Strong Positive Selection and Habitat-Specific Amino Acid Substitution Patterns in *Mhc* from an Estuarine Fish Under Intense Pollution Stress

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Population-level studies using the major histocompatibility complex (*Mhc*) have linked specific alleles with specific diseases, but data requirements are high and the power to detect disease association is low. A novel use of *Mhc* population surveys involves mapping allelic substitutions onto the inferred structural molecular model to show functional differentiation related to local selective pressures. In the estuarine fish *Fundulus heteroclitus*, populations experiencing strong differences in antigenic challenges show significant differences in amino acid substitution patterns that are reflected as variation in the structural location of changes between populations. Fish from a population genetically adapted to severe chemical pollution also show novel patterns of DNA substitution at a highly variable *Mhc* class II B locus including strong signals of positive selection at inferred antigen-binding sites and population-specific signatures of amino acid substitution. Heavily parasitized fish from an extreme PCB-contaminated (U.S. Environmental Protection Agency Superfund) site show enhanced population-specific substitutions in the *a*-helix portion of the inferred antigen-binding region. In contrast, fish from an unpolluted site show a significantly different pattern focused on the first strand of the *B*-pleated sheet. Whether *Mhc* population profile differences represent the direct effects of chemical toxicants or indirect parasite-mediated selection, the result is a composite habitat-specific signature of strong selection and evolution affecting the genetic repetoire of the major histocompatibility complex.

Introduction

The major histocompatibility complex (*Mhc*) binds antigens and presents them to vertebrate T-cells as a key part of the adaptive immune response. Patterns of *Mhc* allelic diversity within vertebrate populations are thought to result from a combination of balancing and directional selection because pathogen pressure varies in space and time (Edwards and Potts 1996; Edwards and Hedrick 1998; Hedrick and Kim 2000). Limited studies of *Mhc* variation in wildlife populations, thus far, have generally focused on allelic diversity measures in threatened and bottlenecked populations, sometimes in comparison with less affected species or populations or with presumably neutral loci (Ellegren et al. 1993; Mikko and Andersson 1995; Sanjayan et al. 1996; Boyce et al. 1997; Hedrick and Parker 1998; Wenink et al. 1998; Hoelzel, Stephens, and O'Brien 1999; Kim, Parker, and Hedrick 1999; Murray, Michaud, and White 1999; Seddon and Baverstock 1999; Madsen et al. 2000). Some studies use DNA sequencing to define alleles and show evidence of selection, by comparing ratios of nonsynonymous (Dn) and synonymous (Ds) DNA substitutions at the antigen-binding region (Wenink et al. 1998; Hoelzel, Stephens, and O'Brien 1999; Seddon and Baverstock 1999). Despite these compelling sequence comparisons, direct evidence in support of the linkage between pathogen selective pressures and *Mhc* allelic variation has mostly come from large-scale studies of human (Hedrick and Kim 2000; McNicholl et al. 2000) or other domesticated mammalian populations (Paterson, Wilson, and Pemberton 1998), where impressive sample sizes are needed to allow statistical comparisons of allele frequencies with disease incidence. Other approaches take advantage of the lowered complexity of the chicken *Mhc* to show disease correlation (Kaufman and Salomonsen 1997). In general, however, complex *Mhc* genomes and the requirement of large sample sizes have presented substantial challenges to the characterization of population-level immunogenetic responses to particular pathogens and to the understanding of how *Mhc* selection varies across the environmental mosaic. An alternative approach to immunogenetic adaptation takes advantage of structural information on the antigen-binding amino acid residues in *Mhc* molecules (Hughes, Yeager, and Carrington 1996; Ou, Mitchell, and Tingle 1998) to compare patterns of protein variation between populations.

