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Microevolution in a Natural Population of *Daphnia longispina* Exposed to Acid Mine Drainage

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Abstract—Persistent chemical contamination of the aquatic environment might alter the genetic diversity of resident natural populations, with evolutionary consequences. For example, regular exposure of a population to a certain pollutant can result in the loss of genotypes fitted to different environmental conditions. To address this problem, we studied a population of *Daphnia longispina* from an aquatic system contaminated with acid mine drainage, a metal-rich effluent. Previous results indicated that the impacted population has developed increased metal resistance and showed signs of genetic erosion, due to the disappearance of the most sensitive lineages from the initial population. Here, we have genotyped eight microsatellite markers in *D. longispina* lineages isolated from the impacted and reference populations. Strikingly, our results indicate that, although the average gene diversity is low, it is higher in the lineages from the impacted population. Furthermore, some genotypes were only present in the mine lineages, suggesting that the loss of genetic diversity due to chronic exposure to pollution might be counterbalanced by mechanisms that restore diversity, such as mutation and sexual reproduction.

Keywords: *Daphnia*, genotyping, microsatellites, microevolution, metal contamination, acid mine drainage

INTRODUCTION

Contaminated environments may influence the genetic structure and evolution of natural populations, either by causing alterations in individuals' DNA and increasing mutation rates, or by altering genetic variability and allele frequencies in the population (Bickham *et al*., 2000; Belfiore and Anderson, 2001; Roelofs *et al*., 2009). Increased mortality rates and selection acting on loci that are critical for fitness in the contaminated environment can result in a reduction of genetic diversity, as the most sensitive genotypes may be eliminated from the initial

population (Bickham *et al.*, 2000; Nowak *et al.*, 2009). Such genetic erosion causes a shift in the resistance of the overall population to the specific contaminants present, however, adaptation to a certain pollutant can result in the loss of genotypes fitted to different environmental conditions. Namely, studies in the crustacean *Daphnia magna* exposed to increasing cadmium concentrations have shown that the population became resistant to this metal, yet a reduction in genetic diversity was also observed that rendered the population sensitive to other contaminants (Ward and Robinson, 2005). Despite this, there are only a few studies on the impact of chronic chemical exposure at the population level (Bickham *et al.*, 2000; Belfiore and Anderson, 2001; Nowak *et al.*, 2009). The study of natural populations exposed to persistent chemical contamination provides a unique opportunity to study divergent genotypes that are usually present at very low frequencies under non-contaminated environments. These genotypes might reflect different mechanisms involved in chemical tolerance and that may be regulated by different alleles, which in turn can alter the direction of the population microevolution. Most studies use model organisms cultured in laboratory, thus neglecting the variability of molecular responses available in natural populations.

To address these questions, we are studying the effects of acid mine drainage (AMD), a metal-rich effluent, on a historically exposed population of *D. longispina*. Samples were collected in the São Domingos Mine aquatic system (Southeast (SE) Portugal), and in a non-contaminated site. From each population, cloned lineages were maintained in laboratory and the phenotype of each lineage was characterized for life cycle parameters, and for lethal and sublethal sensitivity to chemical contamination (metals and AMD). These previous studies revealed that the exposed *D. longispina* population exhibited an increased tolerance to lethal levels of metals comparatively to populations inhabiting non-contaminated aquatic ecosystems, and that such increased tolerance was genetically determined, since, even after acclimation for more that 15 generations under controlled laboratory conditions those differences persisted (Lopes *et al.*, 2004a, 2004b, 2005). Also, the differences in resistance to metals were most probably due to the disappearance of the most sensitive genotypes form the initial population, as the extremely and very sensitive lineages of *D. longispina* were only present in the population inhabiting the non-contaminated site (Lopes *et al.*, 2004b). This loss of genotypes may compromise the resilience of the population when exposed to future environmental changes. For example, if the most tolerant genotypes to metals (those that remained in the population) are the most sensitive to other type of chemical contamination, then the risk of extinction of this genetically eroded population will be increased under the exposure to such contamination (Lopes *et al.*, 2005).

To assess the consequences of long-term exposure to AMD at the genetic level, we have genotyped *D. longispina* lineages from both impacted and reference populations, using microsatellite markers (Fox, 2004; Brede *et al.*, 2006). Here, we present evidence that gene diversity is not decreased in the impacted population, on the contrary, some alleles were found uniquely in mine lineages. These results

Table 1. Microsatellite marker characterization. The repeat motif, the observed allele sizes and corresponding number of repeats is shown for each marker. The last column indicates the populations where the alleles were found. R, reference population. I, impacted population.

Marker	Motif	Allele sizes	Number of repeats	Population
Dgm102	(TTG)n … (TTG)n	122	8 … 4	R, I
		128	10 … 4	R, I
Dgm105	(CAG)n	187	7	R, I
		190	7	I
		197	10	R, I
Dgm106	(CAA)n	125	5	R, I
		134	7	R, I
Dgm107	(TGC)n	130	7	R, I
Dgm109	(ACC)n	252	3	I
		257	5	I
		261	5	R, I
		263	5	R, I
Dgm111	(CGT)n	206	5	R, I
		209	6	R, I
Dgm112	(TGC)n	108	1	I
		115	3	I
		118	3	R, I
		124	5	R, I
Dgm113	(GCT)n	143	7	R, I
		146	8	R, I

suggest that the loss of genetic diversity due to persistent exposure to AMD might be compensated by mechanisms that restore diversity, such as mutation and sexual reproduction.

