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Cytochrome B Gene Partial Sequence and RAPD Analysis of Two *Daphnia longispina* Lineages Differing in their Resistance to Copper

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The loss of genetic diversity of populations exposed to environmental pollution is receiving growing attention among ecotoxicologists, with the increasing availability of easily applicable molecular techniques (van Straalen and Timmermans 2002). Several studies reported an association between contamination and genetic diversity. For instance, genetic variability reduction was found in populations of the isopod *Platynympha longicaudata,* the crustaceans *Leander intermedius* (Ross et al. 2002) and *Balanus glandula* (Ma et al. 2000), the mollusks *Mytilus galloprovincialis* (Ma et al. 2000) and *Littorina brevicula* (Kim et al. 2003), and the fish *Ameiurus nebulosus* (Silbiger et al. 2001), when subjected to various environmental contaminants. Furthermore, molecular techniques can be used to find and characterize markers that may be related to or confer a specific trait, such as the resistance to toxicants. Sturmbauer (1999), based on the 16 rDNA gene, differentiated oligochaete species differing in their cadmium resistance. Contaminant indicative band (CIB), obtained with the Random Amplified Polymorphic DNA (RAPD) technique, were found in plants (Mengoni et al. 2000) and fish (Nadig et al. 1998, Theodorakis 2001), even when no reduction in genetic diversity was found (Mengoni et al. 2000, Nadig et al. 1998). However, these bands, which may include quantitative trait loci (QTL) for metal tolerance, were not yet characterized. The molecular characterization of some metal responsive genes that confer metal resistance has been performed for a few invertebrate species (Baršyte et al. 1999, Beaty et al. 1998, Liao et al. 2002, Tanguy and Moraga 2001).

Lopes et al. (2003) demonstrated that the increased resistance of a *Daphnia longispina* population, historically stressed by acid mine drainage, was mainly due to the elimination of the most sensitive individuals, as tolerant ones were also present in a reference population, though in lower frequency. The ultimate goal of this research is to compare the genetic diversity of these populations, regarding both diversity in single genes and in the whole genome, and to identify genetic markers that could discriminate resistant from sensitive lineages. A first step, which is the objective of the present study, was to compare two cloned lineages, originated from reference and impacted populations and that differed in their genetically-determined resistance to lethal levels of copper. The choice fell on the characterization of a fragment of the Cytochrome B, a gene commonly

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used as a marker of population divergence and genetic diversity, and one of the few that were already characterized in other *Daphnia* species (Schwenk 1993). Furthermore, this work included the adaptation of the RAPD methodology for this species.

MATERIALS AND METHODS

The *D. longispina* O.F. Müller individuals used in this research belonged to cloned lineages derived from field-collected females of two natural populations: one inhabiting a site historically impacted with acid mine drainage and the other inhabiting a nearby reference (unpolluted) pond (Lopes et al. 2003). From each population, nearly 130 lineages were ranked according to their genetically-determined resistance to lethal levels of copper, after acclimation to laboratory conditions for at least 15 generations (Lopes et al. 2003). Lineages were maintained in ASTM hardwater medium (ASTM 2002), enriched with vitamins and the standard organic extract "Marinure 25" (Glenside, Stirling, UK), at 25 ± 1°C under a 14:10 hr light:dark cycle. Daphnids were fed daily with *Pseudokirchneriella subcapitata* (Korshikov) Hindak (formerly known as *Selenastrum capricornutum* Printz) ($3x10^5$ cells/mL/day). The medium was changed every other day. For this study, two cloned lineages, differing on their genetically-determined resistance to lethal levels of copper, from the reference (R) and the impacted (I) populations were used. Respective values of copper $LC_{50,48hr}$ (with the 95% confidence interval) were 75.5 (67-84) and 254.9 (195-396) µg/L (Lopes et al. submitted).

