260 ratios (*15*) using a spectrophotometer (Eppendorf Biophotometer).

Amplification and Sequencing of the 18S rRNA Fragment. The amplification of an 18S rRNA fragment was successfully carried out with primers *Myt18S* F and *Myt18S* R (*11*) in most of the studied species. In particular cases, the use of the degenerate reverse primer *DtCed18S* designed in this work from sequences obtained in the National Center for Biotechnology Information (NCBI) (AJ309018, *Donax trunculus*; and AY570555, *Cerastoderma edule*) was necessary.

All of the PCR amplifications were performed in a final volume of 50 μL containing 100 ng of DNA template, 5 μL of 10× Buffer, 2 mM $MgCl_2$, 0.4 μL of 100 mM dNTP, 4 μL of a 10 μM solution of each primer, and 1 unit of Taq-polymerase (Bioline). In the case of commercial products undergoing thermal treatment, the PCR reactions were carried out using 300 ng of DNA in a MyCycler thermocycler (Bio-Rad). The cycling program was as follows: 95 °C for 3 min, 35 cycles [95 °C for 30 s, temperature annealing (T_a) (**Table 2**) for 30 s, 72 °C for 30 s], followed by a final extension step of 72 °C for 3 min.

The PCR products were mixed with 5 μL of loading buffer and loaded on 2% agarose gels (Sigma) at 1% in TBE buffer with 5 μg/mL of ethidium bromide (Sigma). Electrophoretic separation was performed at 90 V for 40 min, and the resulting DNA fragments were visualized by ultraviolet transillumination in a Gel Doc XR System and analyzed using the software Quantity One 4.5.2 (Bio-Rad). The size of the amplified PCR products was estimated by comparison with pGEM DNA marker (Promega) or 50 bp ladder (Amersham Biosciences). Next, these PCR products were purified with Nucleospin Extract II kit (Machery-Nagel) according to the manufacturer's instructions. The quality and quantity of the DNA obtained was measured in a spectrophotometer (Eppendorf Biophotometer) in the same conditions described above.

PCR products were sequenced with the primers used for PCR amplification (**Table 2**) in an automatic DNA Genetic Analyzer (ABI Prism 310 Genetic Analyzer) using the BigDye Terminator cycle sequencing kit (Applied Biosystems) following the manufacturer's recommendations. The resulting electropherograms were analyzed with Chromas 1.45 software (*16*).

The obtained sequences were aligned with BioEdit 7.0 (*17*), and the haplotype diversity was calculated with the DnaSP 4.0 software (*18*).

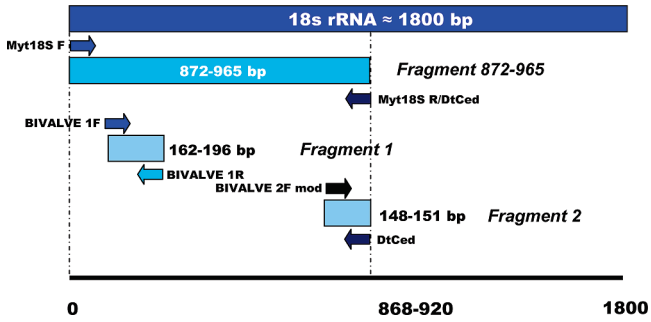

Figure 2. Location and size of the 18S rRNA fragments amplified in this work and position of primer set used.

Subsequently, three new internal primers were designed: BIVALVE 1F, BIVALVE 1R, and BIVALVE 2Fmod (**Table 2**; **Figure 2**). The PCR products obtained from BIVALVE 1F/1R and BIVALVE 2Fmod/DtCed18S primer sets are herein, respectively, called fragment 1 and fragment 2.

Development of the FINS Methodology. The phylogenetic analyses were performed from the alignment of all the obtained sequences with Mega 3.0 (*19*). Genetic distances between sequences were calculated using the Tamura−Nei nucleotide substitution model (*20*) and the neighbor-joining method (*21*). A bootstrap test with 2000 replications was conducted to assess the reliability of the nodes of the phylogenetic tree.

The internal fragments (herein called fragments 1 and 2) were used to identify the species included in food products undergoing thermal treatment (for instance, canned or cooked products). The measurement of genetic distances and the construction of the phylogenetic tree were performed in the same manner as previously described.

Also, the MEGABLAST search available at NCBI was used to assign the bivalve sequences to a particular species (*22*).

