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Molecular differentiation of the species of two squid families (*Loliginidae* and *Ommastrephidae*) based on a PCR study of the 5S rDNA gene

João Bráullio de Luna Sales [a,*], Luis Fernando da Silva Rodrigues-Filho [a], Manuel Haimovici [b], Iracilda Sampaio [a], Horacio Schneider [a]

[a] Universidade Federal do Pará, IECOS, Laboratório de Genética e Biologia Molecular. Alameda Leandro Ribeiro s/n, Bairro Aldeia, Bragança/PA, CEP 68600-000, Brazil
[b] Universidade Federal do Rio Grande (FURG), Laboratório de Recursos Demersais e Cefalópodes, Caixa Postal 474, Rio Grande/RS, CEP: 96201-900, Brazil

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ABSTRACT

The present study aimed to demonstrate the effectiveness of the 5s rDNA gene for the identification of commercially-valuable species of cephalopod belonging to the families *Loliginidae* and *Ommastrephidae*. Our results demonstrate distinct banding patterns in each of the six species sampled (*Loligo surinamensis*; *Loligo sanpaulensis*; *Lolliguncula brevis*; *Sepiotheuthis sepioidea*; *Ornithoteuthis antillarum*; *Illex argentinus*), as well as diagnostic traits at the genus and probably family levels. The results emphasize the efficiency of the 5s rDNA marker as a low-cost and rapid forensic technique, which not only permits the identification of species, but also differentiation of members of the *Loliginidae* and *Ommastrephidae*.

1. Introduction

Cephalopods represent an important fishery resource, corresponding to 3.6% of the total marine tonnage landed worldwide in 2001. Squid – including both coastal and oceanic species – make up around 70% of the cephalopod catch (FAO, 2000). Much of the catch is sold in processed form, which involves removal of the intestines and skin, the separation of the head, arms, tentacles and fins, and in the case of squid, the slicing of the body into rings (calamari). This processing obviously hampers the identification of the species, or even the genus, in some cases (Chapela, Sotelo, & Pérez-Martin, 2003).

This practice also facilitates the substitution of more expensive species (such as those of the family *Loliginidae*) by cheaper ones, generally members of the family *Ommastrephidae* (Chapela et al. 2002). Even considering only the ommastrephids, many species are marketed as higher-quality, more expensive ones, especially in Europe, the principal market. Some of the morphological traits used for the identification of cephalopod species are influenced by sexual maturity, age and gender, while others, such as the hectocotylus, are found only in mature males (Roper & Mangold, 1998).

Recent advances in molecular techniques have greatly enhanced the identification of mollusk species, in particular the cephalopods (Brierly & Thorpe, 1994; Colombo et al., 2002; Santaclara, Espiñeira & Vieites, 2007; Leite, Haimovici, Molina & Warnke, 2008; Warnke, Soller, Blohm & Saint-Paul, 2000). The majority of studies have involved the identification of processed fishery products, based on comparative analyses of fragment length of mitochondrial DNA or nuclear genes, in particular 5S rDNA (Carrera et al., 1999; Céspedes et al., 1999; Cocolin, D'Agaro, Manzano, Lanari & Comi, 2000; Pinhal et al., 2008; Quinteiro et al., 1998; Russel et al., 2000; Wolf, Rentsch & Hübner, 1999).

The 5S ribosomal rDNA is a small molecule forming part of the large subunit of the ribosome. In eukaryotic species it is typically encoded by hundreds to thousands of gene copies located in large arrays of tandem repeats (5S rDNA) at one or more chromosomal sites (Long & Dawid, 1980). The repetitions of the 5S rDNA gene consist of highly-conserved coding sequences of 120 base pairs, which are separated from one another by variable non-transcribed spacers, or NTSs (Long & Dawid, 1980; Wasko, Martins, Wright & Galetti-Jr., 2001). This region is considered to be especially appropriate for PCR-based genetic studies, due to a number of considerations, which include: the gene is highly conserved, even in distantly-related species, allowing the isolations of its repetitions in different species using PCR analysis; given their relatively small size and tandem arrangement, the repetitions may be isolated from DNA of reduced quality (Martins & Wasko, 2004); The 5S rDNA gene copies are organized in tandem array and dispersed all over genome been distributed in different chromosomes (Martins & Wasko, 2004).

