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PRELIMINARY RESULTS ON RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) MARKERS FOR *Callinectes sapidus* AND *C. danae*.

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ABSTRACT

Preliminary results on random amplified polymorphic DNA (RAPD) markers for *Callinectes* (Crustacea, Portunidae) species are presented in this paper. Six ten-base-pair primers were tested in *C. sapidus* and *C. danae* with the aim to find the best conditions of possible RAPDs markers suitable for the discrimination of *Callinectes* species and for future studies on population genetics. Two primers (P1 and P5) were not scorable. Primer P2 was conservative within species and therefore was useful for the discrimination of *C. sapidus* and *C. danae*. A phenetic similarity of 0.250 between both species was calculated according to Nei & Li's (1985) formulation. Primer P3 was successfully resolved only for *C. sapidus*, showing a conservative pattern among individuals. Primer P6 was studied only for *C. sapidus* and was the only variable one, showing 13 bands with different frequencies. It was discussed the use of these RAPDs primers for the discrimination of *Callinectes* species and for future studies on population genetics. It was also recognised the need to increase the number of individuals compared for these primers and the need to test other primers.

Keywords: Portunidae, *Callinectes*, Molecular Markers, RAPDs.

INTRODUCTION

The genus *Callinectes* is well known in all America, because most species of the genus constitute important commercial resources. In the South of Brazil, *Callinectes sapidus* Rathbun, 1896 and *Callinectes danae* Smith, 1869, are the most abundant species. While *C. sapidus* is dominant in the southernmost of Brazil (Rio Grande do Sul), *C. danae* gets more frequent than *C. sapidus* in the State of Santa Catarina and further north. Although both species show morphological characters that allow to distinguish them [e.g. extra-long male gonopods, two-teethed rostrum and white-bluish chelipods in *C. sapidus*; long male gonopods, four-teethed rostrum and violet-purple chelipods in *C. danae*], most fishermen are unable to discriminate them. In the South of Brazil, they are mainly exploited as by-catch of shrimps of the genus *Penaeus* (Viera *et al*, 1996) with the exception of the illegal catch of blue crabs during the reproduction period of *C. sapidus*, which aims the illegal sale of ovigerous females.

The importance of *C. sapidus* and *C. danae* in the South of Brazil is not only due to their artisanal fishery, but also because of their strong influence in the structure of the benthic communities of estuarine and mangrove systems (Hines *et al,* 1987; Martins *et al*, 1989; Fitz & Wiegert, 1990; Garcia *et al*, 1996).

The random amplified polymorphic DNA (RAPD) technique (Williams *et al*. 1990, Welsh & McClelland 1990; Cushwa & Medrano, 1996) chosen for this study, has been widely used in the last years to assess intraspecific genetic variation on a nuclear level

in a number of different organisms such as bacteria (Welsh & McClelland, 1990), aphids (Black *et al.*, 1992), bees (Suaze *et al.*, 1998), snails (Stothard *et al.*, 1997), prawns (Tassanakajon *et al.*, 1997; Meruane *et al.*, 1997), anomuran (D'Amato & Corach, 1997) and fishes (Bielawski & Pumo, 1997; Mamuris *et al.*, 1998). This technique utilises a single short primer of arbitrary sequence to amplify random segments of genomic DNA using the Polymerase Chain Reaction (PCR). This method allows the examination of genome variation without previous knowledge of DNA sequences and is less expensive and time consuming than RFLP, Microsatellite and DNA sequence analysis.

The aim of this work was to adjust conditions for some RAPDs primers, that might be useful as molecular markers for future studies on the population genetics of blue crabs and for the genetic identification of these species.

MATERIALS AND METHODS

Callinectes specimens from two species [*Callinectes sapidus* (N=80) and *C. danae* (N=80)] were collected using beam trawl nets inside the Patos Lagoon estuary. Only mature crabs were selected for analysis. After settling up conditions, five individuals of each species were used for comparisons with primer P2; six individuals of *C. sapidus* were used for primers P3 and P4; and nine individuals of *C. sapidus* for primer P6.

