Food Chemistry 118 (2010) 995–998

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

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

Juan Fernández-Tajes[1], Ruth Freire[1], Josefina Méndez [*,1]

Department of Cell and Molecular Biology, Faculty of Sciences, University of A Coruña, 15071 A Coruña, Spain

ARTICLE INFO

Article history:
Received 28 February 2008
Received in revised form 15 September 2008
Accepted 21 October 2008

Keywords:
Species identification
Food authenticity
Razor clam
Multiplex PCR
Traceability
Seafood

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.

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, & Essink, 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.

* Corresponding author. Tel.: +34 981 167 000; fax: +34 981 167 065.
E-mail addresses: jfernandezt@udc.es (J. Fernández-Tajes), rfreire@udc.es (R. Freire), fina@udc.es (J. Méndez).

[1] All authors contribute equally to the present work.

0308-8146/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2008.10.043

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 $MgCl_2$, 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®2.1-TOPO®, using the TOPO-TA Cloning kit (Invitrogen), and transformed into TOP10F' 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™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

Table 1
Sequence of species-specific primers designed.

Species amplified	Primer	Sequence
Forward primer	Heath et al. (1995)	5′- GTTTCCGTAGGTGAACCTG-3′
E. arcuatus/E. siliqua	ITSArSil-R	5′-CTTCGCGTCGGCAATA-3′
E. directus	ITSDir-R	5′- GACGGAGTGCATAGTGTATAAC-3′
E. macha	ITSMa-R	5′- CGTTGTTTGTGGTAATAAGGC-3′

for *E. arcuatus*/*E. siliqua*, a 223 bp PCR fragment was amplified from *E. directus* individuals, and a 386 bp band was yielded from *E. macha* samples. Amplification of 10 individuals for each razor clam species corroborated the expected product lengths.

Since our aim was to find a simple and straightforward method we carried out a multiplex PCR amplification using the three species-specific oligonucleotides (ITSArSil-R, ITSDir-R, and ITSMa-R) in the same reaction together with the ITS1N-F. The amplification of 26 individuals of *E. arcuatus/E. siliqua*, 27 individuals of *E. directus* and 20 of *E. macha* showed that no disturbance occurred among the primers during the amplification reaction and that the specific primers to each DNA species annealed on their target sequences. Fig. 1 shows the typical amplification pattern obtained with those species-specific primers. Although in this assay *E. arcuatus* and *E. siliqua* could not be identified, this problem could be solved by means of the method described by Freire, Fernández-Tajes, and Méndez (2008). Consequently, a combined protocol that implies the multiplex PCR described here and the PCR–RFLP method of ITS-1 previously developed would allow the identification of the four razor clam species.

The results shown above were obtained from raw (fresh and frozen) samples, and from processed material (canned samples). In fresh products individuals ranging in size from 10 to 15 cm, and in manufactured products 12 commercial cans were purchased at local supermarkets from Galicia and Spain. One of the main problems to apply molecular methodologies to processed products such as canned seafood is that the sterilization procedure could induce DNA fragmentation. Specifically, the sterilization process in razor clams is performed at 112.5 °C for 75 min, what produces DNA fragments about 200–700 bp. For this reason molecular methods used to identify commercial material of razor clams should employ fragments around this size. This criterion is fulfilled for the fragments generated with our specific primers. The methodology developed in this study allowed us to verify the species included in the analysed commercial products and their correct labelling.

ITS-1 and ITS-2 spacers are useful to distinguish between related species (e.g. Fernández et al., 2001; Pérez, Vieites, & Presa, 2005). In bivalves, RFLPs of this region were used for the identification of several *Mytilus* mussels (Heath et al., 1995; Toro, 1998), Veneridae clams (Fernández et al., 2001) and four scallop species (López-Piñón, Insua, & Méndez, 2002); length amplification was used to differentiate several freshwater mussels (White, McPheron, & Stauffer, 1994) and *M. edulis* from two *Modiolus* species (Dixon, Sole-Cava, Pascoe, & Holland, 1995).