Species identification using 5S rDNA is based on the amplification of fragments containing the gene together with non-transcribed

* Corresponding author. Tel.: +55 91 8136 0661.
E-mail address: jbraullio@yahoo.com.br (J. Bráullio de Luna Sales).

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spacers (NTSs). The variation found in the fragments amplified is due to the presence of these NTSs (Martins & Wasko, 2004), which evolve relatively rapidly, with substitution rates comparable to those of pseudogenes or nonfunctional sequences (Li, Luo, &Wu, 1985; Pasolini, Costagliola, Rocco & Tinti, 2006).

Other authors have argued that the interspecific variation in the length of NTSs may also be caused by the presence of insertions and/or deletions, mini repetitive sequences, or even pseudogenes themselves (Sadjak, Reed & Phillips, 1998).

The usefulness of the 5S rDNA gene as a species-specific marker has been assessed for a number of different groups of fishes (Asensio et al., 2001; Imsiridou, Minos, Katsares, Karaiskou & Tsiora, 2007; Karaiskou, Triantafyllidis & Triantaphyllidis, 2003; Pinhal et al., 2008). This marker has not yet been evaluated in the cephalopods, however, which not only present highly-conserved characteristics in some species, but are also often marketed as a processed product, which hinders species identification. Given this, the present study aimed to demonstrate, for the first time, the efficiency of 5S rDNA for the identification of the cephalopod species of two commercially-important families (*Loliginidae* and *Ommastrephidae*).

2. Materials and methods

2.1. Collection of samples and DNA extraction

Tissue samples were collected from six squid species. (Table 1).

Five species were collected locally in northern Brazil, where they were caught as by-catch by trawlers fishing. The sixth species, *Illex argentinus*, was obtained from the local supermarket as a whole frozen specimen and some samples were collected by trawlers fishing in RS state. The samples were preserved in 100% ethanol and stored in a −4 °C freezer until analysis. For extraction, the tissue was first washed, and then centrifuged twice in 600 μl of distilled water. The DNA was then extracted using Sambrook and Russell, (2001) standard phenol/chloroform protocol.

Table 1
Squid families and number of species sampled for forensic analysis in the present study.

Family	Species	Common Name	Origin	Number of samples analyzed in the present study
Loliginidae	*Loligo sanpaulensis* Brakoniecki, 1984	São Paulo squid	Rio Grande, Rio Grande do Sul	25
Loliginidae	*Loligo surinamensis* Voss, 1974	Surinan squid	Bragança, Pará	4
Loliginidae	*Lolliguncula brevis* Blainville, 1823	Western Atlantic brief squid	Caravelas, Bahia	4
Loliginidae	*Sepioteuthis sepioidea* Blainville, 1823	Caribbean reef squid	Barra Grande, Bahia	1
Ommastrephidae	*Ornithoteuthis antillarum* Adan, 1957	Atlantic bird squid	Bragança, Pará	1
Ommastrephidae	*Illex argentinus*, Castellanos, 1960	Argentine sort fin squid	Florianópolis, Santa Catarina and Rio Grande, Rio Grande do Sul respectively	25/5

2.2. Polymerase chain reaction (PCR)

The repetitions of the 5S rDNA gene were amplified using the following primers: 5SA (5′-TACGCCCGATCTCGTCCGATC-3′) and 5SB (5′-CAGGCTGGTATGGCCGTAAGC-3′) (Pendás, Móran, Freije, & Garcia-Vazquez, 1994). Each PCR reaction consisted of a mixture of 0.5 μL of each primer (5SA = 5 pmol/μL, 5SB = 5 pmol/μL), 1 μL of $MgCl_2$ (50 mM), 4 μL of the dNTP mixture (1.25 mM), 2.5 μL of the 10x buffer (Invitrogen, Carlsbad-CA USA − Tris−HCl and KCl, pH 7.8), 0.2 μL of *Taq* polymerase (5 U/μL: Invitrogen), approximately 100 ng of total DNA, and purified water to complete the final volume of 25 μL. The amplification protocol was 4 min at 95 °C for denaturation; 35 cycles of 20 s at 95 °C, followed for 1 min at 55 °C for anneling, 30 s at 72 °C for extension, and a final extension at 72 °C for 7 min.

