DIFFERENTIAL TOLERANCE AMONG CRYPTIC SPECIES: A POTENTIAL CAUSE OF POLLUTANT-RELATED REDUCTIONS IN GENETIC DIVERSITY

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Abstract—Differential mortality of cryptic species (i.e., morphologically similar but genetically distinct sibling species) may contribute to observed reductions in genetic diversity at contaminated sites if the members of a complex of cryptic species exhibit differential responses to the contaminants that are present. We conducted toxicity bioassays with both polynuclear aromatic hydrocarbon and metal contamination on Cletocampus fourchensis and C. stimpsoni from two intensively sampled locations. Previous molecular and detailed morphological analyses segregated these as cryptic species from the cosmopolitan C. deitersi. We found that these species occur together at two field sites and that they exhibit unique toxic responses to heavy metals, suggesting differential tolerances at contaminated sites. These findings suggest that reported losses of genetic diversity at contaminated sites may represent a reduction in species diversity rather than a loss of the presumed less-tolerant genotypes within a species. They also suggest that members of a cryptic species complex should not be used in laboratory toxicity tests unless populations are genetically characterized. Future studies using genetic diversity as a marker of contaminant effects should consider the possibility of undetected cryptic species.

Keywords—Phenanthrene Metals Acute exposure Cryptic species Cletocampus sp.

INTRODUCTION

In several recent surveys of DNA polymorphisms, exposure to contaminants has been associated with a reduction in genetic diversity [1–4]. These findings have led to recent proposals to use genetic diversity of a population as an indicator of contaminant effects [3]. However, a loss of genetic diversity in populations from contaminated sites compared to reference sites may have several causes [5–8], some of which act indirectly by altering the demography or other population-level characteristics of a species. One possible cause is the direct result of exposure to contamination via the process of genotypic selection. Selection may favor some genotypes over others, causing population-level genetic change. Genes that experience a reduction in diversity need not directly function in tolerance. Linkage with genes that are under selection may be responsible for the loss in diversity observed in samples from contaminated sites for both mitochondrial and nuclear genomes [9,10]. Second, a population that is reduced in abundance because of exposure to a toxicant may experience a genetic bottleneck. If so, genetic diversity in exposed populations may be reduced compared to reference populations through the process of genetic drift. In addition to its possible use as an indicator of contaminant effects, loss of genetic diversity in exposed populations is potentially important for both ecological and evolutionary reasons, because genetic bottlenecks are often associated with local population fitness as a result of inbreeding depression [11]. A third possible explanation for reduced genetic diversity in samples from contaminated sites is contaminant-induced reduction in migration rates, which would tend to augment the effect of genetic bottlenecks.

Loss of genetic diversity is not the only observed consequence of contaminant exposure. As reviewed elsewhere [12,13], previous research has sometimes found that exposure to contaminants (heavy metals have been the most frequently studied) is associated with increased or unchanged genetic diversity compared to unexposed populations, particularly for studies of genetically controlled protein variation. These outcomes also have several possible causes, including selection favoring heterozygotes or rare alleles, increased mutation rates, and contamination-induced increases in migration rates [8].

Studies of contaminant-associated shifts in genetic diversity have paid less attention to another potential complicating factor. Reductions in genetic diversity observed in some population samples from contaminated sites may represent a loss of species diversity through local contaminant-caused extinction of one or more members of a cryptic species complex rather than a within-species loss of putatively less-tolerant genotypes or haplotypes. Cryptic species are morphologically similar but genetically distinct sibling species. Such complexes occur within numerous cosmopolitan and previously well-studied (based on classical morphologically based systematics) invertebrates [14]. In fact, many taxa that thrive in polluted habitats belong to complexes of cryptic species [15–19]. Although the possible existence of cryptic species has been mentioned in previous studies of pollution effects (see, e.g., [20]), few investigators have explicitly examined this possibility and its implications. If cryptic species with differing levels of pollution tolerance are common, then the apparent reduction in genetic diversity of polluted environments could represent a loss of species diversity rather than genotypic selection. Alternatively, the unrecognized presence of cryptic species with equivalent pollution tolerance could potentially conceal a pollution-associated reduction in genetic diversity within each

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taxon. Because genetic divergence between species is usually much larger than that within species, the reduction in intraspecific diversity associated with exposure to a contaminant would not be easily detectable against a background of unchanged interspecific diversity.