Total DNA was extracted according to Sambrook et al (1989) after modifications. About 100 mg of muscle tissue from each specimen were ground with glass pestles in 1.5 ml microcentrifuge tubes containing 500 µl of extraction buffer (10mM Tris-HCl, pH 8.0; 100 mM NaCl; 25 mM EDTA; 1% SDS; and 1µg/ml Proteinase K). Samples were incubated at 55°C for 2 h and extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) and twice with chloroform/isoamyl alcohol (24:1). The DNA was precipitated with two volumes of ice-cold absolute ethanol for about 2 h at -20°C. The precipitated DNA was peleted by centrifugation at 10 000 rpm for 10 min. After a wash with 70% ethanol was vacuum dried and resuspended in 100 µl TE buffer (10 mM Tris-HCl, pH 8.0; 1mM EDTA).

Six decamer primers (P1-P6), purchased from *Pharmacia Biotech do Brasil Ltda.*, were tested to assess genetic markers useful for discriminating between species and for estimations of intraspecific variability.

Experiments were run to test the effect of DNA, dNTPs, Mg^{++} and Taq polymerase concentrations and to determine the optimum anneling temperature. Best results were obtained with the following PCR conditions: 50 µl reaction mixture containing 30 µg of template DNA, one unit of Taq polymerase (Pharmacia); 0.2 mM dNTPs; 2µM of primer; and 1x reaction buffer (10mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 0.001% gelatin). Reactions were performed by a termocycler (MJ Research Inc.) programmed for an initial denaturation step at 94°C for 4 min, followed by 35 cycles at 92°C for 1 min; at 36°C for 1 min; at 72°C for 2 min; and a final extension step at 72°C for 5 min. Then 10 µl of the reaction products were separated on a 2% agarose gel containing 0,5 µl ml^{-1} of ethidium bromide in TAE buffer (40 mM Tris-acetate, 1 mM EDTA). Bands were visualised by ultraviolet (UV) fluorescence and photographed. Approximate DNA fragment size (bp) was estimated by using a 1kb ladder.

Similarity between *C. sapidus* and *C. danae* was estimated for P2, using Nei & Li (1985) formula, $S = 2 N_{ab} / N_a + N_b$, where N_{ab} is the number of bands shared by species a and b; and N_a and N_b are the number of bands exclusives of a and b, respectively. This similarity was used analysing data as phenotypic traits using the

presence (presence of an amplificable site) and absence (absence of the amplificable site) of bands as states of a binary character.

RESULTS

The results obtained after testing six primers (P1-P6) are summarised in Table I. Primers P1 and P5 were not scorable, because they did not give reliable results. Primer P2 was scorable for both species, *C. sapidus* and *C. danae*, and was not variable within species for the individuals tested (Fig. 1). Therefore, it represented a good molecular marker for distinguishing between both *C. sapidus* and *C. danae*. Only one (band 7) out of nine bands (see Fig. 1) was shared by both species, giving a Nei & Li's (1985) phenetic similarity of 0.250. Primer P3 was experimented for both species, but only *C. sapidus* gave good results, showing the same five bands in the six individuals compared. Figure 2 shows the pattern of four bands obtained using primer P3. A fifth band, weak in some individuals, was found situated between the first and the second band. Primer P4 was tested only for *C. sapidus* and did not show any variation among the six individuals studied, showing all the same nine bands (Fig. 3). As the bands were not strong enough to be reproduced from the original photograph, it was drawn the pattern obtained and the approximate size of each DNA fragment was given. Primer P6 was successfully resolved for nine individuals of *C. sapidus*, which showed thirteen highly variable markers (bands). Table II shows the approximate size (bp) of each band, and for each individual, if it was present (1) or absent (0).

Table I. Summary results of the primers tested.

