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LABORATORY CULTURING AND SELECTION FOR INCREASED RESISTANCE TO CADMIUM REDUCE GENETIC VARIATION IN THE LEAST KILLIFISH, *HETERANDRIA FORMOSA*

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Abstract—Populations exposed to environmental contaminants can undergo intense selection pressures, which in turn can lead to a loss of genetic variation. We assessed this loss of genetic variation in the least killifish *Heterandria formosa* for laboratory populations that had undergone eight generations of selection for an increased resistance to cadmium. Using microsatellite markers, we compared genetic variation between three selection and three control laboratory populations and between these laboratory populations and the source population. Heterozygosity was lower in each selection population than it was in its paired control population, with this difference being statistically significant in two of the three comparisons. This is evidence that adaptation to environmental contaminants can result in an overall loss of genetic variation. Furthermore, the laboratory populations had much lower heterozygosity than did the source population. The latter loss of genetic variation, probably a result of random drift, did not prevent the laboratory populations from showing a strong response to the selection for cadmium resistance. The loss of genetic variation resulting from maintaining populations in the laboratory demonstrates that it is important to maintain a large population size for such populations and that the potential for loss of genetic variation in laboratory populations is taken into consideration in ecotoxicology when extrapolating from laboratory to natural populations.

Keywords—Cadmium Selection Genetic variation Heterozygosity Effective population size

INTRODUCTION

Environmental contaminants can be strong selective agents, selecting for those phenotypes that have a reduced sensitivity to the contaminants [1–4]. Such directional selection is expected to result in the loss of genetic variation for the trait(s) selected for, as well as for loci linked to the genes under selection [5]. Studies on plants had results consistent with this hypothesis for metal-polluted sites, as well as for sites contaminated with organic pollutants [6,7]. However, studies on natural populations are difficult to interpret because of the various factors that can play a role. For example, some forms of pollution are known to increase mutation rates [8,9]. Furthermore, contaminants can affect genetic variation by altering migration or by causing population bottleneck events as a consequence of contaminant-related mortality or decreased fecundity [4,10]. It is therefore not surprising that exposure to pollutants has been associated with both decreased and increased genetic variation in natural populations [11–14]. In this study, we looked at laboratory populations that had undergone a specific selection regime and were paired with control populations maintained at the same population size, thus allowing us to focus on the effects of directional selection resulting from contaminant exposure.

We used nuclear microsatellite loci to determine whether intense selection for resistance to cadmium (Cd) in the least killifish, *Heterandria formosa*, has resulted in a loss of genetic variation. Microsatellites are DNA sequences comprising tandem arrays of one– to five–base pair repeating units. Microsatellite loci are not expected to be directly under selection, although they might be linked to sites that are involved in resistance mechanisms. More likely, these markers putatively sample across the genome randomly, making them useful for measuring the effects of selection on overall genetic diversity. Microsatellite loci show high levels of polymorphism, making them very useful for many population genetic studies [15,16]. The least killifish is a small, prolific, live-bearing fish common throughout the coastal plains of the southeastern United States. The laboratory populations used in this study were from an eight-generation experiment of Xie and Klerks [17] designed to study the responses to selection for resistance to Cd. They used three selection populations in which fish were selected for an increased resistance to Cd, with resistance quantified as survival time when exposed to a lethal Cd level and with fish contributing to the next generation chosen on the basis of their high resistance. In addition, each selection population was paired with a control population, in which fish contributing to the next generation were not exposed to Cd and in which sex ratios and population sizes were matched to those in the selection population. Additional details on the selection experiments are summarized in the *Materials and Methods* section. A rapid evolution of resistance occurred in that earlier study [17] and specific fitness costs were associated with this adaptation [18].

Our objective in this study was to assess the effects of selection on overall levels of genetic variation. In addition to comparing genetic variation between the control and Cd-adapted populations, we compared genetic variation in these laboratory-maintained populations to that in fish from the original source population. This allowed us to better understand the role of genetic drift on levels of genetic diversity in these populations.