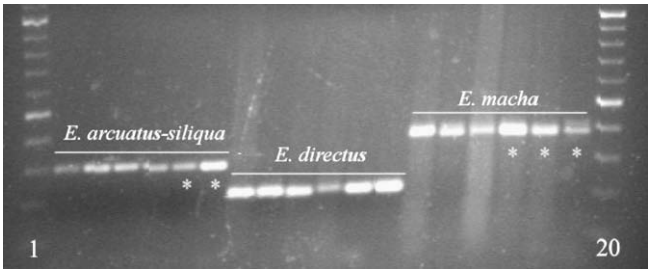

Fig. 1. ITS-1 products generated by multiplex PCR of fresh and canned (*) products with species-specific primers for *E. arcuatus/siliqua*, *E. directus* and *E. macha*. Lanes 1 and 20, 100 bp DNA ladder. Note: DNA was extracted from foot tissue sections both in canned and fresh samples.

A genetic method for the differentiation of these razor clam species, based on PCR–RFLPs of the 5S rDNA gene, was previously described (Fernández-Tajes & Méndez, 2007). Nevertheless, the organisation of ribosomal genes in multiple tandem arrays could be considered as a one of the major drawbacks in the use of these regions as genetic markers due to the intraspecific variation that could exist in the different repeats at individual level, giving rise to possible mutations and, consequently, to the loss or gain of restriction recognition sequences. However, concerted evolution tends to homogenise the repetition units present inside and among individuals of the species (Dover, Coen, & Strachan, 1982). Moreover, availability of more than one genetic marker is convenient as a technical support in the case of a legal requirement to demonstrate fraudulent substitution of species.

The results obtained in this work suggest that multiplex PCR amplification of selected ITS rDNA fragments using species-specific primers ITSArSil-R, ITSDir-R, and ITSMa-R together with ITS-F, is a powerful technique for the identification of razor clam species. Compared to alternative techniques such as direct sequencing of PCR products, PCR–RFLP or PCR–SSCP, multiplex PCR system is less consuming than these other molecular techniques, being easily performed in food inspection laboratories, especially when large numbers of samples are required to be analysed. Thus, the protocol described in this work offers an alternative tool for fraudulent species detention and for food authenticity in the market of razor clam products, as in raw as in manufactured samples.

Acknowledgements

This work was funded by a PFSHARE-90 (Sustainable HARvesing of Ensis (SHARE)) and by a TIMES (Towards a Integrated Management of Ensis stocks) grants from the European Community Initiative Programme INTERREG-IIIB "Atlantic Area" and partially funded by I3 Program 2007–08 "Programa de promoción da intensificación da actividade investigadora no sistema universitario de Galicia" financed by Xunta de Galicia. We are grateful to Mr. Jose García Gil for his technical assistance. We would also like to thank Dr. Irene Lepez for providing *E. macha* samples.

References

Asensio, L., González, I., Fernández, A., Céspedes, A., Rodríguez, M. A., Hernández, P. E., et al. (2001). Identification on nile perch (*Lates nitolicus*), grouper (*Epinephelus guaza*), and wreck fish (*Polyprion americanus*) fillets by PCR amplification of the 5S rDNA gene. *Journal of AOC International, 84*, 777–781.

Baron, P. J., Real, L. E., Ciocco, N. F., & Re, M. E. (2006). Morphometry, growth and reproduction of an Atlantic population of the razor clam *Ensis macha* (Molina, 1782). *Scientia Marina, 68*, 211–217.

Birnboim, H. C., & Dolly, J. (1979). Rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research, 7*, 1513–1523.

Carrera, E., García, T., Céspedes, A., González, I., Fernández, A., Hernández, P. E., et al. (2000). PCR-restriction fragment length polymorphism of the p53 gene as a genetic marker for identification of smoked Atlantic salmon (*Salmo salar*) and rainbow trout (*Onchorhyncus mykiss*). *Journal of AOC International, 83*, 341–346.

Céspedes, A., García, T., Carrera, E., González, I., Fernández, A., Hernández, P. E., et al. (1999). Identification of sole (*Solea solea*) and Greenland halibut (*Reinharius hippglossoides*) by PCR amplification of the 5S rDNA gene. *Journal of Agricultural and Food Chemistry, 47*, 1046–1050.

Council Regulation (EC) No 104/2000 of 17 December 1999 on the common organization of the markets in fishery and aquaculture products. (2000). *Official Journal of European Community*, *L17*, 22–52.

Cross, I., Rebordinos, L., & Díaz, E. (2006). Species identification of *Crassostrea* and *Ostrea* species by polymerase chain reaction amplification of the 5S rRNA gene. *Journal of AOC International, 89*, 145–148.

Dixon, D. R., Sole-Cava, A. M., Pascoe, P. L., & Holland, P. W. H. (1995). Periostracal adventitious hairs on spat of the mussel *Mytilus edulis*. *Journal of the Marine Biological Association of the United Kingdom, 75*, 363–372.

