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Analytical Methods

Species authentication of octopus, cuttlefish, bobtail and bottle squids (families Octopodidae, Sepiidae and Sepiolidae) by FINS methodology in seafoods

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ABSTRACT

In the present work a molecular a method for the authentication of cephalopods products was developed, which allows the genetic identification of about 30 species belonging to the families Octopodidae, Sepiidae and Sepiolidae. This molecular system is based on the phylogenetic analysis of DNA sequences. The molecular marker studied was the *cytochrome b* gene (*cyt b*), that was amplified by PCR and subsequently sequenced. The developed methodology was validated and further applied to 20 commercial samples, detecting 6 that were incorrectly labelled (30%). Therefore, this molecular tool could be applied in questions related to correct labelling, traceability, and import control of products containing the taxonomic groups studied.

1. Introduction

The class *Cephalopoda* contains 21 families, each consisting of a large number of species, many of them of high commercial importance. Industries manufacture different products from these, which entail different transformation processes.

The great morphological similarity among different cephalopod species makes very difficult to identify them. Moreover, in many cases the raw material is commercialised at different transformation levels, impeding or making difficult the specific assignation by means of morphologically-based methods.

On the other hand, the volume of cephalopods commercialised in the world during 2004 was 3775161 t (including squids, sepias, and octopuses) (data from FISHSTAT, FAO). Most of these raw materials are destined to be consumed after a processed step, and this fact makes possible the substitution of species.

Concerning the labelling of fish products, the European Commission Regulation 104/2000 and 2065/2001 (EU, 2000, 2001) establish dispositions relative to the information of consumers about fish products and the traceability control. The EU only allows commercialising fish products if the labelling contains the appropriate information about the commercial denomination and scientific name of the species.

All of these factors point out the need for analytical methods that allow the determination of the authenticity of the raw materials included in commercial products.

Numerous molecular phylogenetic (Anderson, 2000a, 2000b; Bonnaud, Boucherrodoni, et al., 1994; Bonnaud, BoucherRodoni, et al., 1997; Herke & Foltz, 2002; Ito, Yanagimoto, et al., 2006; Piertney, Hudelot, et al., 2003; Zheng, Yang, et al., 2004) and ecological studies exist (Collins, Boyle, et al., 1999; Collins, Pierce, et al., 1997; Moreno, Pereira, et al., 2002). Their main disadvantage being the low number of species studied. No previous studies exist about the genetic identification of octopus, cuttlefish, bobtail and bottle squid species. It is worth highlighting the work of Santaclara, Espiñeira, et al. (2007), who developed a system to identify more than 20 species belonging to the families Ommastrephidae and Loliginidae by PCR-RFLP and FINS methodologies, but did not study the families Octopodidae, Sepiidae and Sepiolidae. In the present study, these taxonomic groups were studied, allowing the development of a genetic method to identify most important species of octopus, cuttlefish, bobtail and bottle squid. This method was applied to different products, from fresh to canned products elaborated in the pilot plant, using as raw material genuine specimens previously characterised, based on their morphological traits. Due to the different commercial value of the species belonging to these families, substitutions between species in seafood products can take place. Thus, the methodology herein developed can be very useful in the normative control of those products, particularly in the authenticity of imported species, the verification of the

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traceability of different fishing batches along the commercial chain, the correct labelling and the protection of consumers.

2. Materials and methods

2.1. Sampling and DNA extraction

Authentic cephalopod samples were collected and provided by Universities and Marine Research Centres from different locations around the world. The number of samples by species ranged from 2 to 10 (Table 1 and Fig. 1). These samples were preserved in 70% ethanol at −80 °C until being processed for DNA extraction. Some commercial samples were provided by importer industries, and others were obtained in local markets. When it was possible, the identification of the specimens was carried out on the basis of morphological characteristics according to different bibliographic references (Roper & Seewney, 1984; Guerra, 1992; Norman, 2000).

Total Genomic DNA from all the samples previously described (Table 1) was extracted from a piece of 30 mg of muscle tissue according to the standard CTAB phenol–chloroform protocol described by Roger and Bendich with slight modifications (Roger & Bendich, 1988). In the case of the products elaborated by us to validate the method and others purchased in the local market, DNA was extracted from a piece of 30–200 mg of tissue. Moreover, when the cephalopods were covered with different sauces, the extracted DNA was cleaned using the kit NucleoSpin Extract II (Macherey–Nagel, Inc., Bethlehem PA, USA) according to the protocol of the manufacturer.

The extracted DNA was visualised on agarose gels (Sigma Aldrich, Saint Louis Mo, USA) at 1% in TBE buffer with 5 μg/mL of ethidium bromide (Sigma Aldrich, Saint Louis Mo, USA) under ultraviolet light using a Molecular Imager Gel Doc XR System transiluminator and the software Quantity One v 4.5.2 (Bio-Rad, Hercules, CA, USA).

The purity and quantity of the DNA obtained was measured with a spectrophotometer at 260 nm (Eppendorf Biophotometer, Germany). The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. The quality of the DNA samples was determined by means of indicators for protein and RNA contamination, calculating the ratio of absorbance at A260/A280 and A234/A260 (Winfrey, 1997).

