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MOLECULAR DIFFERENTIATION OF TWO ANTARCTIC FISH SPECIES OF THE GENUS *Notothenia* (NOTOTHENIOIDEI: NOTOTHENIIDAE) BY PCR-RFLP TECHNIQUE

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Abstract: *The Antarctic fish Notothenia rossii and Notothenia coriiceps were selected as target organisms for studies of biomarker responses of environmental monitoring research of Admiralty Bay, King George Island. In this case, molecular taxonomy analysis of the referred population became an important study subject in order to increase the knowledge of especies diversity. The taxonomy of Antarctic fish has been predominantly based on morphological characteristics rather than on genetic criteria. A typical example is the Notothenia group, which consists of* ***N. coriiceps****,* ***N. neglecta*** *and* ***N. rossii****. The Polymerase Chain Reaction and Restriction Fragment Length Polymorphism (PCR-RFLP) technique was used to determine whether* ***N. neglecta*** *and* ***N. coriiceps*** *are different or whether they are the same species with morphological, physiological and behavioural variability.* ***N. rossii*** *was used as control. Mitochondrial DNA (mtDNA) was isolated from muscle specimens of* ***N. neglecta****,* ***N. coriiceps*** *and* ***N. rossii****, which were collected in Admiralty Bay, King George Island. The DNA was used to amplify a fragment (690 base pairs) of the coding region of the mitochondrial gene for NADH subunit 2. Further, the amplicon was digested with following restriction enzymes: DdeI, HindIII and RsaI. The results showed a variation of the digestion pattern of the fragment amplified between* ***N. rossii*** *and* ***N. coriiceps*** *or* ***N. neglecta*** *species. No differences were found between* ***N. coriiceps*** *and* ***N. neglecta*** *specimens.*

Keywords: *Notothenia* species, DNA mitochondrial, NADH-2, PCR-RFLP

Introduction

The Antarctic fish *Notothenia rossii* and *Notothenia coriiceps* were selected as target organisms for studies of biomarker responses of environmental monitoring research proposed in Module 3 INCT-APA (Instituto Nacional de Ciência e Tecnologia Antártico de Pesquisas Ambientais) for Admiralty Bay, King George Island (Rodrigues *et al.*, 2009). In this case, molecular taxonomy analysis of the referred population became an important study subject to increase the knowledge of the diversity of this species.

The species *Notothenia coriiceps* was first described by Richardson in 1844. Nybelin (1951) described *N. neglecta* as a new species of the genera, contested in 1966 by DeWitt who considered *N. neglecta* a subspecies of *N. coriiceps.* Fischer and Hureau (1988) supported the hypotheses that *N. coriiceps* and *N. neglecta* are distinct species, presenting differences in the number of fin rays of the pectoral and second dorsal fins, interorbital width and head length. Nowadays, most authors consider that *N. coriiceps* and

N. neglecta are the same species (Kock, 1992; Eastman, 1993; Eastman & Eakin, 2000).

Mitochondrial DNA has been used in research at the population level as well as in studies on molecular taxonomy. The present work used Polymerase Chain Reaction and Restriction Fragment Length Polymorphism (PCR-RFLP) techniques of a region of NADH dehydrogenase subunit 2 gene of the mitochondrial DNA (Meyer, 1993), with the purpose of analysing the polymorphism among *Notothenia* species to assess the existence of *N. neglecta* and *N. coriiceps* separate species.

Methods

Specimen collection and DNA extraction

Specimens of *Notothenia coriiceps* Richardson, 1844, *N. neglecta* Nybelin, 1951 and *N. rossii* Richardson, 1844 were collected at different localities near the Comandante Ferraz Brazilian Station (62° 05' S and 58° 24' W) in Admiralty Bay, King George Island, South Shetland Islands. *Notothenia rossii*, a phylogenetically close species was used as control. Thirty-six specimens were used for molecular analyses: 11 *N. coriiceps,* 11 *N. neglecta* and 14 *N. rossii* specimens. Counts of meristic characters and morphometric measurements from the specimens examined in this study following procedures by Fischer and Hureau (1988) (Table 1).

A fragment of muscle tissue (1 cm^3) of the tail region was collected and preserved in absolute ethanol until processing. The Easy-DNA kit extraction (Invitrogen, Carlsbad, CA) was used for DNA extraction, according to the manufacturer's instructions.