Primer	5'-Sequence-3'	Scorable	Polymorphic	Species*	No. of bands
P1	GGTGCGGGAA	N	---	S/D	---
P2	GTTTCGCTCC	Y	N	S/D	6/4
P3	GTAGACCCGT	Y	N	S	5
P4	AAGAGCCCGT	Y	N	S	9
P5	AACGCGCAAC	N	---	S/D	---
P6	CCCGTCAGCA	Y	Y	S	13

*S = *C. sapidus*; D = *C. danae*

DISCUSSION

The RAPD technique was developed in 1990 (Williams. *et al.*, 1990; Welch & McClelland, 1990) and since then it has been widely used with various purposes. Most works on decapod crustaceans dealt with *Penaeus* spp. problems (see e.g. Garcia *et al*, 1994; Garcia & Benzie, 1995; Tassanakajon *et al*, 1997; Meruane *et al.*, 1997), while no papers, as far as we know, has been published using RAPDs on brachyurans. Recently, the anomuran *Aegla jujuyama* was studied by D'Amato & Corach (1997). The present work might be one of the few works done using RAPDs in brachyurans, if not the first one. The preliminary results of this study showed that RAPDs method is a powerful tool to discriminate *Callinectes* species and to obtain genetic markers with high intraspecific variation for population genetic studies. The phenetic similarity obtained,