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Table 1. Microsatellite loci used in this study on the least killifish *Heterandria formosa*, along with primer sequence and identity of species for which the primer had been reported. Polymerase chain reaction cycling conditions were similar except for annealing temperatures (AT)

Locus	Primer sequence	AT (°C)	Source species
HETF01	CCAAGGATATTGCTGACTCC TTCACTGAGCTTAAAGGCAG	53	*Xiphophorus montezumae* [19]
HETF02	CAGGACTTAGAATTAACAGG AGAACCAGTTGGACTGACAG	53	*Xiphophorus montezumae* [19]
HETF03	CATCCAGCCTGCTTAGTGAG TGTTCGTCATTAATTTGCAG	53	*Xiphophorus montezumae* [19]
HETF04	CACTACACTGGCAAACCCATC TCAAACCTCCTAGTATGACAA	60	*Poeciliopsis occidentalis* [20]
HETF05	CATAGATTCTGCAGGCAGTG CTCAGTGACTATAAGGCCAAC	62	*Poeciliopsis occidentalis* [20]
HETF06	TCATCTGGAGCAGGCACATG GCGTTTGGTTTCCTACTGAC	58	*Poecilia latipinna*[a]
HETF07	CGCCGCTTACCAGAACTTAAT TCAGGCTCTCTGTTTGTCCA	58	*Poecilia latipinna*[a]

[a] Kyoko Nakamura, 2001, Master's thesis, Florida International University, Miami, FL, USA.

MATERIALS AND METHODS

Screening of microsatellite markers

We screened microsatellite molecular markers identified in earlier studies on closely related poeciliid fish species. Those sequences were from *Xiphophorus montezumae* [19], *Poeciliopsis reticulata* [20], *Poecilia latipinna* (Kyoko Nakamura, 2001, Master's thesis, Florida International University, Miami, FL, USA), and *Gambusia affinis* [21]. We screened 27 pairs of microsatellite primers on DNA from 36 adult *H. formosa* collected in Louisiana (Lake Martin, St. Martin Parish, USA) and Florida (Wascissa River, Leon County, USA). Screening of polymerase chain reactions (PCRs) was performed by varying the annealing temperature ±4°C from the published temperature. Once a locus amplified, the PCR was repeated twice to confirm amplification. Among the 27 screened loci, 13 loci amplified with the *H. formosa* DNA. The 13 loci were screened a second time by PCR with fluorescently labeled, dinucleotide phosphates (DNTPs) to quantify the degree of polymorphism at each locus for the 36 fish. Among the 13 loci, seven had at least three alleles per locus and were chosen for use with our populations of *H. formosa* (Table 1).

Fish populations

We used the six *H. formosa* laboratory populations from the Xie and Klerks [17] study, in which three replicated populations were selected for an increased resistance to Cd and each selection population was paired with a control population. All the fish used in that laboratory experiment were collected from Lake Martin, Louisiana, in 1998. For each selection population, parents used to obtain the subsequent generation from that population consisted of fish that survived exposure to Cd. For each generation and each selection population, immature fish (9–12 mm standard length) were exposed to 6 mg/L of Cd until at least 50% of individuals had died. Additional post-exposure delayed mortality resulted in an overall average mortality of approximately 74%, such that the proportion selected averaged 0.257 over all generations and all selection populations. This methodology was repeated for eight generations. The number of fish used as parents for each generation was targeted at 60 and ranged from 55 to 64. Before breeding, fish of a selection population were sexed and counted. At this point, composition of the control (i.e., not Cd-exposed) population that was paired with this selection population were adjusted such that sex ratio and population size were the same as for the selection population. Thus, each selection population was paired with a control population, with both populations having the same degree of inbreeding resulting from having laboratory populations with a limited population size and a specific sex ratio. Additional details on the design of the selection experiment are reported in Xie and Klerks [17]. After eight generations of selection, a sixfold increase in resistance (quantified as time-to-death when exposed to a lethal level of Cd) was recorded. Xie and Klerks also looked at possible changes in Cd accumulation and detoxification underlying the increased resistance [22,23] and investigated the occurrence of fitness costs associated with the adaptation [18]. In this study, we further extend the selection experiment by evaluating the effects of selection on neutral genetic variation.