Groups of 25 individuals from each lineage were placed in eppendorf tubes, dried by aspiration and manually homogenated in 25 µL of ethanol, with a glass rod. Ethanol was left to evaporate prior to extraction. DNA extraction was performed according to the phenol/chloroform/isoamylic alcohol extraction protocol (Sambrook et al. 1989). One milliliter of phenol/chloroform/isoamylic alcohol (25:24:1) solution was placed in the tubes and mixed with a vortex. Samples were centrifuged for 10 min at 10,000 rpm in a microcentrifuge. Supernatant was then pipetted to new tubes, to which 500 µL of cold (4°C) isopropanol were added, centrifuged for another 10 min at 10,000 rpm, and resulting supernatant discarded. Then, 500 µL of cold (4°C) ethanol were added and centrifugation followed for another 10 min at 10,000 rpm. Supernatant (isopropanol) and most of the ethanol phase (lower phase) was carefully pippeted off, avoiding pellet disturbance. Excess ethanol was left to evaporate at room temperature. The pellet was then resuspended in 250 µL of autoclaved nanopure nuclease-free water (NFW), and stored at -20°C.

In this work, a fragment of the cytochrome B (CytB) was sequenced accordingly to Brehm et al. (2001). Prior to sequencing, the target gene fragment was amplified by polymerase chain reaction (PCR), using standard primers corresponding to conserved sequences of the gene: CytB1 and CytB2, flanking a ≈ 300 base pairs (bp) sequence near the 5' end of the gene: 5'-CCATCCAACATCTCAGCATGATGAAA-3' and 5'-CCCTCAGAATGATA

TTTGTCCTCA-3', respectively (adapted from Kocher et al. 1989). The reaction mixture for the PCR consisted of 16.5 μL of NFW, 3.5 μL of 10x Taq DNA buffer (Amersham Pharmacia, Uppsalla, Sweden), 2 μL of Mg^{2+}, 3 μL of each oligonucleotide primer (5 pmol/μL), 2 μL of dNTP mix (2 mM of each nucleotide; Promega, Charbonniéres, France), 1.5 units of Taq polymerase (Amersham Pharmacia), and 2.0 μL of template DNA. Amplification was performed in a Biometra (Goettingen, Germany) thermocycler, and consisted of a 2-min denaturation phase at 96°C, followed by 35 cycles of 30 sec at 94°C, 30 sec at 50°C and 30 sec at 72°C, followed by a final 5-min extension phase at 72°C. After amplification, the presence of PCR products was confirmed by running 10 μL of sample in an 1.2%-agarose gel (Sigma, Munich, Germany), at 150 V for 45 min. Bands were visualized under UV light, after soaking in ethidium bromide (Sigma). For samples that successfully amplified, DNA was purified in Sephadex G-50 microcolumns (Sigma), by centrifugation during 4 min at 4,000 rpm. Afterwards, sequencing reaction proceeded with a second PCR reaction, in which fluorescent-labeled nucleotides and the CytB1 primer were added, producing antisense single stranded DNA chains. Reaction mixture for the second PCR consisted of 4.5 μL of NFW, 3.0 μL of primer, 2.0 μL of Big Dye (Applied Biosystems, Foster City, CA, USA), and 2.0 μL of template DNA. Amplification reaction conditions were the same as for the previous reaction. After cleaning in Sephadex microcolumns, water was evaporated and 15 μL of Template Supression Reagent (Applied Biosystems) were added to the DNA. Sequencing was performed in an Applied Biosystem 373 DNA sequencing apparatus. Obtained sequences were aligned and compared with published sequences using the online version of BLAST 2 (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2; Tatusova and Madden 1999).

RAPD reactions were performed according to Freitas and Brehm (2001), using OpA3, OpA4, OpA5, OpA7, and OpA11 primers (Operon Technologies, Alameda, CA, USA). From these, only OpA3, OpA4 and OpA7 primers yielded good resolution banding patterns. To ensure the repeatability, RAPD reaction was repeated with these primers. The reaction mixture consisted of 12 μL of NFW, 2 μL of 10x Taq DNA buffer, 2 μL of Mg^{2+}, 2 μL of the oligonucleotide primer (5 pmol/μL), 2 μL of dNTP mix (2 mM of each nucleotide), 1 unit of Taq polymerase, and 2 μL of template DNA. Amplification was performed in a Biometra thermocycler, and consisted of a 2-min denaturation phase at 96°C, followed by 45 cycles of 1 min at 92°C, 1 min at 36°C and 2 min at 72°C, followed by a final 3-min extension phase at 72°C. Amplified samples were kept at 4°C until being ran in a 1.2%-agarose gel, at 180 V for 30 min. Bands were detected under UV light, after soaking in ethidium bromide. The 100 base pair ladder (Amersham Pharmacia) was used as a molecular size marker.