Species-specific responses to pollutant exposure are the basis of most observational and experimental studies in ecotoxicology. However, some commonly used taxa in laboratory research harbor cryptic species. For example, analyses of *Hyalella azteca*, an amphipod extensively used in toxicological bioassays, have established the existence of genetic groups with levels of differentiation characteristic of distinct species in six North American laboratory stocks [16,21]. Utilizing taxa with such high genetic divergence to determine no-effect criteria will represent an unrecognized source of variation in the response to pollutant exposure if the tolerances among cryptic species differ. Although recent studies have reported examples of cryptic or sibling species that differ in habitat preference [22–25], studies of tolerance to pollution in which the investigators have explicitly examined more than one member of a cryptic species complex are rare [17,26].

The goal of the present study was to determine if cryptic species of a meiofaunal copepod differ in their sensitivity to common pollutants. Harpacticoid copepods (crustaceans ~1 mm in length and ~1 µg in dry mass as adults) and other meiofaunal taxa frequently harbor cryptic species (see, e.g., Volkman-Rocco and Fava [27]), and several species are commonly used as laboratory toxicity-test species (see, e.g., [28,29]). The harpacticoid *Cletocamptus deitersi* (Richard) lives in inland brines (seeps, streams, and lakes) and coastal estuaries and mangroves with a reported cosmopolitan distribution [30]. This taxon is unusually tolerant of salinity [31] and hydrocarbon contaminants, and it is commonly found in disturbed environments and contaminated sediments [32,33]. Recently, molecular [34] and morphological [30] analyses have segregated four cryptic species within the *C. deitersi* complex in North America. Here, we analyze tolerance to a nuclear aromatic hydrocarbon and a mixture of metals in two of these species (*C. stimpsoni* and *C. fourchensis*).

MATERIALS AND METHODS

**Source of Cletocamptus sp.**

Specimens of *Cletocamptus* sp. were obtained from surface-exposed sediment from an inland brine seep near Jackson (AL, USA; 31°23′N, 87°53′W) and from a *Spartina alterniflora* salt marsh near Port Fourchon (LA, USA; 29°05′4″N, 90°05′8″W). Specimens were collected for a phylogenetic analysis, and relative abundances of the two cryptic species (*C. fourchensis* and *C. stimpsoni*) encountered at both sites were determined by analysis of sequence data of the mitochondrial cytochrome *c* oxidase subunit I (COX-I) gene as reported previously [34].

Collections for bioassay experiments were carried out from November 1999 to April 2000. The upper 1 cm of field sediment was skimmed, placed in buckets with water from the collection site, and transported to the laboratory. In the laboratory, overlying water was replaced with 25% seawater (ASW; Instant Ocean Aquarium Systems, Mentor, OH, USA) and maintained at 4°C with aeration. Harpacticoid specimens were transferred to sediment-free cultures, which were maintained in 350 ml of 25% ASW and fed the green alga *Selenastrum capricornutum* twice a week in conditions similar to those described previously [28]. After an acclimation period of at least 5 d, copepods were used in bioassays.

**Contaminant dosage**

*Phenanthrene.* Experimental solutions were prepared from crystallized phenanthrene (98% purity; Aldrich Chemical, Milwaukee, WI, USA) stocks (0.5 and 1 g L⁻¹) dissolved in acetone. Based on the findings of Emery and Dillon [35], an initial range-finding experiment was conducted with *Cletocamptus* organisms from Louisiana (USA) using the procedures described below (see Materials and Methods, Toxicity bioassays) in triplicate at two nominal phenanthrene concentrations (low, 250 µg L⁻¹; high, 1,000 µg L⁻¹; plus solvent control; stock, 1 g L⁻¹), which resulted in very high survivorship in exposed and control treatments (see Results). Given the level of phenanthrene solubility in seawater (1,002–1,290 µg L⁻¹ [36]), we opted to use phenanthrene-saturated ASW in subsequent experiments (1,280 µg L⁻¹, as measured by high-performance liquid chromatography; stock, 0.5 g L⁻¹). Stock solutions were measured before dosage, and experimental solutions were measured at the end of the experiments.