Validation of the Proposed Method. The aim of this process was to evaluate the correct performance of the proposed methodology in all kinds of transformed foodstuffs. Different frozen, canned, and cooked products were manufactured in the pilot plant of CECOPESCA from authenticated individuals. The methodology developed in this work was applied to the above-mentioned products to evaluate the correct operation of the developed methods. The coincidence percentage between the species identified on the basis of morphological traits and the genetic methodology developed was calculated to establish the specificity of the method.

Application to Commercial Samples. Thirty commercial samples subjected to different transformation processes (fresh, frozen, and canned products) were purchased in stores and supermarkets of Pontevedra, Spain. Subsequently, these products were analyzed with the methods herein developed.

RESULTS AND DISCUSSION

In this study a complete methodology for the genetic identification of bivalves was designed, validated, and applied to commercial samples. This method allows one to carry out the genetic identification of different species marketed under the commercial denominations clams, razor clams, and scallops.

The first step of the proposed methodology is the DNA extraction. The methods evaluated for this purpose worked correctly, both in fresh and in processed products. The quality and quantity of DNA obtained (evaluated by means of optical measurements) were optimal to successfully amplify the DNA by PCR in all cases. The spectrophotometric values obtained at 260 nm were higher than 100 ng/μL, the 260/280 ratio was between 1.7 and 1.9, and the 234/260 ratio was less than 0.5 in all cases. These values and ratios show a low amount of proteins and RNA in the DNA extractions.

Amplification and Sequencing of the 18S rRNA Fragment. In this work a partial region of 18S rRNA was amplified in all studied samples (**Table 1**). Previously, several authors used this genomic region to carry out several genetic studies. For instance, Kenchington et al. studied the relationship among several mussel species (*23*), Larson et al. identified six species of bivalve larvae (*24*), and Santaclara et al. designed a methodology for the genetic identification of mussel species (*11*). All of these works point out this region as a possible molecular marker for the genetic identification of bivalve species. For this reason, the primers *Myt18S* F and *Myt18S* R described by Santaclara et al. (*11*) were used in the PCR amplification of the species included in the present work, allowing us to obtain the expected PCR product in all of the studied species except for those belonging to the genera *Cerastoderma* and *Donax*. Because of that, a new reverse primer (DtCed18S) was designed for a successful PCR amplification in these problematic genera. In this manner the PCR products and DNA sequences of the species shown in **Table 1** were obtained. All of the PCR products showed a size between 872 and 965 bp (**Table 2**; **Figure 3**). The sequences were submitted to NCBI database (accession no. EU660728 to EU660825).

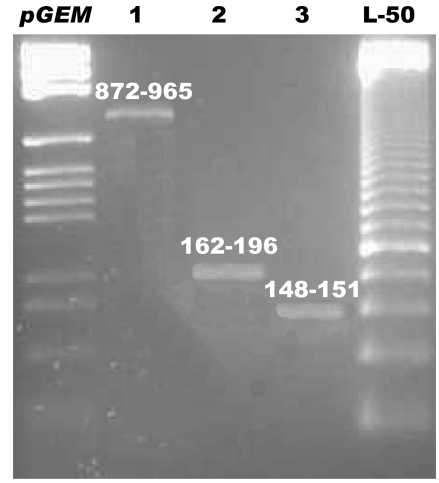

Figure 3. PCR products from bivalve samples amplified in this work (lanes 1−4). *pGEM*, molecular marker p-GEM (Promega); L-50, molecular marker ladder 50 pb (Amersham); lane 1, PCR product obtained with primer Myt18S; lane 2, PCR product of fragment 1 obtained with primer BIVALVE 1; lane 3, PCR product of fragment 2 obtained with primer BIVALVE 2Fmod/DtCed18S.

The main characteristic of this genetic marker was the absence of intraspecific variability [the intraspecific haplotype diversity (Hd) was 0] and a low interspecific variability [although the Hd was 1, there were very few nucleotide changes between species as reflected by the nucleotide diversity per site calculated from all the nucleotide sequences (Pi = 0.07788 ± 0.00582)].

From the sequences mentioned above, three internal primers were designed: BIVALVE 1 and BIVALVE 1R, which amplify a PCR product herein called fragment 1, and BIVALVE 2Fmod, which together with DtCed18S amplifies a PCR product herein called fragment 2. These fragments are located inside the fragment previously described (**Figure 2**) and were designed to make possible the identification of all species included in products undergoing thermal treatment. The DNA obtained from these commercial preparations is partly degraded in fragments of approximately 200 bp (*25*). In this sense, the primers designed in the present work are appropiate in that case because the size of the amplified PCR products varies between 148 and 196 bp (**Figure 2**).