2.3. Electrophoresis

The PCR products were stained with 0.5 μL ethidium bromide and electrophoresed in 1% agarose gel (Agarose D-1 Low EEO, CONDA Laboratories, Madrid-Spain), using the following conditions: $V = 50$, $mA = 110$, and $W = 200$ for 60 min. The gel was subsequently verified and photodocumented in a Vilber Lourmat UV transilluminator coupled to a Vilber Lourmat digital camera with a 51 mm lens (Vilber Lourmat, Marne-la-Valée-France). A 1 kb Plus DNA ladder (Life Technologies, Inc. Carlsbad-CA USA) was used to estimate the width of each band observed during electrophoresis.

3. Results and discussion

The present study confirmed the use of the 5S rDNA gene for identification of species. The repetition pattern of the 5S rDNA gene provided the basis for the identification of six species of squid, using only a single gel run. Similar levels of precision have been recorded in studies which have identified subspecies of mice, species of fish, and plants (Céspedes et al., 1999; Linder, Moore & Jackson, 2000; Suzuki, Moriwaki & Sakurai, 1994). The amplification of the repetitive 5S rDNA sequences generated distinct patterns among species, but no intraspecific variation was found in the present study (data not shown) even when the same species was collected from different localities as in the case of *Illex argentines*. This pattern has also been observed in a number of other studies that used the same marker (Martins & Galetti-Jr., 2001; Martins & Wasko, 2004; Martins et al., 2002; Karaiskou et al., 2003; Pinhal, Araki, Gadig, & Martins, 2009).

In the species analyzed here, fragments varied in size from 200 to 1000 base pairs (bps) (Fig. 1). *Loligo surinamensis* presented five bands, one with 1000 bps, one with more than 650 bps, one with approximately 450 bps, a band with only 200 bps and finally a band with 150 bps. A similar configuration was observed in *Loligo*

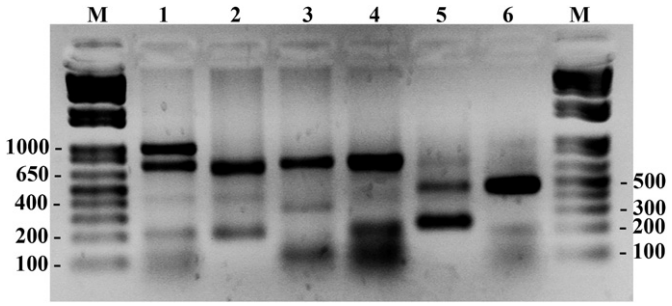

Fig. 1. The PCR analysis of the 5s rDNA gene of the six cephalopod species sampled in the present study. 1- *Loligo surinamensis*; 2-*Loligo sanpaulensis*; 3-*Lolliguncula brevis*; 4-*Sepiotheuthis sepioidea*; 5- *Ornithoteuthis antillarum*; 6- *Illex argentinus*.

sanpaulensis, except for the absence of the largest band (Fig. 1). *Lolliguncula brevis* presents three bands, with 150, 450 and one with >650 bps, while the fourth loliginid, *S. sepiodea*, presented three bands of approximately 150 bps, 200 bps, and >650 bps. The banding pattern was quite distinct in the two species of the family *Ommastrephidae*. Both species presented three bands but the patterns are distinct between them. *Ornithoteuthis antillarum* presented three bands of approximately 250, 500, and 650 bps, whereas *I. argentinus* presented three bands of approximately 100, 200 and 500 bps.

The banding pattern was not only effective for distinguishing species, but also apparently for wider taxonomic groups, i.e. families and genera, based on the number and length of fragments (Fig. 1). In the case of the *Loliginidae*, the >650 bps fragment observed in all four species appears to be diagnostic of the family, whereas differences in the two smallest bands were sufficient to differentiate the three genera. By contrast, the 500 bps band was apparently characteristic of the *Ommastrephidae* family. However, for confirmation of the observed pattern in the present study, the number of samples belonging to other genera and families must be increased.

While the 5S rDNA gene has been used increasingly for the identification of species in recent years, its application to the study of mollusks has been limited primarily to bivalves (Cross, Rebordinos & Diaz 2006; Fang, De Baere, Vandenberghe & De Watcher, 1982). The present study was the first to apply the gene to the identification of cephalopods, and has proved that it can be a precise tool for the identification of species. A characteristic pattern is also noted between genera and families, but unfortunately the number of species used is still not sufficient to confirm these results. The marker clearly has enormous potential for eventual forensic studies of cephalopods, although it would be necessary to analyze additional species, including both those of commercial value and those which are not the direct target of fisheries but which could be used fraudulently.

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