*Metal.* Metal toxicity tests were carried out with a mixture of three metals in proportions similar to those found in the contaminated water brought to the surface through production of crude oil or natural gas (i.e., produced water; Zn: Pb: Cd, 100:10:0.5) [37]. Target concentrations were obtained from stock aequous solutions of ZnCl₂ (Zn concentration: nominal, 1,000 µg L⁻¹; actual, 1,014 µg L⁻¹), PbCl₂ (Pb concentration: nominal, 100 µg L⁻¹; actual, 99 µg L⁻¹), and CdCl₂ (Cd concentration: nominal, 1,000 µg L⁻¹; actual, 820 µg Cd L⁻¹) (Aldrich Chemical). Based on a lethal concentration that kills 50% of test animals (LC50; Zn concentration, 708 µg L⁻¹) found in the harpacticoid *Schizopera knabeni* (J.W. Fleeger, unpublished data), a range-finding experiment was designed for *Cletocamptus* from Louisiana using procedures described below (see Materials and Methods, Toxicity bioassays) in triplicate at three nominal concentrations of Zn (low, 1,000 µg L⁻¹; medium, 1,500 µg L⁻¹; high, 2,000 µg L⁻¹), which resulted in very high survivorships in exposed and control treatments (see Results). Given this result, we opted to use Zn-saturated ASW in subsequent experiments (endpoint mean concentrations: Zn, 6,249 ± 480.8 µg L⁻¹; Pb, 685 ± 67.5 µg L⁻¹; Cd, 62 ± 3.7 µg L⁻¹). All metal concentrations were verified by inductively coupled plasma-emission spectroscopy after correction for interelement interference. Stock solutions were measured before dosage, and experimental solutions were measured at the end of the experiments.

**Toxicity bioassays**

Bioassays measured acute toxicity (96 h) of aqueous exposure to phenanthrene and the metal mixture. Experiments were conducted in 35- x 50-mm crystallizing dishes (Kimble, Toledo, OH, USA) containing 20 ml of 25% ASW without food. Dishes were placed inside loosely covered plastic containers over soaked paper towels to retard evaporation and were incubated at constant temperature (25°C) in total darkness.

Phenanthrene bioassays were conducted with 10 *Cletocamptus* organisms per dish (*n* = 3 replicates). In addition, solvent and ASW controls (*n* = 2 replicates each) were incubated simultaneously. Because of the limited number of co-
pepods available from Alabama (USA), no ASW control was conducted for this treatment. The number of living copepods was tallied at the end of the 96-h incubation period.

Metal bioassays were conducted with 20 Cletocamptus organisms per dish (n = 8 replicates). In addition, ASW controls (n = 2 replicates) were incubated simultaneously. The number of living copepods was tallied every 3 h for 96 h. Dead copepods were removed and placed in 95% ethanol for subsequent identification through molecular analysis.

Species identifications

Specimens were placed in 95% ethanol until genotyped with the multiplex haplotype-specific polymerase chain reaction (MHS-PCR) method [34] applied to the mitochondrial COX-I gene. Briefly, this method uses primers complementary to diagnostic regions of the gene to produce haplotype-specific amplions that can be readily resolved in agarose gel electrophoresis. Methodological details have been described previously [34].

Statistical analyses

Fractions of surviving copepods at the end of 96-h acute toxicity tests were arcsine square-root transformed following Freeman and Tukey, Equation 14.5 of Zar [38] before parametric analyses to conform to normality assumptions. Survivorships of exposed and control treatments were compared using Dunnett’s two-tailed t test (α = 0.05) in range-finding experiments. In bioassays, the effects on survivorship of exposure, species, and their interaction were compared using a two-way analysis of variance (ANOVA) with replication using SAS® PROC GLM routine (Ver 8.2; SAS Institute, Cary, NC, USA) using type III sums of squares in bioassays. Survivorship to metals was also analyzed using Fisher’s exact tests (SigmaStat®, Ver 2.0; Jandel Scientific, San Rafael, CA, USA) to assess the significance of differences between species. In addition, a survival analysis applicable to censored observations was used to compare survivorship curves for the two Cletocamptus sp. exposed to metals with the nonparametric Cox-Mantel test (Statistica®, Ver 5.5A; StatSoft, Tulsa, OK, USA).