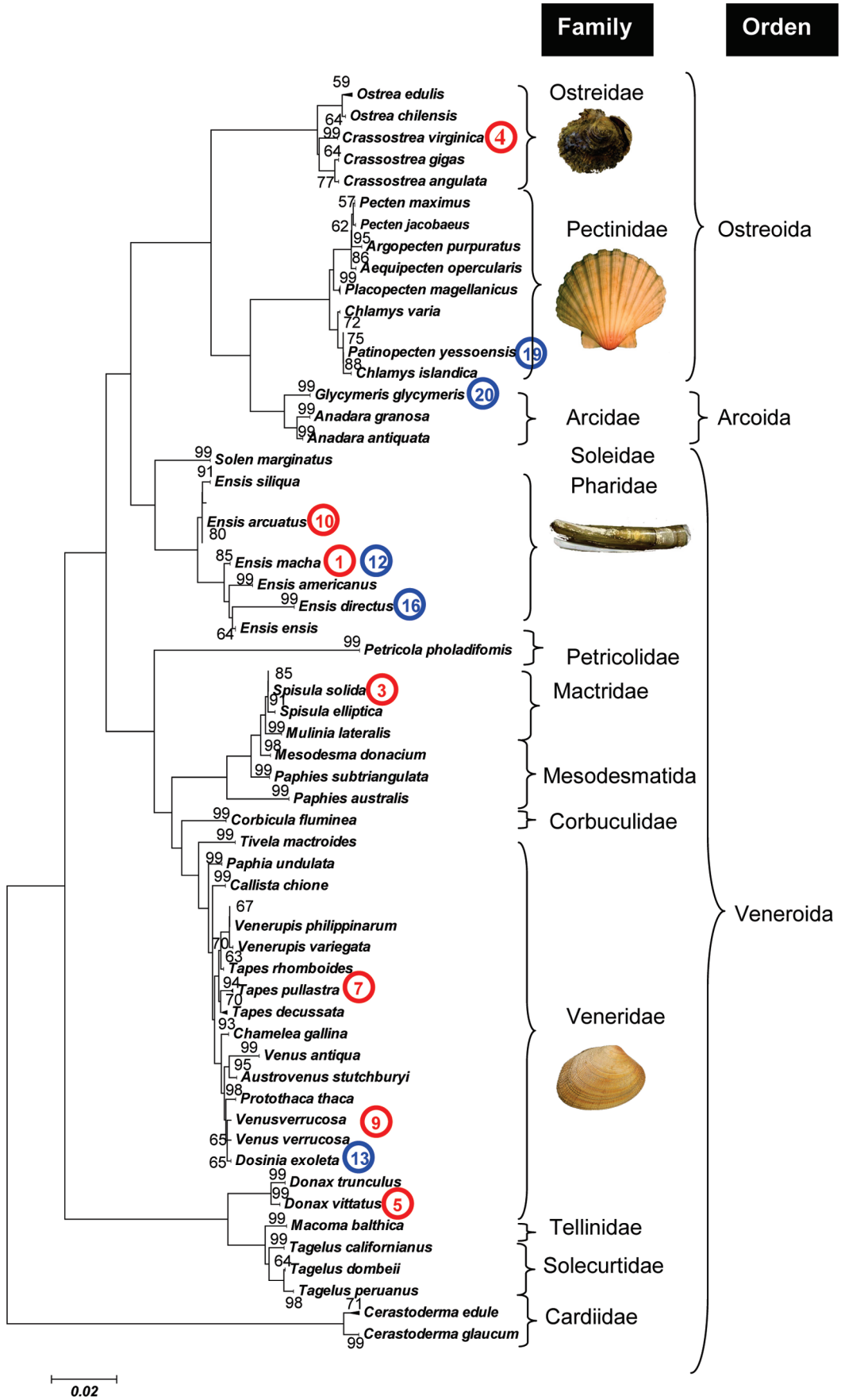

Figure 4. Phylogenetic tree of 872−965 bp fragment of 18S rRNA gene sequences of all species included in this study constructed by the neighbor-joining method. Red and blue circled numbers belong, respectively, to the fresh and frozen commercial samples analyzed for which a mislabeling was detected.