In the absence of information on genetic variability in the source population at the time of the start of the Xie and Klerks study, the population was sampled again in February and October 2003. The need for a large number of fish during the establishment of experimental populations had resulted in collections taking place at a series of closely situated sites in Lake Martin. Although we did not expect spatial differences along the shoreline of this lake over which sampling occurred (~500 m), such differences could potentially influence comparisons of genetic variation of the laboratory populations with the wild source. We compared 16 to 18 fish collected from each of three locations, separated by 100 to 200 m for genetic variation, corresponding to the portion of Lake Martin from which the original laboratory populations were obtained. Because no significant spatial differentiation was observed among the Lake Martin locations, those results are not presented here, and Lake Martin samples were combined to obtain the allele frequencies for the natural source population.

DNA extraction, amplification, and electrophoresis

All DNA from the fish samples was extracted with Gentra's Puregene™ DNA isolation protocol (Qiagen, Valencia, CA, USA). Because least killifish are small and sexually dimorphic with respect to size (with males being significantly smaller than females), we generally used whole fish when working with young individuals or males and tail muscle when working with females.

Microsatellite loci were amplified by PCR with a MyCycler personal thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). The cycling conditions for all loci began with a 20-min denaturation at 94°C followed by 35 amplification cycles with 40-s denaturation at 94°C, 45 s at an optimum annealing temperature for each primer pair (Table 1), a 1-min ramp to 72°C, and a 1-min extension at 72°C and concluded with a 20-min final extension at 72°C. Polymerase chain reaction amplifications were performed in a 15-μl final reaction volume containing 80 to 120 ng of genomic DNA template, 1 pmol of fluorescently labeled forward primer, 10 pmol of reverse primer (Bio-Synthesis, Lewisville, TX, USA), 10 nM of each DNTP, 0.6 U of Amplitaq Gold DNA polymerase and 10× PCR buffer (Applied Biosystems, Foster City, CA, USA).

Fluorescently labeled PCR products were electrophoretically separated and analyzed on an ABI Prism 310 and ABI 3100 Genetic Analyzer (Applied Biosystems). The product

sizes determined with Genescan Genetic Analysis software (Ver 3.1, Applied Biosystems), were used to assign genotypes to each individual.

Population genetic and statistical analyses

We used Tools For Population Genetic Analysis (TFPGA) ([24]; http://www.marksgeneticsoftware.net/) software to generate, for each population and each of the microsatellite loci, Hardy–Weinberg (H-W) expected and observed heterozygosities. Genepop On the Web (GOW) [25] was used to perform exact tests to assess whether the observed and expected heterozygosities differed significantly and to calculate Weir and Cockerham F_{is} values [26]. These F values can range from -1 to $+1$, with values approaching 1 being indicative of a high degree of heterozygote deficiency. We used Genetic Data Analysis (GDA) ([27]; http://hydrodictyon.eeb.uconn.edu/people/plewis/software.php) software to calculate allele frequencies for each population and to obtain estimates of overall expected heterozygosity in each population. Genetic Data Analysis was also used to assess the occurrence of linkage disequilibrium among loci. Paired t tests, conducted with SAS® (Statistical Analysis System Institute, Cary, NC, USA) were used to compare heterozygosities among populations.

RESULTS

Population heterozygosities did not deviate from those expected on the basis of H-W equilibrium for the Lake Martin source population (Table 2). However, they did differ from H-W expectations at many of the microsatellite loci in most of the experimental populations, with differences being statistically significant ($p < 0.05$) in 64% of the cases (71 and 57%, respectively, in selection and control populations). All but one of the 27 instances of statistically significant differences were cases of heterozygote deficiencies, with observed heterozygosities on average 39% lower than expected. The lower-than-expected population heterozygosities were also reflected in the Weir and Cockerham F_{is} statistic, with most values being well above zero. In the selection populations, F_{is} values averaged 0.512 while averaging 0.338 in the control populations. In contrast, F_{is} values for the Lake Martin population averaged -0.018.

