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Rapid identification of crustacean species by pcr amplification of the ribosomal internal transcribed spacer region (*Its-1*)

Abstract

The internal transcribed spacer 1 region (*ITS*-1) is located between the 18S and 5.8SrDNA nuclear genes and although it has been applied as a molecular marker for genetic identification, phylogeny and population studies in different organisms, it was only begun to be used in crustaceans in the last 15 years. The shrimp (*Pleoticus muelleri*) and the crab (*Lithodes santolla*) are two natural resources in Argentina of high commercial value with great demand in international markets (whole or processed). The Benthic Crustaceans INIDEP´s Subprogram has initiated studies to promote artisanal and coastal fishing of the swimmer crab, *Ovalipes trimaculatus* (De Haan 1833), whose commercialization is based on cooked crab claws and pulp. In this framework, *ITS-1* assays were carried out to identify crustaceans species and provide a tool that could be used as a first approximation to authentication of fishery products. Results showed that the amplification of *ITS-1* has been effective in distinguishing crabs and swimmer crabs with simple visualization of their molecular weight band, 480pb and 650bp, respectively. No amplification was achieved in shrimp under the same PCR conditions. The distinction of both species allowed, in addition, saving time and inputs as it did not need further treatment with restriction enzymes. This trial was the first attempt to authenticate fishery products and its optimization in other argentine crustaceans would serve as a tool for future traceability.

Keywords: crustacean, PCR, *ITS*, crabs, identification, traceability

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Introduction

In eukaryotes, the first internal transcribed spacer 1 region (*ITS-1*) is located between the 18S and 5.8S rDNA nuclear genes and although it has been applied as a molecular marker for genetic identification,[1–3] phylogeny[4,5] and population studies in different organisms,[6–9] it began to be used only in the last 15years in crustaceans.[2,6,10,11] All of these studies have shown that *ITS-1* is a robust molecular marker for the identification of organisms at the genus-species level and its popularity is due to the use of highly conserved primers; it is very variable and has a fast evolution rate.[12] The shrimp (*Pleoticus muelleri*) and the crab (*Lithodes santolla*) are two natural resources in Argentina of high commercial value. The pleasant food texture and flavor of these species are features that have made them the target of great demand in international markets. Exportation of these fisheries (as whole or meat) in 2016 corresponded to 160.742T and 1899T, respectively, mainly stationed to Spain, China, the United States, Japan, Vietnam and Italy, among others.[13] On the other hand, in the Benthic Crustaceans Subprogram of INIDEP, studies were started to promote the artisanal and coastal fishing of the swimmer crab, *Ovalipes trimaculatus*,[14,15] due to its high quality meat and excellent acceptance for affordable market prices. Its commercialization is based on cooked crab claws and pulp.[16] It is necessary to develop analytical methods for the identification of species in order to detect and avoid unintentional or deliberate substitutions of fishery products, particularly when the product is not marketed as whole but manufactured or canned, losing in this process the morphological characteristics that it distinguishes (eg, exoskeleton).[17] For this reason it was decided to carry out tests with the *ITS-1* as molecular marker to identify species with the aim of providing a tool that serves, not only to distinguish crustacean species, but also to be used as a first approach to authentication of fishery products or traceability.