RESULTS

Relative abundance patterns

Cletocamptus fourchensis and C. stimpsoni are not readily distinguishable (i.e., body length and width, rostrum shape, caudal rami shape, and leg segmentation are essentially identical under observation in a dissection microscope [39]). The individual species can be identified by detailed inspection of dissected body parts [30], genetic characterization [34], or optical analytical methods using digitized images subject to position-invariant classification algorithms [39]. All specimens used in the present study, including surviving individuals in bioassays, were genotyped to be assured of identity. The MHS-PCR analysis of the COX-I gene showed that C. fourchensis and C. stimpsoni were sympatric at collecting sites in both Louisiana and Alabama. Relative abundance patterns, however, differed at the two sites. Cletocamptus fourchensis dominated collections from Louisiana, comprising 92.3%, but was rare in collections from Alabama (7.2%). Cletocamptus stimpsoni was the most common taxon in collections from Alabama, comprising 92.8%, but was rare in collections from Louisiana (7.7%).

Table 1. Two-way analysis of variance with replication for effect of species identity and contaminant exposure on arcsine-transformed percentage of test subjects surviving to 96 h

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Source</th>
<th>Sum of squares</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenanthrene</td>
<td>Species</td>
<td>0.0001</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Exposure</td>
<td>0.1340</td>
<td>8.08</td>
</tr>
<tr>
<td></td>
<td>Species × exposure</td>
<td>0.0051</td>
<td>0.30</td>
</tr>
<tr>
<td>Metals</td>
<td>Species</td>
<td>0.0237</td>
<td>5.46</td>
</tr>
<tr>
<td></td>
<td>Exposure</td>
<td>0.3548</td>
<td>81.67***</td>
</tr>
<tr>
<td></td>
<td>Species × exposure</td>
<td>0.1414</td>
<td>32.54***</td>
</tr>
</tbody>
</table>

*All mean squares have one degree of freedom and so are identical to their respective sum of squares.

**All effects are fixed. Species refers to Cletocamptus fourchensis versus Cletocamptus stimpsoni.

***p < 0.001 (adjusted for multiple tests).

MHS-PCR genotyping

All copepods from bioassays were successfully genotyped using MHS-PCR of the COX-I gene. Copepods collected in Alabama were all C. stimpsoni, the predominant species found at that locality. Similarly, all those copepods examined in bioassays but collected in Louisiana were C. fourchensis.

Toxicity bioassays

Range-finding experiments revealed very high levels of survivorship and no significant treatment effect of exposure to phenanthrene (87.0%, q’ = 0, p > 0.5) and metals (94.6%, q’ < 0.03, p > 0.5) with respect to controls. Therefore, toxicity bioassays were carried out at near-saturation phenanthrene and metal concentrations.

Endpoint survivorship levels in phenanthrene-saturated ASW also revealed high tolerance of C. fourchensis and C. stimpsoni to this xenobiotic. Approximately 80% of each species survived 96-h experimental exposures. The ANOVA (Table 1) indicated that neither species nor exposures were significant sources of variation in survival rate, and the interaction term was also not significant (p > 0.05 in each case) (Fig. 1).

The two species were also highly tolerant to metals. However, ANOVA (Table 1) indicated that exposure and species × exposure effects were each significant (p < 0.001) sources of variation in survival rate, whereas species alone was not (p > 0.05) (Fig. 1). Time-course observations revealed that survivorship of both lineages was very similar until 70 h of incubation. Thereafter, survivorship in C. stimpsoni (all collected from Alabama) decreased markedly, to slightly less than 50%, whereas more than 75% of C. fourchensis (all collected from Louisiana) survived at the end of the experiment (Fig. 2). Both the endpoint survivorships (Fisher’s exact test) and the survival curves (Cox-Mantel test) were significantly different (p < 0.001) between the two species.

DISCUSSION

Our results suggest that a mechanism involving cryptic species exists by which apparent genetic diversity at contaminated sites may be reduced. For cryptic species to contribute to losses in genetic diversity at contaminated sites, three observations must be true. First, the taxa studied must form a cryptic species complex or be easily misidentified (to, in effect, act as cryptic species). Harpacticoid copepods in the cryptic species complex associated with C. deitersi (a taxon found at contaminated sites [32]) were selected for intensive study. The
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Fig. 1. Percentage survival of Cletocamptus fourchensis and C. stimpsoni for individuals exposed and unexposed (control) to saturated phenanthrene (top) and a mix of heavy metals (bottom; see text for composition) for 96 h. Error bars show ± one standard error of the mean. No variation was observed among replicates for the C. fourchensis metals-control individuals.