The overall population heterozygosity estimate for the Lake Martin source population (0.732) was substantially higher than estimates for the experimental populations (averaging 0.475), with this difference being statistically significant for each of the six experimental populations. The lower genetic diversity in the experimental populations (relative to the Lake Martin population) was also reflected in the average number of alleles per locus (Table 3). This value was about twice as high in the Lake Martin population than it was in the experimental populations. Each of the experimental populations had lost one or more alleles (relative to the Lake Martin population) for each of the seven loci analyzed, whereas Lake Martin fish had three alleles that were not found in any of the experimental populations (not shown here; see N.R. Giridhar Arthrey, 2004, Master's Thesis, University of Louisiana at Lafayette, Lafayette, LA, USA, for a complete listing of allele frequencies).

The overall heterozygosity was in general a bit lower for the selection populations (averaging 0.454) than it was for the control populations (averaging 0.496), with the difference being statistically significant in two of the three pairwise comparisons (Table 3). The selection and control populations showed no clear difference with respect to the average number of alleles per locus; for none of the three sets of populations was the average number of alleles per locus significantly different between the selection and the control populations.

Table 2. Hardy–Weinberg expected (H_{HW}) and observed (H_{obs}) heterozygosities for each of seven loci for the source population (Lake Martin, Louisiana, USA), cadmium-selected and control populations. The p values are from exact tests determining deviations from Hardy–Weinberg expectations, whereas the F_{is} Weir and Cockerham inbreeding coefficient is based on the difference between expected and observed heterozygosities

Locus	Population	H_{HW}	H_{obs}	p	F_{is}
HETF01	Lake Martin	0.720	0.700	0.9448	0.028
	Selection 1	0.505	0.420	0.2847	0.173
	Control 1	0.500	0.327	0.0217	0.349
	Selection 2	0.416	0.200	<0.0001	0.952
	Control 2	0.483	0.227	0.0002	0.534
	Selection 3	0.435	0.117	<0.0001	0.731
	Control 3	0.428	0.110	<0.0001	0.785
HETF02	Lake Martin	0.615	0.560	0.4714	0.085
	Selection 1	0.481	0.455	0.7792	0.055
	Control 1	0.502	0.384	0.1015	0.235
	Selection 2	0.243	0.400	<0.0001	0.837
	Control 2	0.447	0.208	0.0002	0.538
	Selection 3	0.455	0.216	0.0003	0.529
	Control 3	0.480	0.260	0.0012	0.462
HETF03	Lake Martin	0.745	0.800	0.4961	−0.074
	Selection 1	0.503	0.330	0.0143	0.352
	Control 1	0.505	0.590	0.2626	−0.183
	Selection 2	0.389	0.120	<0.0001	0.693
	Control 2	0.464	0.302	0.0157	0.352
	Selection 3	0.472	0.078	<0.0001	0.835
	Control 3	0.503	0.315	0.0076	0.376
HETF04	Lake Martin	0.671	0.640	0.1814	0.047
	Selection 1	0.494	0.310	0.0061	0.376
	Control 1	0.451	0.442	>0.9999	0.019
	Selection 2	0.285	0.140	0.0018	0.511
	Control 2	0.383	0.283	0.0679	0.263
	Selection 3	0.363	0.274	0.1154	0.247
	Control 3	0.359	0.278	0.1249	0.228
HETF05	Lake Martin	0.754	0.840	0.3021	−0.115
	Selection 1	0.323	0.254	0.1938	0.213
	Control 1	0.503	0.443	0.4104	0.122
	Selection 2	0.447	0.180	<0.0001	0.599
	Control 2	0.447	0.170	<0.0001	0.622
	Selection 3	0.461	0.157	<0.0001	0.662
	Control 3	0.487	0.370	0.0924	0.242
HETF06	Lake Martin	0.790	0.900	0.3901	−0.141
	Selection 1	0.502	0.382	0.1075	0.241
	Control 1	0.496	0.404	0.2590	0.187
	Selection 2	0.500	0.140	<0.0001	0.722
	Control 2	0.500	0.301	0.0051	0.399
	Selection 3	0.489	0.235	0.0002	0.522
	Control 3	0.504	0.426	0.2808	0.157
HETF07	Lake Martin	0.834	0.800	0.9595	0.042
	Selection 1	0.671	0.545	0.0687	0.189
	Control 1	0.648	0.403	0.0008	0.379
	Selection 2	0.476	0.240	0.0006	0.498
	Control 2	0.655	0.264	<0.0001	0.599
	Selection 3	0.626	0.118	<0.0001	0.814
	Control 3	0.661	0.370	0.0004	0.442