RESULTS AND DISCUSSION

No genetic diversity was found for the CytB gene in the two lineages. The obtained sequence has 308 bases and is accessible via GenBank (http://www.ncbi.nlm.nih.gov/genbank), under accession number AY453690. It

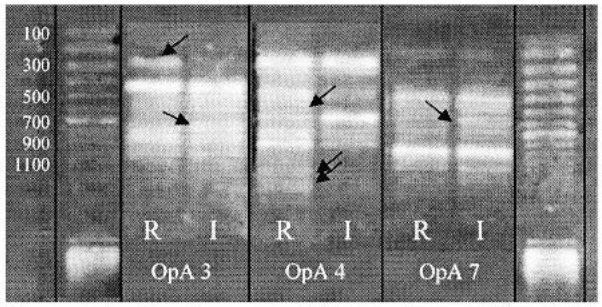

Figure 1. Random amplified polymorphic DNA profiles of the two studied *Daphnia longispina* cloned lineages, one sensitive (R) and one resistant (I) to copper contamination, using the OpA3, OpA4 and OpA7 primers. Arrows indicate differentially amplified bands. First and last lanes are the 100 base pairs DNA ladder.

presents 73% homology with the published sequence for the same gene of *D. pulex* (Weider et al. 1999) and 89% for *D. galeata* (Schwenk 1993). The later author pointed out that, within the *D. longispina* subgroup species, CytB variability is very low, since species divergence is relatively recent. For this species, this author suggested the study of other faster evolving mtDNA regions, like the adenine-thymine rich region of the mitochondrial genome. Low variability for CytB was also found in a similar study by Dahl et al. (2001) in black crowned heron (*Nycticorax nycticorax*) populations subject to genotoxic contaminants. The authors referred that this low variability might have been due to a previous genetic bottleneck or to a high conservativeness of the gene in this species. Kim et al. (2003) found in a population of the gastropod *L. brevicula*, subjected to heavy metal pollution, a reduction in the variability of the ND6 gene when compared to a reference population, whereas for the CytB gene no genetic differences were found. This may indicate that the study of the ND6 gene could be more informative and relevant, regarding metal resistance, than the CytB gene, although this gene is commonly used in phylogenetic studies (Brehm et al. 2001, Schwenk 1993). Mitochondrial DNA haplotype polymorphisms enable to trace the genetic history or phylogenetic divergence of the studied populations. Populations along a pollution gradient should share an evolutionary history, rather than being polyphyletic, otherwise a convincing argument cannot be made that pollution adaptation has evolved within these populations. The study of mitochondrial haplotypes of CytB denoted that both populations probably share a common phyletic origin.

Obtained RAPD profiles are depicted in Figure 1. For the three primers with which good resolution banding patterns were obtained, RAPD profiles yielded at least one unshared band in each lineage. Probably due to a poor amplification with primer OpA4, lineage I yielded a smaller number of bands than lineage R. With primers OpA 3 and OpA7, all bands except one were common, having each lineage one specific band. Lineage specific (R- and I-) bands were the following: with primer OpA3, the 300-bp R-band and the 800-bp I-band; with primer OpA4,

the 600- and the two >1100-bp R-bands; with primer OpA7, the 800-bp I-band. Although these differences have little biological significance, since it is unknown what genes, if any, they code for, this confirmed that each lineage corresponds to a different clone. Comparing the two used methodologies, RAPD analysis proved to be more useful than the direct CytB gene sequencing in discriminating the two lineages. However, other methodologies of whole-genome comparison, like AFLP or TE-AFLP (van Der Wurff et al. 2000), are to be considered in future works, as they are more robust and reproducible than RAPD analysis.

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