Materials and methods

The CTAB technique, according to Milligan's protocol 5[18] and phenol: chloroform: isoamyl alcohol method, according to Sam brook et al.[19] were used for the extraction of total genomic DNA from shrimp, crab and swimmer crab muscles. The extracted DNA was resuspended in 100µL of ultrapure water and stored in freezer until use. Dilutions were standardized at 1:20 to obtain a working concentration of 15ng DNA /µL. The PCR reaction mixture (15µL total volume) contained, in final concentration, 0.5un*its* of Go Taq DNA polymerase (Promega), 1.2mM $MgCl_2$, 0.3µM of each primer, 200µM each dNTP, and 15ng of genomic DNA. PCR amplification was performed in a temperature gradient thermo cycler programmed with a first denaturation step of 1min 30s at 94°C, 33 cycles of 20s at 94°C, 30s at variable temperature from 56° to 60° and 30s at 72°C. At the end of these cycles, a final extension step was performed at 72°C for 5min (to complete any partial amplification) and a final cooling at 6°C. Negative controls were included in each amplification assay. The amplified product was electrophoresed with 0.5 X TBE as buffer medium at 60mA on a 1.5% agarose gel with ethidium bromide (0.5mg / mL). Gels were visualized and photographed under ultraviolet light and a 1Kb molecular weight marker (Invitrogen) was used as a band size reference. The SP-1-58 (5'-CAC ACC GCC CGT CGC TAC TA-3 ') which is located in the 18S gene and SP-1-38 (5'-ATT TAG CTG CGG TCT TCA TC-3 '), located in 5.8SrDNA,[6] were used as primers for *ITS-1*. The crab, shrimp and swimmer crab specimens used belong to the Molecular Biology and Microbiology

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Lab collection of different research campaigns carried out by the Crustaceans Fisheries Program.

Results and discussion

In this work the swimmer crab could be distinguish from the king crab *L. Santolla* simply by *ITS-1* fragment size. This fact is not usual in the *ITS-1* amplification that normally exhibits a single band for all the species studied and must be differentiated from each other after a subsequent treatment of the product with restriction enzymes.[2,20] In the crab *Ovalipes trimaculatus*, PCR´s product band was approximately 650bp while for *L. santolla* was 480bp. There was no amplification band corresponding to shrimp (Figure 1). The same PCR conditions were repeated with other different dilutions and undiluted shrimp DNA, but with the same result, no amplification. Assays are now continued with this species as it is known that the same primers have worked in other shrimp species such as *Penaeus japonicus*, showing bands of 500 to 515bp.[6] This same molecular marker is currently been assayed in other Lithodidae such as the false southern king crab, *Paralomis granulosa* and in canned or manufactured products of the studied crustacean species.

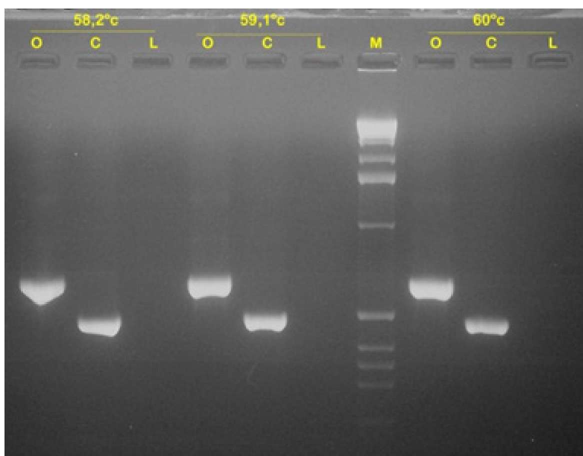

Figure 1 *ITS-1* amplification in swimmer crab *Ovalipes trimaculatus* (O), crab *Lithodes santolla* (C) and shrimp *Pleoticus muelleri* (L) with temperature gradient. (M), 1kb molecular weight marker.

Conclusion

i. Amplification of the ribosomal DNA internal transcribed spacer 1 region (*ITS-1*) has been effective in crab (*Lithodes santolla*) and swimmer crab (*Ovalipes trimaculatus*) but not in shrimp (*Pleoticus muelleri*), with the same PCR conditions.

ii. The successful amplification of the *ITS-1* allowed the distinction between two genera of decapods such as the swimmer crab (*Ovalipes trimaculatus*) and the crab (*Lithodes santolla*), with bands of different molecular weight, 650pb and 480pb, for each species. The exact length of each region will soon be confirmed by sequencing.

iii. The distinction of both species with rapid and simple visualization of its molecular weight also allowed saving time and inputs as no subsequent treatment with restriction enzymes was needed.

iv. This test constitutes the first approach to the authentication of fishery products in argentine crustaceans and would serve as a molecular tool for future traceability.

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None.

Conflict of interest

Author declares that there is no conflict of interest.

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