The 18S rRNA fragment selected has a high degree of conservation in all studied species, and this feature makes this marker very suitable for the genetic identification of bivalve species because interspecific variability is enough

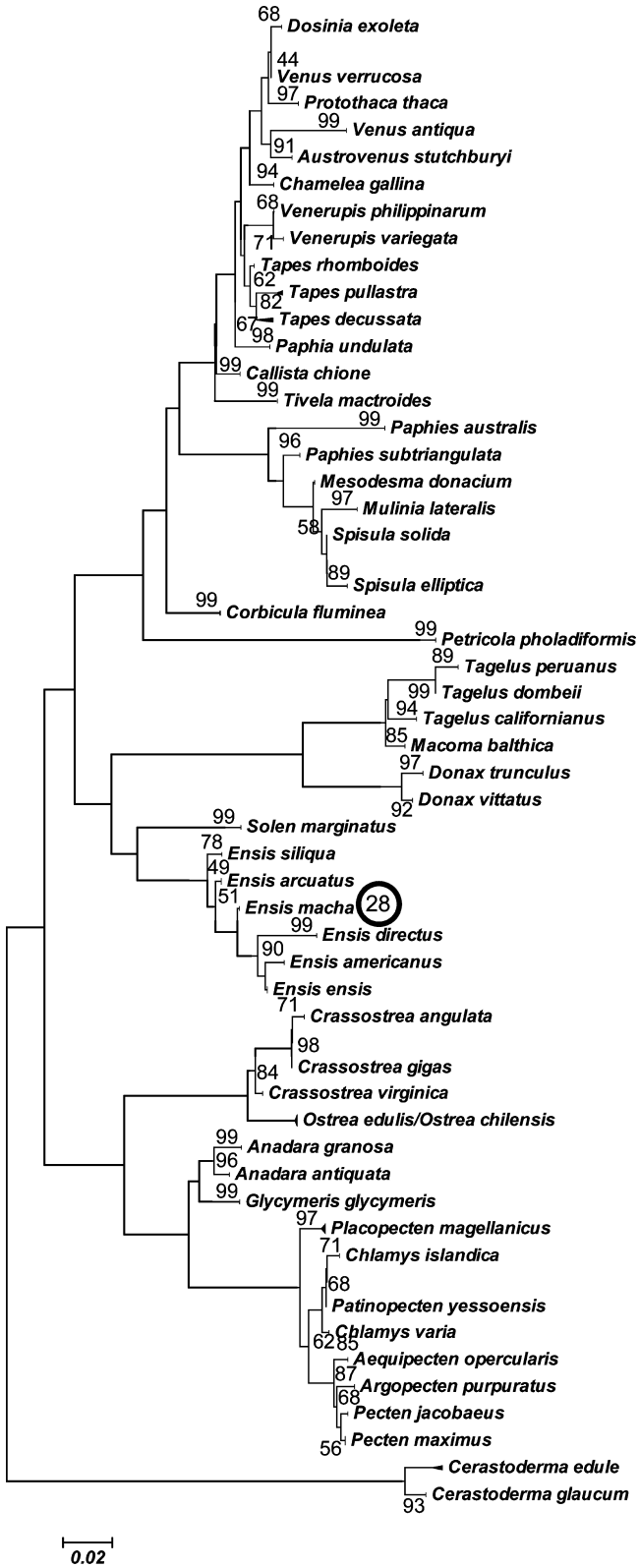

Figure 5. Phylogenetic tree of the two fragment (fragment 1 and 2) of 18S rRNA gene sequences of all species included in this study constructed by the neighbor-joining method. Circled number belongs to a canned commercial sample analyzed for which a mislabeling was detected.

to allow the genetic identification and the intraspecific one was not detected. All of these properties make possible the genetic identification of a high number of species belonging to different families and orders using only one methodology.

Table 3. Commercial Samples Analyzed with the Methods Herein Proposed that Showed an Incorrect Labeling[a]

products	species labeled	species identified
fresh	*Tapes pullatra*	*Spisula solida*
	Crassostrea gigas	*Crassostrea virginica*
	Donax trunculus	*Donax vittatus*
	Tapes decussata	*Tapes pullatra*
	Cerastoderma edule	*Venus verrucosa*
	Ensis ensis	*Ensis arcuatus*
frozen	*Ensis arcuatus*	*Ensis macha*
	Spisula solida	*Dosinia exoleta*
	Ensis ensis	*Ensis directus*
	Pecten maximus	*Patinopecten yessoensis*
	Cerastoderma edule	*Glycymeris glycymeris*
canned	*Ensis ensis*	*Ensis macha*

[a] Ten samples of each group (fresh, frozen, and canned) were analyzed.

