Molecular differentiation by PCR-RFLP technique shows that Antarctic fishes of the genus *Notothenia*, claimed to be two species, are phenotypic varieties with the same genetic pattern

Cintia Machado [1], Marcia Kiyoe Shimada [2], Stênio Perdigão Fragoso [2], Edith Fanta [1,5], Helena G. Kawall [3], Edson Rodrigues [4], Lucélia Donatti [1 *]

(1) Universidade Federal do Paraná (UFPR-PR) - Departamento de Biologia Celular; Av. Cel. Francisco H. dos Santos, s/n, Jardim das Américas, 81531-970, Curitiba, PR, Brasil.

(2) Instituto Carlos Chagas (ICC-PR) - Rua Prof. Algacyr Munhoz Mader, 3775 - CIC 81350-010 Curitiba, PR, Brazil.

(3) Instituto Oceanográfico, Universidade de São Paulo, Praça do Oceanográfico 191, Cidade Universitária, 05508-900, São Paulo, SP, Brazil.

(4) Universidade de Taubaté (UNITAU-SP) - Av. Tiradentes 500, Centro, 12030-180, Taubaté, SP, Brazil.

(5) in memorian

* Corresponding author: Lucélia Donatti

Mailing address: Universidade Federal do Paraná (UFPR); Departamento de Biologia Celular; Av. Cel. Francisco H. dos Santos, s/n, Jardim das Américas, Curitiba, PR, Brasil. Caixa Postal: 19031; CEP: 81531-970.

E-mail: donatti@ufpr.br

Abstract

The taxonomy of Antarctic fishes has been predominantly based on morphological characteristics rather than on genetic criteria. A typical example is the *Notothenia* group, which includes *N. coriiceps* Richardson, 1844, *N. neglecta* Nybelin, 1951 and *N. rossii* Richardson, 1844. The Polymerase Chain Reaction and Restriction Fragment Length Polymorphism (PCR-RFLP) technique was used to determine whether *N. coriiceps* Richardson, 1844 and *N. neglecta* Nybelin, 1951 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. coriiceps* Richardson, 1844, *N. neglecta* Nybelin, 1951 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 mitochondrial gene coding region of NADH dehydrogenase subunit 2. Further, the amplicon was digested with the following restriction enzymes: *Dde*I, *Hind*III and *Rsa*I. The results showed a variation of the digestion pattern of the fragment amplified between *N. rossii*, and *N. coriiceps* Richardson, 1844 or *N. neglecta* Nybelin, 1951. However, no differences were found between *N. coriiceps* Richardson, 1844 and *N. neglecta* Nybelin, 1951, on the grounds of the same genetic pattern shown by the two fish.

Key words: *Notothenia* species, mitochondrial DNA, NADH-2, PCR-RFLP.

1. Introduction

The fish fauna that inhabits the Antarctic Southern Ocean is dominated by species of the suborder Notothenioidei. This group represents 45% of all species known in the Antarctic region and it comprises 95% of fish biomass (Eastman, 1993). No other oceanic ecosystem is so dominated by a single taxonomic group of fish (Clarke and Johnston, 1996), thus it is a region of poor species diversification. The Nototheniidae family is the most diverse group in the Notothenioidei suborder, composed of 12 genera and 48 species (Tokita et al., 2002).

The species *Notothenia coriiceps* was first described by Richardson in 1844. Nybelin (1951) described *N. neglecta* as a new species of the genus contested in 1966 by DeWitt who considered *N. neglecta* Nybelin, 1951 a subspecies of *N. coriiceps* Richardson, 1844. Fisher and Hureau (1988) supported the hypothesis that *N. coriiceps* Richardson, 1844 and *N. neglecta* Nybelin, 1951 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* Richardson, 1844 and *N. neglecta* Nybelin, 1951 are the same species (Kock, 1992, Eastman, 1993).

For many years our research on physiology, morphology and behaviour of fishes collected in Admiralty Bay has been done on what, based on Fischer and Hureau (1988), were considered two species, *N. coriiceps* Richardson, 1844 and *N. neglecta* Nybelin, 1951, allowing even the establishment of comparisons between data from both species (Fanta et al., 1989a; 1990; Lucchiari et al., 1989). In Admiralty Bay we find specimens with significant differences in mouth length and width and shape of the head, but the head length and the intraorbital distance are similar (Fanta et al., 2000).