Amplification reaction

The coding region of the mitochondrial gene for subunit 2 of the NADH (ND2) was amplified using the following primer pairs: ND2F (5' - ACCACCCCCGGGCAGTTGAAG - 3') and ND2R (5' - GCGGTGGGAGCTAGCTCTTGTTTA - 3'). These primers were designed from conserved regions obtained from the alignment of the sequences of the ND2 gene of Antarctic fish deposited in GenBank (NCBI, 2004).

PCR-RFLP technique

The amplicons of the ND2 were digested with 5 U of the following restriction enzymes: *Dde*I, *Hin*dIII and *Rsa*I (New England BioLabs, Beverly, MA). The treated samples were subjected to electrophoresis in 10% acrylamide gel. Molecular size of restriction DNA fragments were estimated by comparison with 1 Kb Plus Ladder (Invitrogen).

Results

Digestion profiles of ND2 amplified fragments showed that *N. rossii* does not possess a site for restriction enzymes *Hin*dIII and *Rsa*I, whereas the amplicon of *N. coriiceps* and *N. neglecta* exhibited one restriction site for *Hin*dIII and *Rsa*I (Figure 1b, c). The fragment patterns produced by digestion with restriction enzyme *Dde*I indicated three restriction sites in N. *rossii* and two for *N. coriiceps* and *N. neglecta* (Figure 1a).

The molecular differentiation between *N. rossii* and *N. coriiceps* was possible using the NADH2 gene of the mitochondrial DNA by PCR-RFLP technique. However, no difference was found within *N. coriiceps* and *N. neglecta*

Table 1. Meristic counts and morphometric measurements of the *Notothenia* specimens (n = 36) captured from Admiralty Bay, with sample separated by species according Fischer and Hureau (1988). Numbers of specimens are 11 for *N. coriiceps*, 11 for *N. neglecta* and 14 for *N. rossii*.

Characteristics	Species		
	N. rossii	*N. coriiceps*	*N. neglecta*
N° of first dorsal fin rays	4 - 7	4 - 6	3 - 7
N° of second dorsal fin rays	32 - 35	35 - 37	37 - 40
N° of pectoral fins rays	22 - 24	16 - 18	16 - 19
N° of anal fin rays	27 - 30	27 - 30	29 - 32
interorbital width / head length	29 - 31	23 - 25	26 - 33