DISCUSSION

The overall patterns observed in the present study were: allele frequencies in the laboratory populations being out of H-W equilibrium and exhibiting substantial heterozygote deficiencies, a lower genetic diversity in the laboratory selection populations (selected for an increased resistance to Cd) than in their matched laboratory control populations, and a lower genetic diversity in all the laboratory populations than in the Lake Martin source population.

Table 3. Overall genetic variation in the source population (Lake Martin, Louisiana, USA) and the selection and control laboratory populations, expressed as the average number of alleles per locus and the Hardy–Weinberg expected heterozygosity (H_{HW}). About 50 fish (n) were analyzed for each population. Paired t tests (with results reported as T and corresponding p values) compared natural versus experimental populations ($T_{N\Leftrightarrow E}$, $p_{N\Leftrightarrow E}$) and control versus selection populations ($T_{C\Leftrightarrow S}$, $p_{C\Leftrightarrow S}$)

Population	n	Alleles/locus	H_{HW}	$T_{N\Leftrightarrow E}$	$p_{N\Leftrightarrow E}$	$T_{C\Leftrightarrow S}$	$p_{C\Leftrightarrow S}$
Lake Martin	50	4.2	0.7323				
Selection 1	55	2.1	0.4969	6.37	0.0007	0.64	0.5440
Control 1	52	2.1	0.5150	10.16	<0.0001		
Selection 2	50	2.0	0.3937	26.72	<0.0001	2.96	0.0252
Control 2	53	2.1	0.4827	12.42	<0.0001		
Selection 3	51	2.1	0.4718	12.82	<0.0001	2.73	0.0342
Control 3	54	2.1	0.4891	12.60	<0.0001		

Deviations from H-W equilibrium

Hardy–Weinberg equilibrium assumes random mating, a very large population size, and the absence of migration, mutation, and selection. The deviations from H-W expectations for microsatellite allele frequencies observed for both selection and control populations might be due to violations of any of these assumptions. Furthermore, in the case of microsatellite loci, such deviations can also result from the presence of null alleles (which result from mutations in the primer binding sites that fail to produce an allele product). The occurrence of null alleles is not a likely explanation for the observed deviations because these would also occur for fish in the Lake Martin source population (for which allele frequencies did not deviate from H-W expectations). Similarly, it is unlikely that migration and mutation were significant contributors because of the nature of the study design (we maintained closed laboratory populations) and the relatively short timescale on which this experiment was conducted. This leaves selection, inbreeding or nonrandom mating, and genetic drift as potential causes for the deviations from H-W equilibrium. The selection for an increased resistance to Cd is likely to be responsible for the more frequent deviations from H-W equilibrium in the selection populations than in the control populations. However, it does not explain why deviations were also observed in control populations. All experimental populations could have been (inadvertently) selected for optimum performance under laboratory conditions, whereas the selection study's approach of exposing individuals in batches as groups of offspring were raised could have favored specific family groups. Both processes are common in laboratory populations [28,29].

Reduction in genetic variation

The selection populations had heterozygosities that were 10 to 20% (depending on the specific parameter) lower than those found for their associated control populations. It is well established that selection for a quantitative trait (such as Cd resistance) results in lower genetic variation for this trait [30]. However, microsatellites are selectively neutral, such that selection for increased Cd resistance should not directly favor specific microsatellite alleles and thereby reduce genetic variation. One possibility for explaining decreased heterozygosity would be physical linkage of some of the genetic markers we examined with loci involved with increased metallothionein induction and reduced Cd uptake [22,23]. This phenomenon, known as genetic hitchhiking [31], has been observed in insecticide-resistant mosquito populations [32]. We find this explanation unlikely in our case because of the low probability that one or more of the seven microsatellite loci, chosen without knowledge of their location in the genome, would be located close enough to a locus under selection to have its diversity significantly affected. Furthermore, examination of heterozygosity estimates for individual loci suggest that losses of heterozygosity in the selected lines, relative to controls, occurred across multiple loci and did not occur at the same loci in each of the selected lines (Table 2). This is not the pattern that would be expected if one or two of the microsatellite loci were physically linked loci responsible for Cd resistance.