Development of the Genetic Identification Methodology by Sequencing and Phylogenetic Analysis. In the past, a high number of DNA-based methods were purposed for the identification of some bivalve species (*1*−*11*). The main disadvantages of these previous works are that they include a low number of species and use highly conserved molecular marker combined with techniques that evaluate a small number of nucleotide positions, such as PCR-RFLP. These facts could lead to misidentifications because substitute species are not taken into account, and the restriction profiles generated in unstudied species could coincide with those obtained in the studied species. Another important disadvantage of previous methods is that these cannot be applied to highly processed products such as canned food, one of the most common formats in which bivalves are commercialized. These difficulties were overcome with the design of two new primer sets that amplify fragments 1 and 2, which have a size lower than 200 bp. Moreover, the phylogenetic analysis was selected because of the fact that this technique uses all of the nucleotide positions of a PCR product. This technique was called FINS (forensically informative nucleotide sequencing) by Bartlett and Davidson, who proposed the genetic identification of species using phylogenetic analysis of DNA sequences (*26*). This method compares sequences belonging to standard individuals and the sequence of an unknown species. The analysis includes two steps: the calculation of genetic distances between sequences obtaining a distance matrix and the construction of a phylogenetic tree.

In this work two phylogenetic analyses were performed on the following data sets: fragment 872−965 and fragment 1 + fragment 2. The intraspecific distances were 0, and interspecific ones were, respectively, 0.085 ± 0.006 and 0.159 ± 0.020.

Fragment 872−965 was proposed to analyze fresh and slightly transformed products (cooked, fried, or other similar preparations) (**Figure 4**). Fragments 1 and 2 were jointly selected to analyze samples that underwent intensive thermal treatment due to the appropriate size of these amplicons (**Figure 5**). Fragments 1 and 2 did not individually allow carrying out the genetic identification of all studied species (data not shown). For this reason, the phylogenetic analysis was performed on a matrix that contains these two fragments together (the size of these sequences is between 279 and 307 bp). Fragment 1 + 2 allows the genetic identification of all studied species except for *Ostrea edulis*/*Ostrea chilensis*, but these species are marketed mainly fresh and frozen. For this reason, these species can be identified using fragment 872−965 (**Figure 5**).

The phylogenetic trees constructed from the two data sets previously mentioned showed that individuals belonging to the

same species were grouped in the same clade. Bootstrap values were in general higher than 70 at the species level (**Figures 4** and **5**). In exceptional cases the values obtained are low to carry out a reliable assignation of species, but it was confirmed that there are one to three nucleotide differences between the species which have these low bootstrap values. These nucleotide differences were determined using the software Mega 3.0. Also, a high number of individuals across the whole geographical distribution range for each species were considered to rule out the intraspecific variability. Moreover, the high degree of conservation of the 18S rRNA reinforces the use of this molecular marker. Therefore, the differentiation of species is possible and reliable on the basis of the proposed DNA fragments.

Another technique used for the genetic identification of species is the homology search in local or Web databases. The most common database used for this purpose is the NCBI. In this database a large number of DNA sequences belonging to multiple organisms are available. The homology search tool of NCBI is called BLAST (basic local alignment search tool) (*27*) and was initially developed to find regions of similarity between sequences; nowadays BLAST is used for the genetic identification of species.

The phylogenetic assignments generated were contrasted with the results of the BLAST analysis, and both fit in the species assignation (data not shown).

Methodological Validation. The samples elaborated in the pilot plant of the Technological Center CECOPESCA were analyzed with the methodologies proposed in the present work. This task was performed to check if after the thermal process the methodology proposed is useful for the identification. The developed techniques showed a specificity of 100%, because all of the samples analyzed with the genetic tool herein developed were assigned to the species identified on the basis of morphological features.

Application to Commercial Samples. The methodology developed was applied to 30 commercial samples. The samples were classified in three different groups depending on the level of processing: fresh, frozen, and highly processed products (products underwent high temperature and/or pressure conditions). Ten samples of each group were analyzed. These analyses allowed us to assess the compliance with labeling requirements of these products. All of the analyzed samples were identified as some of the species included in this work. Twelve of them were incorrectly labeled, a percentage of 40% (**Table 3**). It was observed that the percentage of incorrect labeling was different depending on the degree of processing of the product considered. In this manner the least transformed food products have the higher percentage of incorrect labeling, because 50% of the fresh products were incorrectly labeled. This can be due to the fact that the labeling rules for processed products are stricter than those regarding fresh and frozen products.

In conclusion, the lack of methods for the genetic identification of bivalve species makes this methodology the only useful alternative for traceability or labeling studies. Mussel species were not included in this work because the methodology described by Santaclara et al. (*11*) complements that developed in the present study. Both methods allow the genetic identification of the main commercialized bivalve species, in fresh, frozen, precooked, or canned products. Also, this method could be useful to certify the authenticity of commercial products by laboratories dedicated to this type of analysis, and the shellfish industries could guarantee the identity and authenticity of the commercialized products.

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