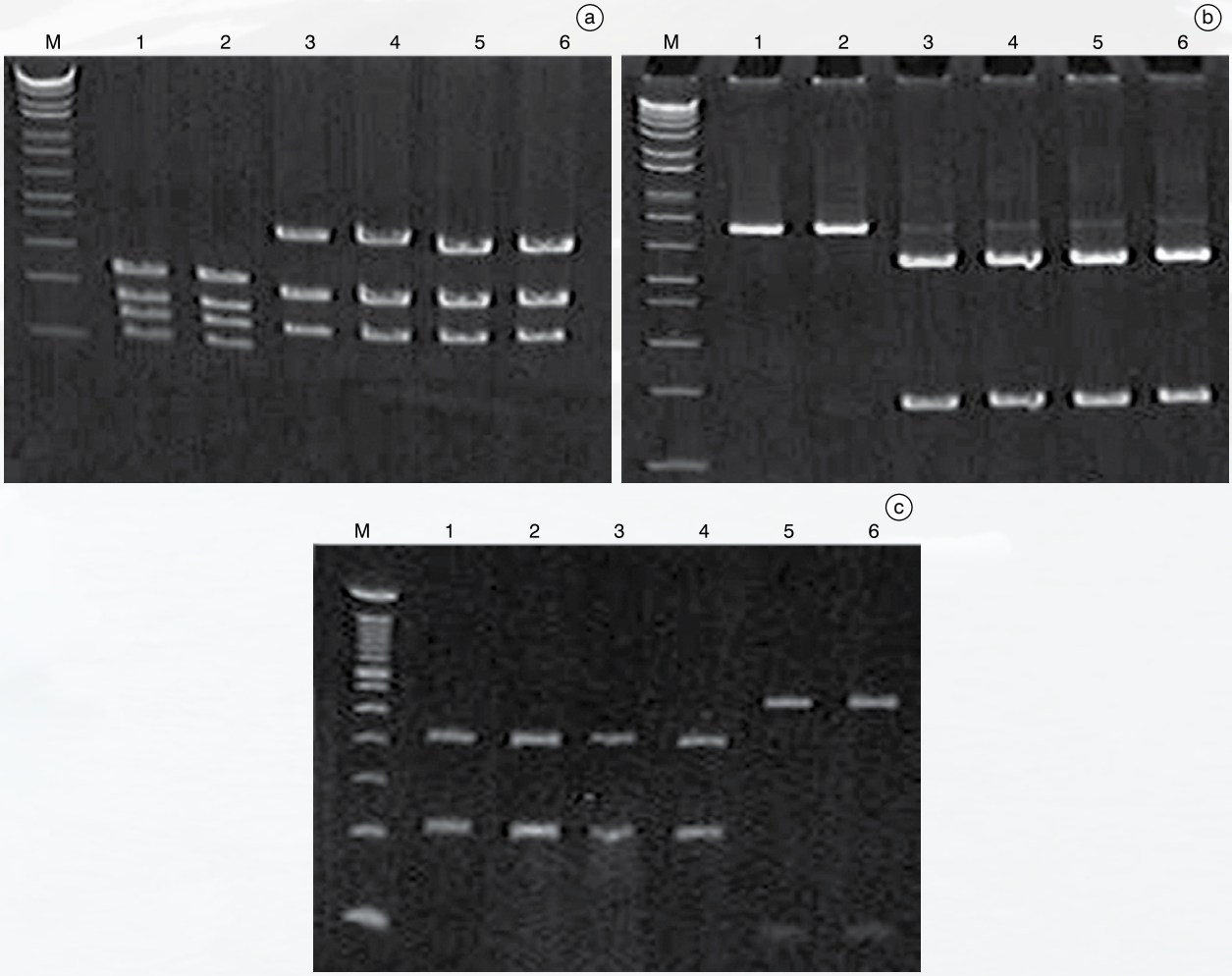

Figure 1. Digestion profile of a fragment (690 base pairs) of the coding region amplified of the mitochondrial gene of the subunit 2 of the NADH using PCR-RFLP technique. a) Amplicon digested by restriction enzyme *Dde*I. Lines 1 and 2 corresponding to *N. rossii* specie. Lines 3 and 4: *N. coriiceps*. Lines 5 and 6: *N. neglecta*; b) Amplicon digested by restriction enzyme *Hind*III. Lines 1 and 2 corresponding to *N. rossii* specie. Lines 3 and 4: *N. coriiceps*. Lines 5 and 6: *N. neglecta*; c) Amplicon digested by restriction enzyme *Rsa*I. Lines 1 and 2 corresponding to *N. coriiceps* specie. Lines 3 and 4: *N. neglecta*. Lines 5 and 6: *N. rossii*. M: 1kb Plus Ladder (Invitrogen).

specimens, by the digestion profile obtained for the *DdeI*, *Hind*III and *Rsa*I restriction enzymes (Figure 1).

Discussion

The species *N. coriiceps,* described by Richardson 1844, is largely distributed in shallow waters of the Southern Ocean and found in high densities in Admiralty Bay. It presents a great deal of morphological variation. Nybelin (1951) described *N. neglecta* as a new species of the genera. Fischer and Hureau (1988) considered *N. coriiceps* and *N. neglecta* as a distinct species, showing differences in the number of fin rays of the pectoral and second dorsal fins, interorbital width and head length. In 1966, DeWitt considered *N. neglegta* as a subspecies of *N. coriiceps* justifying that Nybelin had used a small number of samples to present its classification (Gon & Heemstra, 1990).

Conclusion

The results of the study presented here confirmed that *N. coriiceps*is genetically different to *N.*rossii, being two

distinct species, while there was no evidence of genetic divergence between *N. neglecta* and, *N. coriiceps*. However, additional information on independent genetic loci (nuclear markers) will be required to reject the hypothesis of Nybelin that these two morphotypes are separate species.

Also, in addition to the information from Meyer (1993), we have shown that the gene ND2 is a good gene to differentiate the species of fish of the same genus. In the comparison between *N. coriiceps* and *N. rossi*, by the RFLP technique, the bands pattern was clear and presented good reproducibility.

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