This approach makes use of the specific role of *Mhc* as a mediator of the adaptive immune response by way of antigen binding, taking advantage of some of the strongest signals of selection of any known genetic locus. Small human populations offer one of the strongest pictures of rapid *Mhc* change and selection in novel environments (Parham and Ohta 1996). But although unusual allelic compositions in these cases are presumed to result from a combination of strong selection and drift, the actual selective forces are historical inferences at this point. Here, large wildlife populations, less subject to drift and currently experiencing known differences in strong selective forces, can be compared. Alarming environmental toxicant loads in an Environmental Protection Agency (EPA) Superfund site offer a "natural" experimental comparison with cleaner environments because the toxicant loads are well characterized and are frequently associated with altered vertebrate immune responses, in general (U.S. Environmental Protection Agency 2000, chapt. 4, p. 1), and at this site are

Abbreviations: DLC, dioxin-like compound; PBR, peptide-binding region; LC20, modeled estimate of the concentration killing 20% more than the control.

Key words: *Mhc,* major histocompatibility complex, positive selection, *Fundulus heteroclitus*, chemical stress.

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associated with unusual parasite loads and altered stressor responses (Nacci et al. 1999; Bello et al. 2001; Nacci et al. 2002*b*).

The human *Mhc* class II molecule has been crystallized (Brown et al. 1993; Stern et al. 1994), and correlations between residue variation and antigen-binding ability have been elucidated using a variety of binding affinity assays (Davenport and Hill 1996; Ou, Mitchell, and Tingle 1998) and computer analysis (Sturniolo et al. 1999). In the extra-membrane portion of *Mhc* class II molecules, an open cleft between alpha and beta subunits selectively binds antigens on the basis of the affinities of the particular amino acid residues lining the binding cleft (Stern et al. 1994). A particular portion of the cleft, the B1 subunit, has been shown in a variety of vertebrates to contain variable regions with extremely high rates of amino acid substitution. In this region, high ratios of nonsynonymous substitution to synonymous substitution (Dn/Ds) appear to result from positive selection to recognize diverse antigens (Hughes, Ota, and Nei 1990; Hughes, Yeager, and Carrington 1996). Within the B1 subunit, particular residues have been identified as highly variable, and Dn/Ds comparisons between residues suggest that these highly variable sites are the specific residues driven by positive selection for diverse antigen recognition and binding. Thus, the relationship between residue variation within the *Mhc* antigen-binding region and ability to recognize and bind different antigen motifs is well established (Jardetzky 1996). This relationship may be used to make population-level comparisons of functional immunogenetic repetoires. Populations that have experienced different suites of immune challenges might show different amino acid replacement patterns at antigen-binding sites on the basis of the different binding affinities of various pathogens. Rather than looking for specific allelic correlations with particular pathogens, by using this approach it is possible to assess the immunogenetic status of an entire population relative to other populations experiencing different suites of antigenic challenges.

In this study, the immunogenetic profiles of two fish populations that differ dramatically in exposure to environmental toxicants and parasite loads are compared. *Fundulus heteroclitus* is an extremely abundant resident estuarine teleost fish (Pisces: Cyprinodontidae) in salt marshes along the entire western North Atlantic coast (Bigelow and Schroeder 1953, p. 164). These fish are tolerant to a wide range of environmental conditions, including salinity, temperature, and chemical contaminants. *Fundulus heteroclitus* is a well-established model system for studies of biochemical, physiological, ecotoxicological, and genetic adaptation to natural and artificial environmental variations (Mitton 1997, p. 44; Powers and Schulte 1998), including adaptation to an extreme accumulation of dioxin-like compounds (DLCs) in the EPA Superfund site at New Bedford Harbor, Mass. (Nacci et al. 1999). Functional variation in immune system parameters has been measured in a variety of pollutant assays using *F. heteroclitus* (Rozell and Anderson 1996; Armknecht, Kaattari, and Van Veld 1998); however, immunogenetic *Mhc* variation has not been examined previously in this fish. DLCs cause a variety of immunological, endocrine, reproductive, tumorigenic, and other disorders as well as lethality in vertebrates, and exposures are linked to higher incidences of disease in mammals, birds, and fish (Luebke et al. 1997; Monosson 2000).