The evolution of molecular genetic techniques during the last decades has contributed to an understanding of natural genetic diversity, biogeography and speciation

issues (Oleinik et al., 2004). Analyses of molecular genetics have been helpful in phylogenetic investigations within families, subfamilies and genera of suborder Notothenioidei (Derome et al., 2002; Near et al., 2003; Sanchez et al., 2007). Genetic studies of Antarctic fishes have also revealed that species defined by morphological characters are complexes of cryptic or sibling species (Bernardi and Goswami, 1997; Patarnello et al., 2003; Rogers, 2007).

Mitochondrial DNA has been used mainly in research at population level and in studies of molecular relationships among related species. Most of the research on population relationships of fishes using mitochondrial DNA data was based on restriction fragment length polymorphism (RFLP) (Meyer, 1993). The present work used Polymerase Chain Reaction and Restriction Fragment Length Polymorphism (PCR-RFLP) techniques on a region of NADH dehydrogenase subunit 2 gene of mitochondrial DNA, with the purpose to analyze the polymorphism among Notothenia species, with the aim to establish the existence of *N. coriiceps* Richardson, 1844 and *N. neglecta* Nybelin, 1951 as distinct species, or to establish whether they are the same species.

2. Methods

Specimen collection and DNA extraction

Specimens of *Notothenia coriiceps* Richardson, 1844, *N. neglecta* Nybelin, 1951 and *N. rossii* Richardson, 1844 were collected in Admiralty Bay (62°09`S, 58°26`W) King George Island, South Shetland Islands/Antarctic Peninsula. *Notothenia rossii*, a phylogenetically close species was used as control. Fifty specimens were used for molecular analyses: 11 *N. coriiceps* Richardson, 1844, 11 *N. neglecta* Nybelin, 1951 and 14 *N. rossii*

specimens. These specimens were classified by morphometric features according to Fisher and Hureau (1988) (Table 1).

(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) was used for DNA extraction, according to the manufacture.

Amplification reaction

The coding region of the mitochondrial gene of the subunit 2 of the NADH (ND2) was amplified using the following primers 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 fishes deposited in GenBank (www.ncbi.nlm.nih.gov).

PCR reaction mixtures were as follows: 10 pmol of each pair of primers, 100 ng of total DNA, 200 μM dNTPs, 1.5 mM $MgCl_2$, 20 mM Tris-HCl pH 8.4, 50 mM KCl and 2.5 units of *Taq* DNA polymerase (Invitrogen). The reactions were performed on the thermalcycler model GeneAmp PCR System 9700 (Applied Biosystems) with a denaturation step at 94ºC for 4 minutes, followed by 35 cycles at 94 ºC for 30s, 55 ºC for 30s and 72 ºC for 30s, and the final extension of 72 ºC for 7 minutes. Amplified DNA fragments were purified using the highly pure PCR product purification kit (Roche).

PCR-RFLP

The amplicons of ND2 were digested with 5U of the following restriction enzymes: *DdeI*, *Hind*III and *Rsa*I (Biolabs). The treated samples were subjected to electrophoresis in 10% acrylamide gel. The profile of the sizes of resulting DNA fragments were estimated by comparison with 1 Kb Plus Ladder (Invitrogen).

Results

The digestion profile of an amplified fragment of the NADH dehydrogenase subunit 2 showed that the amplicon of *N. rossii* does not possess a cleavage site for restriction enzymes *Hind*III and *Rsa*I. Therefore, the amplicon of *N. coriiceps* Richardson, 1844 and *N. neglecta* Nybelin, 1951 exhibited one restriction site for *Hind*III and *Rsa*I (Fig. 1 – B and C). The fragment patterns produced by digestion with restriction enzyme *Dde*I indicated three restriction sites for *N. rossii* and two for *N. coriiceps* Richardson, 1844 and *N. neglecta* Nybelin, 1951 (Fig. 1 – A).