Dover, G., Coen, E., & Strachan, T. (1982). The dynamics of sequence turnover and concerted evolution of families of rDNA, histone genes, and non-coding DNA of *Drosophila*. *Heredity, 49*, 141.

Fernández, A., García, T., Asensio, L., Rodríguez, M. A., González, I., Hernández, P. E., et al. (2001). PCR–RFLP analysis of the internal transcribed spacer (ITS) region for identification of 3 clam species. *Food and Chemical Toxicology, 66*, 657–661.

Fernández-Tajes, J., & Méndez, J. (2007). Identification of the razor clam species *Ensis arcuatus*, *E. siliqua*, *E. directus*, *E. macha*, and *Solen marginatus* using PCR–RFLP analysis of the 5S rDNA region. *Journal of Agricultural and Food Chemistry, 55*, 7278–7282.

Freire, R., Fernández-Tajes, J., & Méndez, J. (2008). Identification of the razor clams *Ensis arcuatus* and *E. siliqua* by PCR–RFLPs analysis of ITS-1 region. *Journal of Fisheries Science*.

Gaspar, M. B., Castro, M., & Monteiro, C. C. (1999). Effect of tooth spacing and mesh size on the catch of the Portuguese clam and razor clam dredge. *ICES Journal of Marine Science, 56*, 103–110.

Hayward, P. J., & Ryland, J. S. (1998). *Handbook of the marine fauna of North–West Europe*. Oxford: Oxford University Press.

Heath, D. D., Rawson, P. D., & Hilbish, T. J. (1995). PCR-based nuclear markers identify alien blue mussel (*Mytilus* sp.) genotypes on the west coast of Canada. *Canadian Journal of Fisheries and Aquatic Sciences, 52*, 2621–2627.

López-Piñón, M. J., Insua, A., & Méndez, J. (2002). Identification of four scallop species using PCR and restriction analysis of the ribosomal DNA internal transcribed spacer region. *Marine Biotechnology, 4*, 495–502.

Luczak, C., & Dewarumez, J. M. (1992). Note on the identification of *Ensis directus* (Conrad, 1843). *Cahiers de Biologie Marine, 33*, 515–518.

Luczak, C., Dewarumez, J. M., & Essink, K. (1993). First record of the American jack knife clam *Ensis directus* on the French coast of the North Sea. *Journal of the Marine Biological Association of the United Kingdom, 73*, 233–235.

Nicholas, K. B., Nicholas, H. B., Jr., & Deerfield, D. W. (1997). Genedoc: Analysis and visualization of genetic variation. *EMBNEW News, 4*, 14.

Pérez, M., Vieites, J. M., & Presa, P. (2005). ITS-1 rDNA-based methodology to identify world-wide hake species of the genus *Merluccius*. *Journal of Agricultural and Food Chemistry, 53*, 5239–5247.

Presa, P., Pardo, B. G., Martínez, P., & Bernatchez, L. (2002). Phylogeographic congruence between mtDNA and rDNA ITS markers in Brown trout. *Molecular Biology and Evolution, 19*, 2161–2175.

Rego, I., Martínez, A., González-Tizón, A., Vieites, J., Leira, F., & Méndez, J. (2002). PCR technique for identification of mussel species. *Journal of Agricultural and Food Chemistry, 50*, 1780–1784.

Rodríguez, M. A., García, T., González, I., Asensio, L., Mayoral, B., López-Calleja, I., et al. (2003). Identification of goose, mule duck, chicken, turkey, and swine in Foie Gras by species-specific polymerase chain reaction. *Journal of Agricultural and Food Chemistry, 51*, 1524–1529.

Rozen, S., & Skaletsky, H. J. (2000). Primer3 on the WWW for general users and biologist programmers. In S. Krawetz & S. Misener (Eds.), *Bioinformatics methods and protocols: Methods in molecular biology* (pp. 365–386). Totowa, NJ: Humana Press.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The CLUSTAL_X windows interface. flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research, 25*, 4876–4882.

Toro, J. E. (1998). Molecular identification of four species of mussels from southern Chile by PCR-based nuclear markers: The potential use in studies involving planktonic surveys. *Journal of Shellfish Research, 17*, 1203–1205.

White, L. R., McPheron, B. A., & Stauffer, J. R. (1994). Identification of freshwater mussel *Glochidia* on host fishes using restriction fragment length polymorphisms. *Molecular Ecology, 3*, 183–185.