



## Analytical Methods

## A simple one-step PCR method for the identification between European and American razor clams species

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## ABSTRACT

A specific multiplex polymerase chain reaction (PCR) was applied to differentiate samples of razor clams *Ensis arcuatus*, *Ensis siliqua*, *Ensis directus*, and *Ensis macha*. Universal primers were used for the amplification of internal transcribed spacer 1 (ITS-1) in each species. The alignment of the obtained sequences was the basis for the specific design of species-specific reverse primers (ITSArSil-R, ITSDir-R, and ITSMa-R) located in the ITS-1 region. A multiplex PCR using each specific primer together with a common forward primer allowed identification of razor clam species by means of the different sizes of the species-specific amplicons separated in an agarose gel electrophoresis. This work provides a simple, reliable and rapid protocol for the accurate identification of *Ensis* species. The present methodology can be very useful for traceability of the species and to reinforce labelling regulations.

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## 1. Introduction

Nowadays, there is an important growth in demand of quality food. Correct labelling of aquaculture products offered for sale is important in order to assure consumers of the identity and quality of the seafood they purchase. Authentication of fishery and aquaculture products in the European Community is based on the premises indicated in regulation (EC) 104/ 2000: all fishery and aquaculture products must be correctly labelled with the commercial name of the species, the production method, and the capture zone before retail. In fact, commercial fraud by involuntary or deliberate mislabelling and by substitution of expensive species by similar cheaper ones is one of the most important concerns for consumers. This type of fraud has become much easier to perform in the canning sector where most of the morphological characters necessary to recognise the species are removed. Thus, the development of analytical methods for species authentication is necessary to detect and avoid willful, as well as unintentional substitution of different species and to reinforce labelling regulations.

Razor clams are a group of infaunal bivalve mollusks that constitute a considerable component of infaunal soft-bottom communities, inhabiting fine sand, silt or sandy-mud ocean floors and

forming extensive and dense beds (Gaspar, Castro, & Monteiro, 1999). They are widely distributed from tropical to temperate areas. Among native species in Europe, *Ensis* genus is the most representative, being *Ensis arcuatus* and *Ensis siliqua* the main commercialised species. Their distribution centre is the Northeast Atlantic, spreading out from Norway to Mediterranean and Northwest of Africa (with South of Spain as southern limit for *E. arcuatus*) (Hayward & Ryland, 1998). Important fisheries exist in Spain, Portugal and Ireland being Spain the main razor clam producer in European Union with a production of 267 ton in 2007 (data provided for *Consellería de Pesca e Asuntos Marítimos, Xunta de Galicia, www.pescadegalicia.com*). Although the extraction rate is not too elevated, razor clams reach high sale prices (12.5 €/kg). There are two main market sectors for razor clams in Europe, canneries and fresh (live) market. In the last few years, another two species have been newly brought into the European markets: *Ensis directus* and *Ensis macha*. The former is a species introduced into Europe in 1978, by means of larvae in the ballast water of the ships (Luczak & Dewarumez, 1992). It was initially detected in the German coast, reaching France and the English channel in 1991 (Luczak, Dewarumez, & Es-sink, 1993). *E. macha* is a native species from South America distributed throughout the coasts of Southern Argentina and Chile and represents a valuable fishery resource (Baron, Real, Ciocco, & Re, 2006).

Due to their similarity in morphological features, razor clam species cannot be easily discriminated and this requires the presence of the shell and a detailed analysis of the muscle impressions.

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However, when specimens are small in size and once the shell is removed (as in canned products), they are generally unidentifiable. Hence, replacement with the cheaper razor clam species or mislabelling can occur, and consequently the development of a procedure for species authentication should be of the highest priority.

Genetic approaches based on DNA techniques are widely used nowadays to overcome this problem. Advantages of the use of DNA in these methodologies are: this molecule is very stable, contains more information than proteins and it is present in all tissues of all organisms. Thanks to the development of polymerase chain reaction (PCR) the molecular methods have been simplified being more easily applicable and less time-consuming. PCR amplification of species-specific fragments has been useful in fish traceability (Carrera et al., 2000; Céspedes et al., 1999) and in species identification (Asensio et al., 2001; Cross, Rebordinos, & Díaz, 2006; Rego et al., 2002).

The aim of this work was to develop an appropriate and reliable methodology for identification of five razor clams species, by means of a multiplex PCR methodology of ITS-1 region. The protocol, involving a one-step procedure, is rapid, accurate, and sensitive, enabling its use to identify razor clams in fresh and canned samples in an accurate and prompt manner.

## 2. Materials and methods

### 2.1. Materials

Samples of *E. arcuatus* and *E. siliqua* were collected by local fishermen in Rodas (Pontevedra, Spain) and Finisterre (A Coruña, Spain), respectively. Samples of *E. directus* (Denmark) were purchased from local fish suppliers and *E. macha* individuals (Chile) were supplied by Dr. Irene Lepez. All specimens were morphologically identified. A piece of foot was dissected and immediately stored in 95% ethanol until DNA extraction (less than five days). Canned and frozen products were purchased at local supermarkets.