A more likely possibility for the observed reduction in heterozygosity in selection populations relative to their controls might be higher variance in reproductive success in selected populations. This would occur if some families were more resistant to Cd than others. The greater the differential survival of families, the more likely that the genetic effective population size will only be a fraction of the actual population size [33].

In addition to the differences in heterozygosity between selection and control population, we observed a significant reduction in genetic variation in all experimental populations compared with the natural source population. This is similar to the situation in the Ward and Robinson [34] study, in which *D. magna* was selected for an increased Cd resistance, with genetic variation also decreasing in the control population. Laboratory populations tend to be relatively small, increasing the chance that genetic variation is lost randomly as a consequence of genetic drift [35,36]. In the least killifish selection experiment, we aimed to avoid substantial inbreeding and genetic drift by starting each generation with approximately 60 fish [17] at a sex ratio of approximately 1:1. The observed loss of genetic variation in the control lines is substantially higher than would be expected in a randomly mating population of this size at H-W equilibrium. It is possible however that effective population sizes were much lower than 60 because individual fish could have differed greatly in their reproductive success, thereby reducing the effective population size to a fraction of the actual population size [33]. To estimate what the effective size of the laboratory populations might have been, we used the equation $H_T = H_0(1 - 1/2n)^T$, where H_T is the heterozygosity at generation T, H_0 is the initial heterozygosity, n is the effective population size, and T is the number of generations [37]. With gene diversity (H_{HW}) of the Lake Martin population as the initial heterozygosity H_0, with H_{HW} of each experimental population as H_T at $T = 8$, we calculated the effective population size that would result in the heterozygosity observed for each experimental population. The loss of heterozygosity in the experimental lines relative to the Lake Martin source population indicates that effective population sizes could have been as low as 20% of the numerical population sizes. In comparison, if 60 individuals re-

produce and contribute equally to the next generation during eight generations, genetic variation would decrease by only approximately 10% because of small population size. Low genetically effective population size could be a result of loss of genotypes because of mortality during selection and laboratory culturing, resulting in fewer genotypes available for the next generation. However, there appeared to have been sufficient additive genetic variation in our laboratory lines, in spite of the substantial loss of genetic variation documented here, for a continued response to selection during the eight generations of the selection experiment [17].

In recent literature, some have speculated that exposure and selection for resistance to toxicants in nature might not result in a reduction in genetic diversity. Some studies appear to indicate that the level of contamination in natural environments is not correlated with genetic diversity or with alteration in allele frequencies [38,39]. Past studies of natural populations might have failed to detect decreased genetic diversity in natural populations experiencing selection for Cd resistance because, in natural populations, gene flow is more difficult to estimate or control and hence remains a confounding factor when measuring actual reduction of genetic diversity. Furthermore, most past studies have examined the effects of selection for resistance to toxicants on genetic diversity with the use of allozymes. Highly polymorphic microsatellite loci are more sensitive to losses of genetic diversity than are allozymes [40].

The results from this study have two main implications. First, the loss of genetic variation at genes not directly under selection means that populations that have managed to genetically adapt to an environmental pollutant might be less able to adapt to other environmental stressors. This could be exacerbated by negative effects on fitness-associated characteristics that are sometimes associated with contaminant adaptation, as shown earlier for example for the Cd-adapted lines used in this study [18]. Second, the loss of genetic variation in our laboratory lines that appeared to be due to effective population sizes being much lower than actual population sizes points out the necessity of maintaining large laboratory population sizes of organisms used in environmental toxicology studies that aim to reflect the behavior of natural populations from a population genetic standpoint.

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