To test this population-level approach to immunogenetic variation, a highly variable *Mhc* class II B locus was isolated from *F. heteroclitus* using degenerate PCR methods. Patterns of variability at this locus were characterized to determine (1) if this fish locus shows similar variability and positive selection in comparison with other vertebrate *Mhc* class II loci responding to pathogen pressure, and (2) if patterns of amino acid substitution at the antigen-binding locus vary between populations experiencing strongly differing environmental stresses. This information is used to determine if populations that differ strongly in immune challenges also show differences in immunogenetic profiles at a highly variable *Mhc* locus. Such differences found by looking at composite patterns of unique amino acid substitutions in the antigen-binding region may provide a useful technique for evaluating population adaptation to extreme environmental stress. To control for *Mhc* differences that may reflect a signal of geographic isolation and drift rather than selection, complimentary information from the hypervariable region I of the mitochondrial control region, a noncoding locus thought to reflect nonselective evolutionary forces, was obtained for the same populations.

Materials and Methods

Study Sites

Four populations were sampled in this study with two sampled extensively for the comparison of the immunogenetic profiles of healthy and stressed populations. The highly stressed population for this study lives in an EPA Superfund site in New Bedford Harbor (NBH), Mass., that is characterized by PCB sediment levels of up to four orders of magnitude greater than guideline levels for probable adverse biological effects (Long et al. 1995). This site has a history of contamination with PCBs from industrial sources, dating back to the 1940s. Historical records and sediment cores establish that PCB levels in sediments exceeded levels associated with probable ecological effects from the mid-1940s up to the present time (Long et al. 1995; Nacci et al. 2002*a*). Conservatively, this would represent 15 generations of *Fundulus*. Fish in this population, collected around a location named Hot Spot, have evolved extraordinary tolerance to high levels of DLCs (LC20 PCB126 = 23,770 ng/liter [Nacci et al. 1999], where LC20 is the modeled estimate of the concentration killing 20% more than the control) in comparison with a nearby reference population (West Island, LC20 PCB126 = 304 ng/liter) also sampled in this study. In laboratory DLC exposures, West Island embryos show approximately 60% mortality at DLC levels matching those found in NBH-collected embryos (Nacci et al. 1999). In addition to chemical exposure, Hot Spot fish

show higher incidences of a variety of pathologies including unusual rates and incidences of parasitism (M. Huber and R. Overstreet, personal communication). For a healthy population comparison, fish were sampled from salt marshes around the Anisquam Inlet in Gloucester, Mass. Several fish from a distant healthy population (Beaufort, N.C.), which has been extensively sampled and characterized for other ecotoxicological studies (P. McClellan-Green, personal communication), were also used in this study as a southern genotype comparison. West Island, outside the mouth of New Bedford Harbor and approximately 15 km from Hot Spot, has served as a reference location for many studies related to the Superfund site. Levels of DLCs at West Island currently fall within the NOAA acceptable guidelines, and these fish show a lack of tolerance to DLCs in laboratory exposures relative to Hot Spot fish, although they do show some tolerance differences when compared with populations that are relatively free of PCBs (Nacci et al. 2002*a*). Parasite incidence in West Island fish is lower and does not show the extremely anomalous patterns found at the Hot Spot (M. Huber and S. Cohen, unpublished data).

Samples

Fish were collected from field sites with minnow traps or seines and fatally anesthetized with MS-222 (Sigma). Hot Spot, NBH, Mass., and Beaufort, N.C., fish were dissected before vouchering to confirm general patterns of parasitism. Hot Spot fish used in this study all showed two unusual and elevated levels of internal digenean trematode infestation as encountered in previous sampling (M. Huber and R. Overstreet, personal communication), relative to reference populations in West Island and Gloucester. All Hot Spot fish showed evidence of swim bladder infestation by an adult stage hemiurid digenean tentatively identified as *Stomachicola rubea* (an unusual organ location and life-history stage for this organism to appear in *F. heteroclitus*) as well as high incidences of heterophyid digenean metacercaria heart parasites (identified as *Ascocotyle tenuicollis* by R. Overstreet) in comparison with reference populations (M. Huber, R. Overstreet, and S. Cohen, unpublished data). Beaufort, N.C., fish used in this study showed no evidence of heart, swim bladder, or any other parasitic infestation in a gross examination of the internal and external anatomy, excluding gills. All sampled fish were either frozen as a whole at −80 or a posterior section including a portion of the muscle and tail was removed and stored in 95% ethanol.