(Fig. 1)

The molecular differentiation between *N. rossii* and *N. coriiceps* Richardson, 1844 was possible using the NADH2 gene of mitochondrial DNA for PCR-RFLP.

No difference was found between *N. coriiceps* Richardson, 1844 and *N. neglecta* Nybelin, 1951 specimens, by the digestion profile obtained for the *DdeI*, *Hind*III and *Rsa*I restriction enzymes (Fig. 1).

Discussion

Considering some phylogenetic trees, proposed on the basis of some mitochondrial genes of Antarctic fishes (Bargelloni et al, 2000; Near and Cheng, 2008), the species *N. rossi*

and *N. coriiceps* Richardson, 1844 are very close, proving the reliability of the methodology utilized on this research.

Species of the *Trematomus* genus were the subject of studies and discussions regarding morphological variability. The occurrence of of two morphs of *T. newnesi*, the typical morph and the wide-mouth morph, found close to McMurdo Station, led Eastman and DeVries (1997) to investigate the occurrence of phenotypic plasticity on this species. McDonald et al. (1992) through allozyme markers, suggested that the species *T. bernacchii* is a complex of cryptic species. By the analysis of two mitochondrial genes, 12S and 16S, Bernardi and Goswami (1997) investigated the occurrence of cryptic species of *T. bernacchii* found in the area, but it was not possible to differentiate through the genes studied.

Patarnello et al. (2003) used two mitochondrial genes (D-loop and 16rRNA) to distinguish two closely related species of the genus *Chionodraco*. The distinction between *C. hamatus* and *C. rastrospinosus* is based on few morphological differences and on areas of distribution (Fisher and Hureau, 1988).

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

In addition to the information from Meyer (1993), the ND2 gene used in this investigation is very useful to differentiate species of fishes of the same genus. In the

comparison between *N. coriiceps* Richardson, 1844 and *N. rossi* by RFLP, the band pattern was clear and reproductive.

The results presented here demonstrate that the species we have previously proposed as *N. neglecta* Nybelin, 1951 is actually a phenotypic variety of *N. coriiceps* Richardson, 1844, showing the same genetic pattern. They also confirm that *N. coriiceps* Richardson, 1844 is genetically different from *N. rossii*.

Acknowledgements

The authors wish to thank the Conselho Nacional de Pesquisa e Desenvolvimento (CNPq) for financial support to the Projects nrs. 52.0125/2008-8 (API), 57.4018/2008-5 (INCT-APA) and a Productivity in Research stipend for L. Donatti nr. 305562/2009-6; Fundação de Amparo a Pesquisa (FAPERJ) n E-26/170.023/2008; REUNI/SESU for a Doctorate Stipend to C. Machado; the PROANTAR/SECIRM of the Brazilian Navy, and the staff of the Brazilian Antarctic Station Comandante Ferraz for all logistical support

References

Bargelloni, L.; Marcato, S.; Zane, L.; Patarnello, T. 2000. Mitochondrial Phylogeny of Notothenioids: A Molecular Approach to Antarctic Fish Evolution and Biogeography. Systematic Biology; 49, 114-129

Bernardi, G.; Goswami, U. 1997. Molecular evidence for cryptic species among the Antarctic fish *Trematomus bernacchii* and *trematomus hansoni*. Antarctic Science. 9(4), 381-385.

Clarke, A.; Johnston, I. A., 1996. Evolution and adaptive radiation of Antarctic fishes. Trends in ecology & evolution 11(5), 212 – 218.

Derome, N.; Chen, W.; Dettai, A.; Bonillo, C.; Lecointre, G. 2002. Phylogeny of antarctic dragonfishes (Bathydraconidae, Notothenioidei, Teleostei) and related families based on their anatomy and two mitochondrial genes. Molecular Phylogenetics and Evolution. 24, 139-152.

Dewitt, H. H. 1966. A revision of the Antarctic and southern genus *Notothenia* (Pisces, Nototheniidae). Ph. D. Dissertation. Palo Alto, California: Stanford University. 469 pp.

Eastman, J. T., 1993. Antarctic fish biology. Academic Press, San Diego, 322 pp.