### 2.2. DNA isolation and PCR amplification of ITS-1 region

Total genomic DNA was isolated from 25 mg of raw or processed foot sections using the protocol described by Fernández-Tajes and Méndez (2007).

Amplification of ITS-1 sequences were carried out using primer forward 5'-GTTTCCGTAGGTGAACCTG-3' designed by Heath, Rawson, and Hilbish (1995) and reverse 5'-TGTGCGTTCAAGATGTCG-3', designed in this work based on 5.8S rRNA gene sequences of several bivalve species. Amplification reactions were performed in 25 µl of reaction volume; the reaction mixture contained 15 ng of genomic DNA, 0.24 µM of each dNTP, 2 mM of MgCl<sub>2</sub>, 1 µM of each primer, 0.625 U of Taq polymerase (Roche Applied Science, Barcelona, Spain) and the buffer recommended by polymerase suppliers. The thermal cycler profile consisted of an initial denaturation step of 5 min at 95 °C, 35 cycles at 94 °C for 20 s, 56 °C for 20 s and 72 °C for 1 min, and a final extension step at 72 °C for 5 min. PCR products were visualised by electrophoresis on a 2% w/v agarose gel.

### 2.3. Purification, cloning and sequencing of PCR products

The ITS PCR product was ligated into the plasmid pCR<sup>®</sup>2.1-TOPO<sup>®</sup>, using the TOPO-TA Cloning kit (Invitrogen), and transformed into TOP10F<sup>®</sup> cells. Plasmid DNA purification was carried out using QIAprep Miniprep Kit (Qiagen, Barcelona, Spain), which employs the modified alkaline method of Birnboim and Doly (1979). Sequencing of both strands of the insert of 4–6 clones/individual was performed with an automatic capillary DNA sequencer

(CEQ<sup>™</sup>8000 Genetic Analysis System). The nucleotide sequences have been deposited in the EMBL database under accession numbers J966667-97 and AM933615-31.

### 2.4. Analysis of sequences and design of species-specific primers

Nucleotide sequences were aligned using ClustalX software (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997), and alignments were edited with GeneDoc software (Nicholas, Nicholas, & Deerfield, 1997). Interspecific differences found at ITS-1 region of razor clams were employed for designing reverse species-specific primers using Primer3 software (Rozen & Skaletsky, 2000). Selection was done in such a way that each species-specific primer with a universal primer identical to the four species (in this case the forward described above) would produce a PCR amplification product of species-specific size. The PCR mix and thermal conditions are the previously described.

### 2.5. Multiplex PCR

Once all primers were tested, multiplex PCR was performed in order to simplify the species identification. Multiplex PCR was carried out using 0.24 µM of each primer, and with identical conditions as described above.

### 2.6. Market study

In order to evaluate the usefulness of our methodology in commercial samples 12 commercial cans were acquired in shops and stores from Galicia and Spain to check the correct labelling of the analysed products.

## 3. Results and discussion

The aim of this work was to develop a reliable and efficient method for genetic differentiation between European and non-European razor clam species. This identification was achieved by a multiplex PCR-based method based on ITS-1 region. The ITS spacers show more variability than their flanking coding regions, and they can be easily amplified with universal primers (Presa, Pardo, Martínez, & Bernatchez, 2002). Fresh, frozen and canned products were analysed using this methodology.

With this aim, amplification and sequencing of ITS-1 spacer of *E. arcuatus*, *E. siliqua*, *E. directus* and *E. macha* was done. Thus, PCR products of three individuals from all species were cloned and sequenced; the complete ITS-1 sequences obtained were determined to be 566 bp in *E. arcuatus* and *E. siliqua*, 535 bp in *E. directus*, and 560 bp in *E. macha*. Although there are some small differences in the length of obtained amplicons among species, they could not be detected by agarose electrophoresis.

The alignment of the sequences obtained with those from databases showed 314 variable sites, 248 nucleotide substitutions and 66 indels in a total of 585 bp, all of them in the spacer sequence. The number of species-specific sites was 11 for *E. arcuatus*, 12 for *E. siliqua*, 89 for *E. directus* and 74 for *E. macha*. The presence of this several species-specific sites allowed us to design reverse species-specific primers for razor clams characterisation. In order to make an unequivocally identification of the species, species-specific PCR can be developed because a specific sequence can be detected very sensitively in a pool of sequences of different organisms (Rodríguez et al., 2003). Table 1 shows the designed primers. No specific primers for *E. arcuatus* and *E. siliqua* were obtained, probably due to the small number of species-specific sites in both species.

Using each specific primer together with a common forward primer (ITS-F from Heath et al., 1995), a 296 bp band was obtained

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