Molecular Methods

DNA was extracted from the muscle tissue by standard phenol-chloroform methods (Duda and Palumbi 1999) or by using Nucleospin tissue columns (Clontech). For MHC sampling, degenerate PCR primers were designed from an alignment of guppy, topminnow, cichlid, and bass *Mhc* class II B sequences found in GenBank. Primers were designed using coding sequences from exons 1 and 2 as well as noncoding sequence from cichlid intron 1. This region was chosen for amplification because it includes nearly all of exon 2 in the B1 unit, where the highly variable portion of the peptide-binding region (PBR) is located (as well as the preceeding intron and a small portion of the leader peptide). Using these primers, the B1 subunit lacks 14 bases at the 3′ end of the exon because these nucleotides are located in the reverse primer. This portion of the exon includes all the inferred peptide-binding codons for this region (24 residues) and 92% (60/65 residues) of the non–peptide-binding codons. PCR primers used were forward XIS (sense, 3′ exon 1 and 5′ intron 1) CASCSTCHRCWCWGCAGGT and reverse MRS (antisense, 3′ exon 2) RCTYACCTGATTTAKMCAGA. The PCR reaction followed the manufacturer's recommended conditions using TaKaRa Ex *Taq* (Promega) with the modification of one-half of the lowest recommended *Taq* concentration in a 25-μl hot-started reaction. PCR cycling conditions were usually one cycle at 94°C for 2 min; 35 cycles at 94°C for 30 s, 50–53°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 0–5 min. PCR conditions were varied to search for additional related loci. Different concentrations of magnesium, *Taq,* primers, templates, and dNTPs were tested; none produced additional *Mhc* alleles beyond those found with the standard conditions. Annealing temperatures and extension times were varied as well and also did not produce additional *Mhc* alleles. Extension times below 15 s sometimes produced additional artifactual alleles by recombination during PCR of incompletely extended products. These recombinant alleles were not found when the reaction was repeated with longer extension times.

PCR products were ligated into TOPO or TA (Invitrogen), or pGEM (Promega) vectors using TOPO or TA kits. Clones were screened in PCR reactions with the cloning primers T7 and M13R. PCR products for clones of appropriate sizes were cleaned using a shrimp alkaline phosphatase–exonuclease reaction or with Qiaquick PCR purification columns (Qiagen) before cycle sequencing with Big Dye for analysis on an ABI 377 or ABI 310 sequencer. Clones of many sizes were sequenced to look for additional alleles and loci; clones between 500 bp and 1 kb were routinely screened, and clones outside this size range (both smaller and larger) were occasionally sequenced. All sequences were submitted to BLAST searches against the GenBank database. All alleles shown in this study were confirmed by sequencing a minimum of two, but in many cases more independent clones (frequently 3–5, occasionally up to 10 or more). As an additional means of searching for additional loci and alleles, interior degenerate and nondegenerate primers were designed using the *Fundulus* sequence obtained in this study, in alignment with other GenBank sequences. Interior primers provided the same alleles as those obtained with the standard outer primers named above. DNA sequences were aligned in Sequencher. Inferred amino acids and Dn/Ds values were calculated with MEGA (Kumar, Tamura, and Nei 1993) 1.02 using Jukes-Cantor distance measures. A phylogenetic neighbor-joining distance tree was constructed

using all alleles in the program using PAUP* (Swofford 2000) version 4.0b3a with Jukes-Cantor distances.