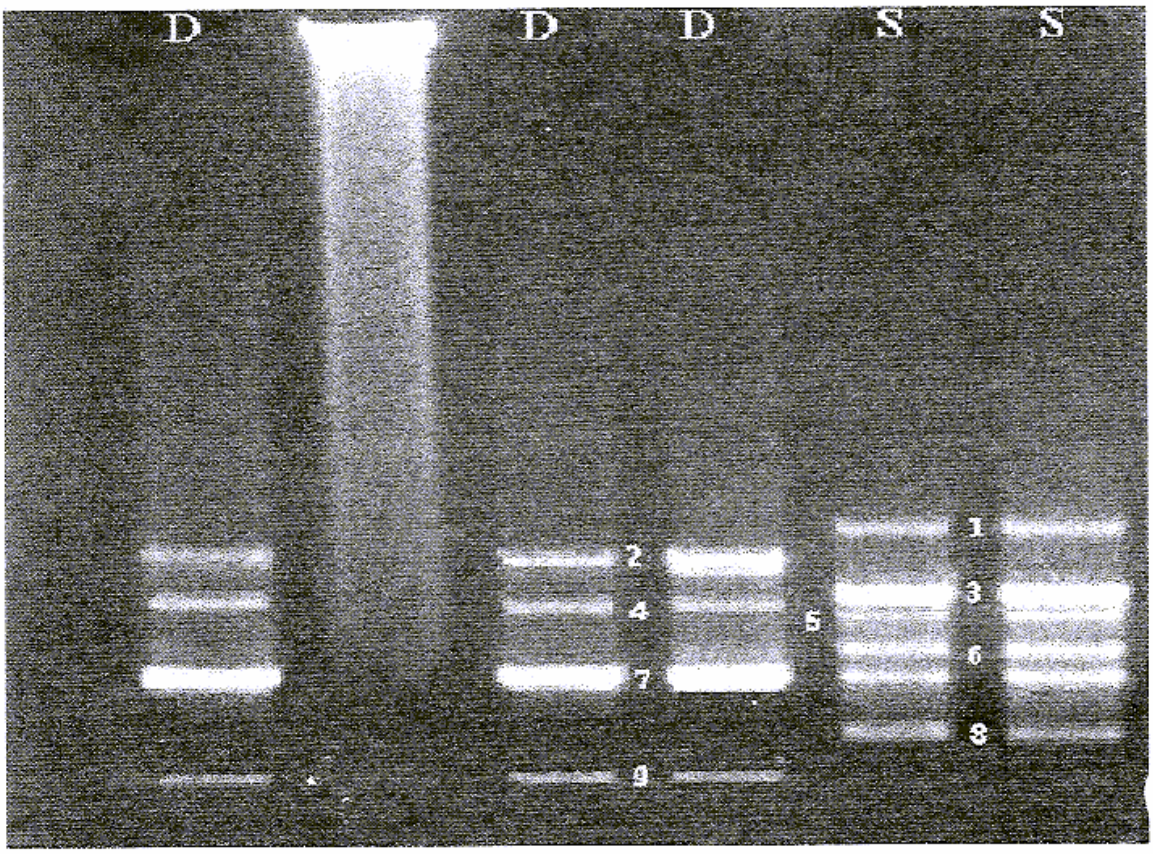

Figure 1: *Callinectes sapidus* and *C. danae*. RAPDs pattern after using Primer P2.

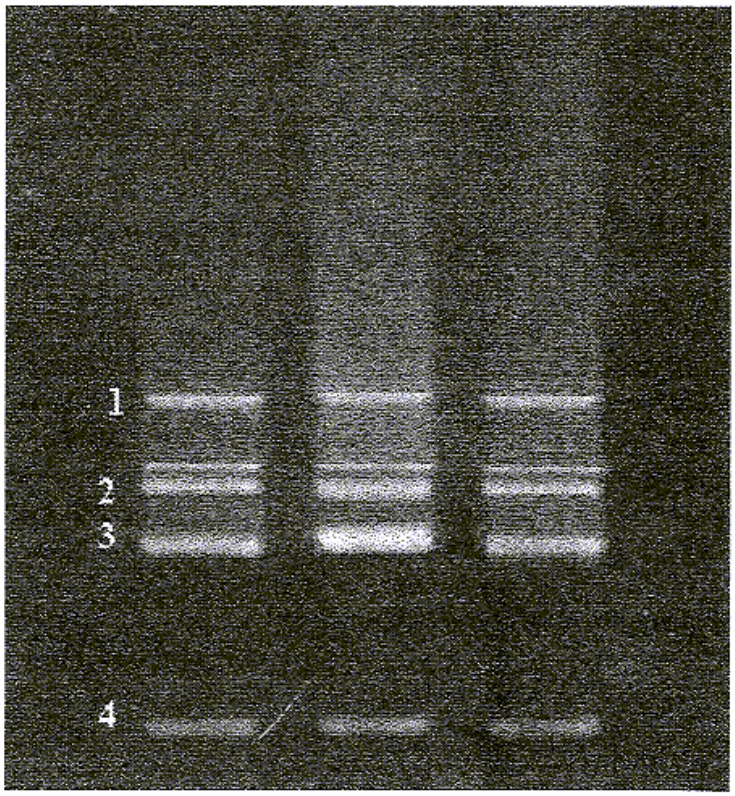

Figure 2: *Callinectes sapidus*. RAPDs pattern after using Primer P3.