Table 1
Samples included in this work and the location of collection.[a]

Family	Species	Samples	Location
Octopodidae	*Octopus vulgaris*	18	SPA, ZAF, JAP, MOR, SEN, PHI, POR, EGY
	Octopus defilippi	3	MOR, EGY
	Octopus dollfusi	2	VIE, CHN
	Octopus fangsiao	2	JAP, CHN
	Octopus aegina	3	JAP, CHN
	Octopus macropus	2	EGY, SPA
	Octopus maya	2	MEX
	Octopus membranaceus	2	EGY, CHN
	Eledone cirrhosa	3	SPA, IRISH Sea
	Eledone moschata	2	GBR, POR
	Bathypolypus pugniger	1	CAN
	Bathypolypus bairdii	1	CAN
	Enteroctopus dofleini	2	JAP, USA
Sepiidae	*Sepia officinalis*	7	SEN, SPA, North Sea
	Sepia orbignyana	3	SPA, GRE
	Sepia berthelothi	3	SEN, MAU
	Sepia hierredda	3	SEN, MAU
	Sepia aculeata	2	CHN, JAP
	Sepia elegans	4	FRA, SPA, ITA
	Sepia esculenta	2	JAP
	Sepia lycidas	2	JAP
	Sepia apama	2	AUS
	Sepia dollfusi	2	EGY
	Sepia pharaonis	2	EGY, IND
Sepiolidae	*Inioteuthis japonica*	2	JAP, CHN
	Sepiola atlantica	5	NOR, BEL, North Sea
	Sepiola rondeletii	2	MOR, GRE
	Rossia palpebrosa	2	CAN, USA
Loliginidae	*Uroteuthis duvauceli*	3	EGY
	Lolliguncula diomedeae	2	ECU
Architeuthidae	*Architeuthis dux*	2	IRL
Gonatidae	*Gonatus fabricii*	2	NOR

[a] Location abbreviations: AUS, Australia; CAN, Canada; CHN, China; ECU, Ecuador; EGY, Egypt; BEL, Belgium; GRE, Greece; FRA, France; ITA, Italy; IRL, Ireland; JAP, Japan; MAU, Mauritania; MEX, Mexico; MOR, Morocco; NOR, Norway; PHI, Philippines; POR, Portugal; SEN, Senegal; SPA, Spain; USA, United States; VIE, Vietnam; ZAF, South Africa.

2.2. Amplification and sequencing of the PCR products

The primers CEF H (5′ TTA TGG KTG RGT RYT DCG TTA T 3′) and CEF L (5′ TAC HCC YCC WAR TTT WYT AGG AAT 3′) designed by Santaclara et al. (2007) were used for the PCR amplification. In the case of processed products' analysis used for the validation and application to commercial products an internal fragment was amplified using the primers CEF H and H15149AD as previously described (Santaclara et al., 2007).

The amplifications were carried out in a final volume of 50 μL containing 100 ng of DNA template, 5 μL of 10X 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 T*aq*-polymerase (Bioline, London UK). PCR of the samples that underwent thermal treatment was carried out with 400 ng of DNA. PCR was performed in Bio-Rad® MyCycler™ thermocycler (Bio-Rad, Hercules, CA, USA). The cycles program was the following: a preheating step of 3 min at 95 °C, then 35 cycles of 30 s at 95 °C, 1 min at 50 °C, 1 min and 30 s at 72 °C, and a final extension step of 7 min at 72 °C.

The PCR amplifications in the processed products were carried out in the following conditions: a preheating step of 94 °C for 5 min was followed by 35 cycles of amplification (94 °C for 20 s 52 °C for 20 s and 72 °C for 20 s) and a final extension step of 7 min at 72 °C.

To verify the proper working of PCR amplification, PCR products were loaded on agarose gels (Sigma Aldrich, Saint Louis Mo, USA) at 2% in TBE buffer and 5 μg/mL of ethidium bromide to allow band detection. The size of the amplified fragments was estimated from the molecular marker 100–1500 bp (Dominion-MBL, Córdoba, Spain) and a 50 bp ladder (GE HealthCare, NJ, USA).

PCR products were cleaned before the sequencing reaction using NucleoSpin Extract II (Macherey–Nagel, Inc., Bethlehem PA, USA) according to the protocol of the manufacturer.

Both DNA strands were sequenced on the CEQ 8800 Genetic Analysis System (Beckman Coulter, Fullerton CA), with the primers described previously (CEF H, CEF L, and H15149AD) and using CEQ Dye Terminator cycle sequencing Quick Start kit (Beckman Coulter, Fullerton, CA) according to the recommendations of the manufacturer. Nucleotide sequences obtained were corrected with Chromas 1.45 (Mc Carthy, 1996) and subsequently aligned with Clustal W, available within the program pack BioEdit 7.0 (Hall, 1999). From this alignment, a polymorphism analysis was carried out using DnaSP 4.0 (Rozas, Sánchez-DelBarrio, et al., 2003).