Other summary statistics were obtained using PAUP* and DnaSP (Rozas J. and Rozas R. 1999), version 3.14.3.

Mitochondrial Hypervariable Control Region Sequences

Genomic extracts of *F. heteroclitus* were used in 25-μl PCR reactions using Perkin-Elmer PCR buffer and Amplitaq (Applied BioSystems) with the following conditions: one cycle at 94°C for 2 min and 35 cycles at 94°C for 1 min, 51°C for 1 min, 72°C for 1.5 min. The following PCR primers were used to obtain the sequence for hypervariable region I of the mitochondrial control region: forward primer Pro-5′ (CTA CCC CTA ACT CCC AAA GC) (Palumbi 1996) and reverse primer M-3b (AGA AAT AGG AAC CAG ATG CCA G) (courtesy, C. Reeb). PCR products were cleaned with a shrimp alkaline phosphatase–exonuclease reaction and cycle sequenced with Big Dye for analysis on an ABI 3100 sequencer. Sequences were aligned in Sequencher, and Fst values between populations were estimated with the program HEAP (courtesy, S. Palumbi).

Results

Mitochondrial Control Region Sequences

Mitochondrial PCR primers produced an approximately 250-bp product in West Island ($n = 20$), Gloucester ($n = 20$), and New Bedford Harbor Hot Spot fish ($n = 22$). The alignment had no indels (except for a 1-bp insertion in a North Carolina individual compared with the northern fish analyzed in this study). For analysis, the alignment was trimmed to exclude a 3′ variable region associated with a terminating string of C's. The resulting 225-bp alignment included six polymorphic sites, nine haplotypes total, and three informative sites (GenBank accession numbers AF529626–AF529634, alignment submitted to GenBank). No fixed differences between populations were found. In an analysis of within- and between-population genetic distances between individual genotypes, no significant difference was found between any population, and the average within-population genetic diversity (0.28%) was equal to the average between-population genetic diversity (0.28%) using HEAP. Tajima's D, a conservatively biased test for neutrality using DNA sequence information from a single locus, is estimated to be -1.0944, $P > 0.10$, confirming the expectation of neutrality for this locus.

Mhc Sequences and Polymorphism

A 400- to 500-bp PCR product (including PCR but not cloning primers and vectors) from all populations (Beaufort, N.C. [$n = 3$], Gloucester, Mass. [$n = 10$], Hot Spot, New Bedford Harbor, Mass. [$n = 17$], West Island, Mass. [$n = 5$]) showed similarity to GenBank *Mhc* sequences in BLAST searches. The *Mhc* matching clones were inferred to include all of intron 1 and most of exon 2 (i.e., the entire exon minus the final 14 bases at the 3′ end, which are included in the reverse primer) on the basis of primer design and matches with various fish class 2 DB1 sequences and cichlid class 2 B group 4 sequences in GenBank. The number of clones sequenced per individual ranged from 2 to greater than 10. In one case where an unusual motif (ERW NDP) could be identified by an RFLP assay, additional clones were screened by PCR and RFLP. No more than two functional alleles were found in any individual. An inferred pseudogene (containing a stop codon) was found in a single individual from Gloucester. The pseudogene sequence was excluded from further analysis. No indels were found in the coding sequence.

For a subset of individuals, the initial PCR and cloning was repeated, and identical alleles for that individual were always obtained. Also, interior primers only located alleles identical to the sequences obtained using the external primers that amplified the entire intron-exon piece. Thus, the alleles found in this study are inferred to represent a single functional DB locus in *F. heteroclitus*. One or more additional class II B loci have been found in a variety of fish (McConnell, Godwin, and Cuthbertson 1998), sometimes in high numbers, e.g., cichlids (Malaga-Trillo et al. 1998). But other species, including those most closely related to *Fundulus* and with characterized *Mhc* class II, show evidence of only a single locus in assays using cDNA, Southern blotting, or family genotype comparisons, e.g., topminnows (Hedrick and Parker 1998) and guppies (Sato et al. 1995), and McConnell, Godwin, and Cuthbertson (1998) provide evidence for a second highly divergent DXB locus. Alternatively, the primers used in this study may be sampling only a limited subset of the existing alleles at a single locus, thus providing a limited sample of the actual amino acid replacements occurring in these populations at this locus. There is a low frequency of nulls (4%, 2/48 individuals subjected to PCR, one each from West Island and Gloucester). But in either case, comparison of amino acid replacement profiles is appropriate because populations were consistently sampled with the same primer set.