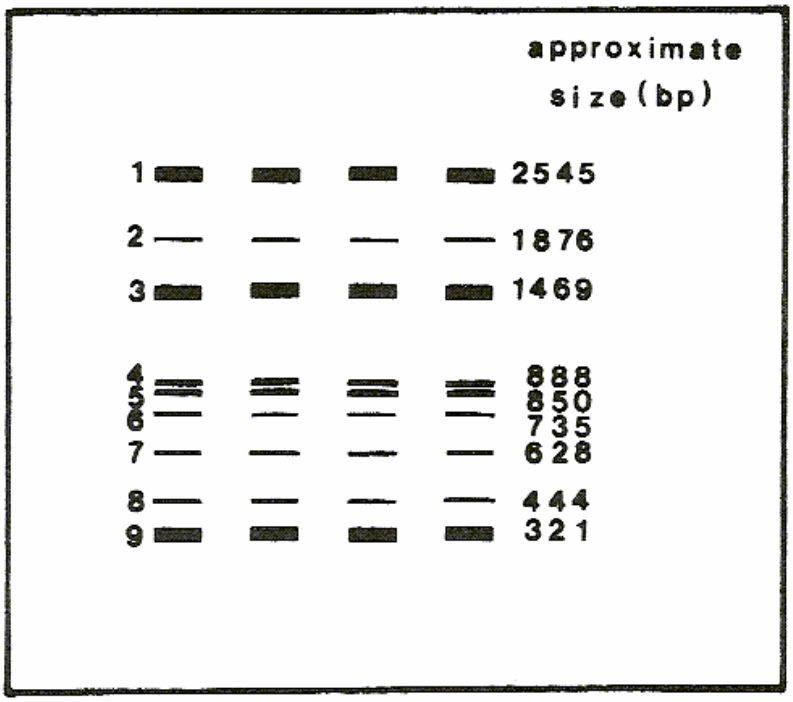

Figure 3: *Callinectes sapidus*. RAPDs pattern after using primer P4.

Table II: Primer P6 in *C. sapidus*. Approximate size of DNA markers (bp), their presence (1) or absence (0) for each presumptive locus in the nine individuals studied.

MARKER		INDIVIDUAL								
	APPROX. SIZE	1	2	3	4	5	6	7	8	9
1	1492	0	0	0	0	1	0	0	0	0
2	1279	0	0	0	0	1	0	0	0	0
3	1142	0	1	1	0	0	0	0	0	1
4	950	0	1	1	0	0	0	0	0	0
5	881	0	1	0	1	1	0	0	0	0
6	830	1	1	1	1	0	1	1	1	1
7	711	0	0	0	0	1	0	0	0	0
8	595	1	1	0	0	1	0	0	0	1
9	506	1	0	0	1	0	0	1	0	0
10	478	0	1	1	0	1	0	0	0	1
11	380	1	1	1	0	1	1	0	1	1
12	316	0	1	1	0	0	0	1	0	0
13	<298	0	0	0	0	1	0	0	0	0

according to Nei & Li (1985) method, between *C. sapidus* and *C. danae* seems low when compared with Nei (1978) genetic similarity (0.530) obtained for these species by allozymes (Weber, L., unpublished data). This is an evidence that higher number of primers should be used for getting more accurate estimates of phenetic and genetic similarities between species. Nevertheless, this fact, does not diminish the potential use of RAPDs for distinction of species. In this study, Primer P2 showed three markers exclusive of *C. danae* and five exclusive of *C. sapidus*, indicating their potential for future studies on postlarval species identification. Although, primers P3 and P4 were only resolved for *C. sapidus*, they appear to be conservative from the individuals compared. They seem to be good candidates for comparisons between species.

Primer P6 showed intraspecific variation, therefore it may be useful for studying the population genetics of blue crabs, since allozymes have revealed very low genetic diversity in *C. sapidus* [see e.g. 0.077 (Cole & Morgan, 1978); 0.040 (McMillen-Jackson *et al*, 1994); 0.023-0.028 (Berthelemy-Okazaki & Okazaki, 1997)]. Meehan & Scoles (1988) recommended the use of mtDNA for studying the population genetics of the blue crab, because it generally evolves more rapidly than nuclear DNA and therefore it is a sensitive indicator of geographic structuring of populations. However, genotypes obtained from mtDNA are unrecombinant characters (haplotypes) transmitted usually through females, and therefore, it describes only maternal lines in most species (Avise, 1994).

The RAPD technique has the advantage of working with nuclear DNA and being less expensive and time-consuming than other techniques, but it also has serious disadvantages. The main one, is the problem while analysing data. Although, Lynch & Milligan (1994) developed a statistical framework for analysing population genetic data, it is based on two assumptions (bi-allelic system with a dominant marker and a null allele; and that the population is in Hardy-Weinberg equilibrium) that might not be realistic in most cases. The alternative analysis, used mainly for taxonomy and

phylogeny, analyse data only as phenetic traits (presence and absence of an amplificable site). The increasing used of RAPDs for screening cultured species and for taxonomic identification of problematic species, indicate that the technique is a good tool for solving problems, and in many cases, more variable than previous techniques (e.g. allozymes).

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