Eastman, J. T.; Devries, A. L. 1997. Biology and phenotypic plasticity of the antarctic nototheniid fish *Trematomus newnesi* in McMurdo Sound. Antarctic Science. 9(1), 27-35.

Fanta, E.; Lucchiari, P. H. Bacila, M. 1989. The effect of environmental oxygen and carbon dioxide levels on the tissue oxygenation and behavior of Antarctic fish. *Comp. Biochem. Physiol*. 93A (4), 819-831.

Fanta, E.; Lucchiari, P. H. Bacila, M. 1990. Cardian rhythm of oxygen consumption and oxygen levels in the muscle of *Nothotenia neglecta* (Pisces, Teleostei*).* Comp. Biochem. Physiol. 96C (1), 151-155.

Fanta, E., Kawall, H. G., Donatti, L. 2000. On the Phenotypic plasticity of the Antarctic fish *Notothenia neglecta/coriiceps* (?) in the Admiralty Bay, King George Island In: International Symposium on Biology of Polar Fish, Cambridge.

Fischer, W.; Hureau, J. C. 1988 Oceano austral. 1985. Vol II. Roma*:* Organizacion de las Naciones Unidas para Alimentacion e la Agricultura, 471 pp.

Gon, O.; Heemstra, P. C. 1990. Fishes of the Southern Ocean. J. L. B. Smith Institute of Ichthyology. Grahamstown, South Africa, 462 pp.

Kock, K. H. 1992. Antarctic fish and fisheries; studies in polar research. Cambridge Univ. Press, Cambridge, 359 pp.

Lucchiari, P. H.; Fanta, E.; Bacila, M. 1989. The effect of temperature on the oxygen levels in Antarctic fish. *Proc. NIPR Symp. Polar Biol. 2, 117*-122.

Mcdonald, M. A.; Smith, M.H.; Smith, M. W.; Novak, M.J.; Johns. P.E; Devries. 1992. Biochemical systematics of Notothenioid fishes from Antarctica. Biochem. Syst. Ecol. 20, 233-241.

Meyer, A. 1993. Evolution of mitochondrial DNA in fishes. In: Hochachka and Mommsen, Biochemistry and molecular biology of fishes, vol.2. Elsevier Science Publishers, New York, pp. 1-38.

Near, T. J.; Pesavento, J. J.; Cheng, C. H. C. 2003. Mitochondrial DNA, morphology, and the phylogenetic relationships of Antarctic icefishes (Notothenioidei: Channichthyidae). Molecular Phylogenetics and Evolution 28, 87-98.

Near, T. J.; Cheng, C. C.-H. 2008. Phylogenetics of notothenioid fishes (Teleostei: Acanthomorpha): Inferences from mitochondrial and nuclear gene sequences. Molecular Phylogenetics and Evolution. 47, 832-840.

Patarnello, T.; Marcato, S. Zane, L.; Varotto, V. Bargelloni, L. 2003. Phylogeography of the Chionodraco genus (Perciformes, Channichthydae) in the Southen Ocean. Mol. Phyl. Evol. 28, 420-429.

Rogers, A. D. 2007. Evolution and biodiversity of antarctic organisms: a molecular perspective. Phil. Trans. R. Soc. B 362, 2191-2214.

Sanchez, S.; Dettai, A.; Bonillo, C.; Ozouf-Costaz, C.; Detrich Iii, H. W.; Lecointre, G. 2007. Molecular and morphological phylogenies of the antarctic teleostean family Nototheniidae, with emphasis on the Trematominae. Polar Biology. 30, 155-166.

Oleinik, A. G.; Skuiikhina, L. A.; Frolov, S. V.; Brykov, V. A. & Chereshnve, I. A. 2004. Differences between two subspecies of Dolly Varden, *Salvelinus malma*, revealed by RFLP-PCR analysis of mitochondrial DNA. Environmental Biology of Fishes. 69, 449-459.

Tokita, M.; Ishii., S.; Iwami, T.; Miyazaki, J. 2002. Phylogenetic analysis of Antarctic notothenioid fishes based on two-dimensional gel electrophoresis. Polar Biology 25, 163-168.