The B1 exon alignment of 254 nucleotides consisted of 41 unique alleles found across all populations (GenBank accession numbers AF529585–AF529625, alignment included in supplemental material and submitted to GenBank). A few alleles were found more than once in different individuals, including a total of three duplicated alleles from the Hot Spot location (alleles H150c4, H6c5, and H6c4 all had identical matches within the population), two duplicated alleles in Gloucester (G10c4 had an identical match), and one allele shared between Hot Spot and West Island (an allele identical in nucleotide sequence to W5c2 was also found in Hot Spot). The DNA sequences were translated using MEGA (Kumar, Tamura, and Nei 1993) into the 84 residues shown in figure 1. All inferred amino acid alleles were encoded by only a single DNA sequence, i.e., there were no differing DNA alleles redundant for the same amino acid allele.

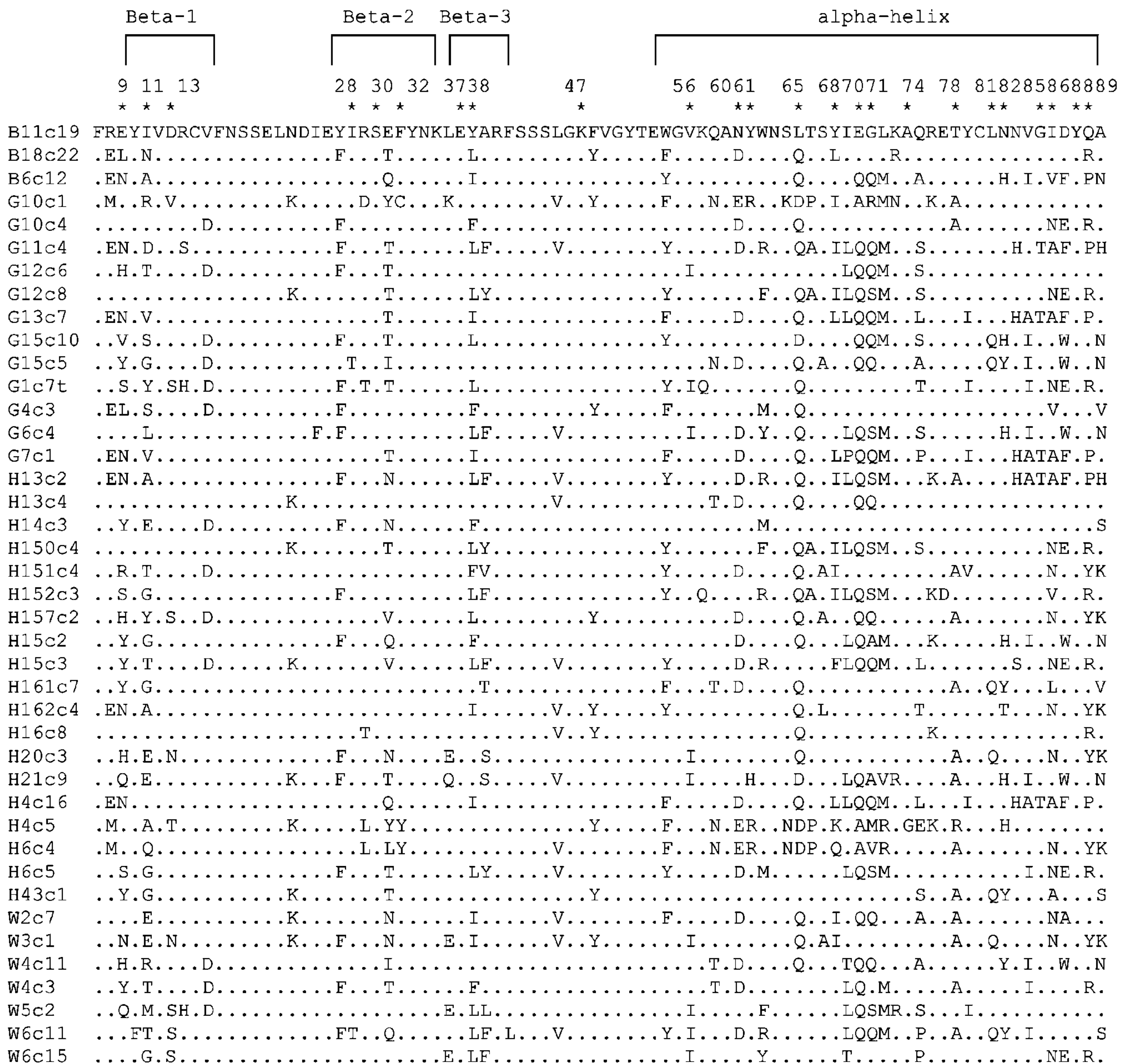


FIG. 1.—Amino acid sequences inferred from DNA sequences of *F. heteroclitus* class II B exon 2 from four populations. Sequence names indicate geographic location (capital letter) and clone. B, Beaufort, N.C.; G, Gloucester, Mass.; H, Hot Spot (Superfund area), NBH, Mass.; W, West Island, mouth of NBH, Mass. Inferred PBR sites are indicated at the top of the alignment with asterisks. Numbering corresponds with the mature protein (Ono et al. 1993).

Mhc Sequence Variation

Alleles are highly variable with large Jukes-Cantor genetic distances (PAUP*) between most alleles (ranging from 0.008 to 0.344 within the most variable population, Gloucester) and with mean ranges between populations of 0.150 ± 0.016 nt to 0.182 ± 0.018 nt (DnaSP). Shared motifs between populations are evident in the amino acid alignment, e.g., HATAF, ERW, NDP, and IEGL and variations of these motifs. Disjunct motif sharing within similar alleles is also evident and may be a result of recombination. For example, alleles with ERW NDP–like motifs in the *a*-helix also share a similar pattern of replacement (i.e., the LXXY motif) in the second strand in the *B*-sheet–encoding portion of the molecule, although the intervening sequence may differ. The ERW NDP alleles also show large genetic distances (up to 34%) from other alleles that do not have these motifs.

Mhc Variation in Amino Acid Replacement Patterns Related to Structure

The PBR codons were inferred from the human model of the *Mhc* class 2 DRB1 locus (Brown et al. 1993) (fig. 1). Comparison with zebrafish (Ono et al. 1992) and salmonids (Dixon et al. 1995; Miller, Withler, and Beacham 1997) (truncated coding region) shows similar patterns of generally higher variability at inferred antigen-binding codons.