MATERIALS AND METHODS

Natural populations of the crustacean *Daphnia longispina* were field-collected at the São Domingos Mine aquatic system (Southeast (SE) Portugal), which has been exposed to long-term contamination with acid mine drainage (N lineages); an unpolluted pound nearby was used as reference (E lineages). Cloned lineages of *D. longispina* were cultured in ASTM hard water, supplemented with seaweed extract, at 20°C and under a photoperiod of 14:10 hours (light: dark cycle), for more than 500 generations. Daphnids were fed everyday with 3×10^5 cells/ml of the green algae *Pseudokirchneriella subcapitata*. Twenty-two clones, eleven from each population, were used for microsatellite genotyping with eight markers

Fig. 1. Sequence analysis of the flanking regions of Dgm112. Sequence alignments show the number of repeats in each allele, and also highlight insertions/deletions (indels) that can account for the observed size differences in alleles with the same number of repeats.

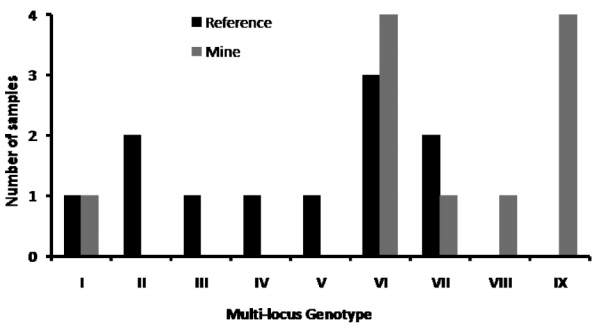

Fig. 2. Multi-locus genotype of the *D. longispina* lineages. Lineages of both reference and mine populations were distributed in multi-locus genotypes according to their allelic profile. Each category was classified arbitrarily by assigning a Roman numeral.

(Dgm102, Dgm105, Dgm106, Dgm107, Dgm109, Dgm111, Dgm112, and Dgm113) selected from previous reports (Fox, 2004; Brede *et al*., 2006). DNA was extracted from isolated individuals using Chelex (InstaGene Matrix, BioRad). Primers were labelled with 6-FAM, VIC, NED, and PET Standard Dye Sets (Applied Biosystems) for multiplex PCR reactions. The PCR-amplified products were run in an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems), using the GeneScan 500 LIZ size standard (Applied Biosystems), and fragment size analysis was carried out using the GeneMapper 4.0 software (Applied Biosystems). For sequencing reactions, PCR products were treated with ExoSAP-IT (USB Corporation) and used as a template for the sequencing reactions that were performed with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The DNA was purified with Sephadex G-50 Fine (GE Healthcare) and sequenced in an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). The results were analysed with the Sequencing Analysis 5.2 software from Applied Biosystems. Alignments of allele sequences were obtained using the MUSCLE software (Edgar, 2004), and were edited in Geneious (http://www.geneious.com).

RESULTS AND DISCUSSION

A total of 22 *D. longispina* lineages collected in a metal contaminated site (mine) and a non-contaminated site (reference) were used in this study for microsatellite genotyping, based on a panel of eight markers already described for the *D. longispina* group (Fox, 2004; Brede *et al.*, 2006). The list of all microsatellite alleles observed in our dataset is described in Table 1.

Overall, the genetic diversity was low, with some markers presenting only one (Dgm107) or two alleles (Dgm102, Dgm106, Dgm111 and Dgm113), while the others showed three (Dgm105) or four (Dgm109, Dgm112). Surprisingly, some alleles were only present in the lineages collected from the impacted population, such as the alleles Dgm105_190, Dgm109_252, Dgm109_257, Dgm112_108 and Dgm112_115 (Table 1), suggesting that the chronic exposure to AMD did not significantly reduce the genetic diversity of the *D. longispina* population.

In addition, when considering the number of repeats observed in each allele, we found that different alleles had a similar number of repeats (Table 1). As an example, the alignment of all allele sequences of Dgm112 is shown in Fig. 1. The alleles 115 and 118 have 3 repeats each, despite a difference in size of 3 base pairs. In order to determine the causes of such size differences, we have sequenced the flanking regions of all the alleles and found several insertions/deletions (indels), most frequent in mine lineages as illustrated in Fig. 1 (full data not shown). These results highlight the importance of combining fragment size determination with sequencing analysis in microsatellite studies of *D. longispina*.

A total of nine genotypes (identified by Roman numerals) were found in all lineages (Fig. 2). Interestingly, only three genotypes were found shared among individuals of both populations (I, VI and VII), while most genotypes were unique for one of the populations. Despite the presence of specific genotypes in the reference population (II to V), all alleles observed in the reference population were also observed in the mine lineages. On the other side, genotypes restricted to the mine population include five private alleles (Table 1).

In conclusion, microsatellite genotyping of *D. longispina* shows that both impacted and reference populations have low levels of genetic diversity, although sequencing analysis of microsatellite repeat regions reveals a higher variability in terms of base substitutions and indels. Additionally, the lineages isolated from the aquatic system contaminated with AMD were more diverse than lineages from a reference population, and the most divergent genotypes are found in the population historically exposed to metal stress. Consistent with our results, previous studies using AFLP markers found that AMD impacted populations do not have a reduction in their genetic diversity in comparison to reference populations (Martins *et al.*, 2009). Therefore, the loss of genetic diversity in natural populations under persistent exposure to pollution might be counterbalanced by mechanisms that restore diversity, such as mutation and sexual reproduction.

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