Fig. 2. Survivorship curves of Cletocamptus fourchensis and C. stimpsoni in 96-h exposures to heavy metals. C. f. exposed = C. fourchensis exposed to metals; C. f. control = C. fourchensis not exposed to metals; C. s. exposed = C. stimpsoni exposed to metals; C. s. control = C. stimpsoni not exposed.

two chosen Cletocamptus spp. exhibit large genetic distances for two mitochondrial genes, COX-I and 16S rDNA, suggesting they diverged at least 38 million years ago [34]. The large mitochondrial distances separating C. stimpsoni and C. fourchensis are accompanied by concordant differentiation in nuclear gene sequences [34] as well as significant differences in morphology, validating the taxonomic status of these two species [30]. Second, cryptic species must co-occur at uncontaminated sites. Our research examined this possibility with Cletocamptus at two locations. Third, cryptic species must exhibit different responses to contaminants such that differential mortality occurs at contaminated sites. We examined this possibility by conducting toxicity bioassays with C. fourchensis and C. stimpsoni with both hydrocarbon and metal contamination. If these three conditions are met, then apparent losses in intraspecific genetic diversity at contaminated sites may actually be the result of losses in species diversity because of a reduction in the number of cryptic species.

The taxonomy of small crustaceans including harpacticoid copepods is based on morphological characters that must be visualized with high-power microscopy after whole-body dissection. Modern microscopy allows the use of subtle features associated with body ornamentation and mouthpart structure in systematics, and extremely rigorous criteria for the erection of harpacticoid species now exist [40]. These standards suggest that apparent cryptic harpacticoid species can be resolved morphologically [30,41]. However, the dissection required for morphological observation destroys tissue in very small animals, precluding DNA extraction and taxonomic analysis on the same individuals. Furthermore, studies using restriction fragment length polymorphisms may underestimate the true amount of divergence between sequences, because restriction enzyme digestion samples only a portion of the nucleotide site diversity in a particular gene region [42]. We used direct sequencing of PCR products as our primary molecular technique, and each individual used in bioassays was subjected to genotyping to clarify species status to avoid these pitfalls.

Based on our molecular data, two cryptic species co-occurred at each of two intensively studied field sites. Similarly, Fleeger [43] found two morphological patterns in dissected specimens of Cletocamptus from Louisiana corresponding to C. stimpsoni and C. fourchensis, suggesting that coexistence of sympatric species occurs over long periods of time. If a study of genetic structure in Cletocamptus sp. using restriction fragment length polymorphisms was conducted from either site, and if many individuals were processed for genetic analysis without examination of relevant morphological features, both species would likely be included and coded as a single species.

Our research suggests that C. stimpsoni and C. fourchensis from two locations have similar tolerances to the polycyclic aromatic hydrocarbon phenanthrene but different tolerances to a mixture of heavy metals. The results of 96-h bioassays reveal that both species are very resistant to phenanthrene. Mortality in control populations did not differ from that in populations exposed to concentrations of phenanthrene at the limit of solubility in aqueous solution. Both C. fourchensis and C. stimpsoni appear to be more tolerant than other estuarine species. Phenanthrene causes mortality at sediment concentrations less than saturation in other benthic harpacticoids [28,29], and Carmean et al. [32,33] found that nominal C. deitersi survives diesel contamination when most other harpacticoid species suffer severe mortality in microcosms. Both C. stimpsoni and C.
Several species of harpacticoid are used in toxicity bioassays, and some are members of cryptic species complexes. For example, *Schizopera knabeni* was described from collections in California, although nominal specimens from Louisiana have been used in toxicity tests [29]. Additionally, *Tisbe* is known to harbor cryptic species [27]. *Microarthridion littorale* shows variations in genetic composition over space that suggest the taxon is composed of cryptic species, and tolerances have been shown to vary strongly within intraspecific lineages [49]. These findings suggest that laboratory populations of small metazoans should be characterized genetically before tests that measure no-effect criteria are conducted. Our research suggests that cryptic species may potentially contribute to the loss of genetic diversity at contaminated sites. This type of reduction would be caused by a loss of species at contaminated sites, and reductions in species diversity are a generally acknowledged consequence of contamination in benthic communities [50]. A loss of genetic diversity associated with a reduction in species diversity at contaminated sites is fundamentally different than adaptation-related changes in genetic diversity within species and has an ecological rather than an evolutionary basis. If genetic diversity is used as a marker for contamination effects in the future, then the possible contributions of cryptic species (or taxa that act like cryptic species, because identification requires dissection) should be considered.

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