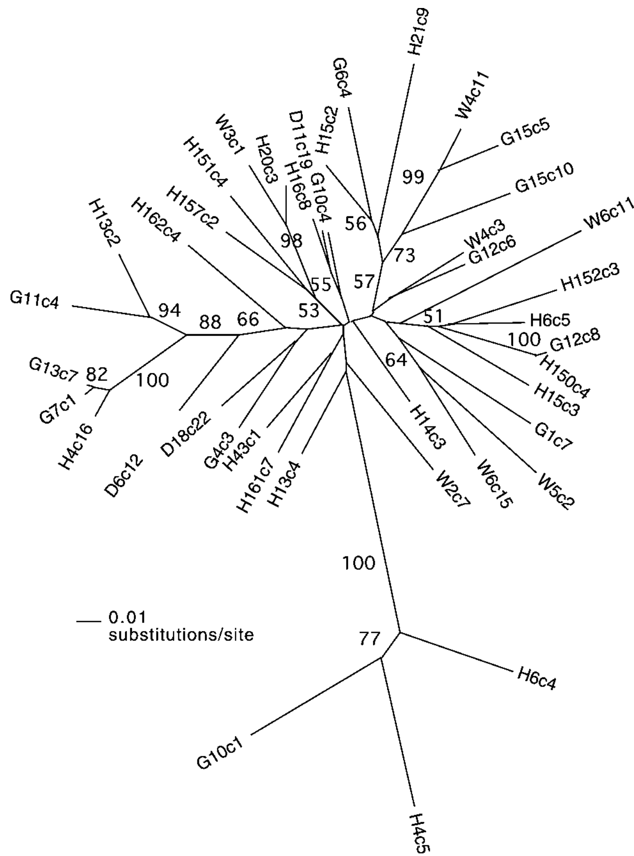
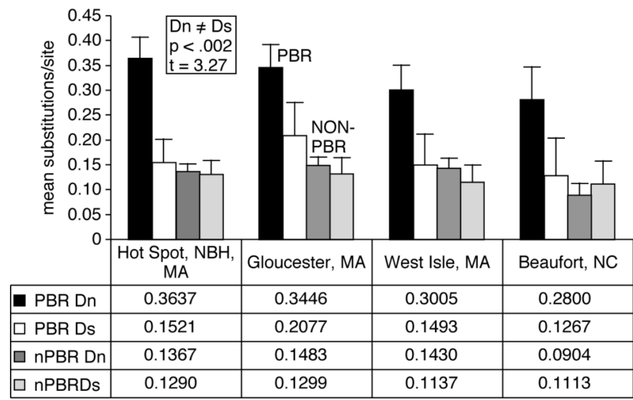


FIG. 2.—Unrooted neighbor-joining phylogram of all unique DNA alleles using Jukes-Cantor genetic distances. Bootstrap support >50 based on 100 bootstraps is indicated at appropriate branches.

Population-specific replacements for the Gloucester fish are concentrated at residue 11 located in the first strand of the *B*-pleated sheet. Although fish from the Hot Spot do show variation at this residue, few of the replacements are population specific, i.e., unique to Hot Spot in this study. Instead, Hot Spot population-specific replacements predominantly occur in the *a*-helix. The southern *Mhc* alleles found in this study include only a single population-specific amino acid replacement (at residue 11). Residue 11 is among the most variable in other organisms including primates and other teleosts (Klein and O'hUigin 1995).

Phylogenetic analysis of DNA alleles using a neighbor-joining algorithm and Jukes-Cantor distances places Beaufort alleles separated from each other within clusters containing other localities (fig. 2). Indeed, as predicted for highly variable *Mhc* PBRs under balancing selection and undergoing recombination (Jakobsen, Wilson, and Easteal 1998; Martinsohn et al. 1999; Edwards, Nusser, and Gasper 2000), there is no phylogeographic signal to this exon either in analyses of the entire region or of the *a*-helix and *B*-pleated sheet sections analyzed separately under the supposition that they may be experiencing separate evolutionary histories (Bergstrom et al. 1998).

Figure 3 shows the distribution of Dn and Ds values for all populations in the PBR and non-PBR regions inferred from the human model. Below the histogram



	Hot Spot, NBH, MA	Gloucester, MA	West Isle, MA	Beaufort, NC
PBR Dn	0.3637	0.3446	0.3005	0.2800
PBR Ds	0.1521	0.2077	0.1493	0.1267
nPBR Dn	0.1367	0.1483	0.1430	0.0904
nPBRDs	0.1290	0.1299	0.1137	0.1113

FIG. 3.—The Dn/Ds values per population for PBR and non-PBR regions. The Dn/Ds values are calculated using all unique alleles obtained per population and excluding repeat alleles within a population: Hot Spot (n = 20), Gloucester (n = 12), West Island (n = 7), and Beaufort (n = 3).

are values of synonymous and nonsynonymous substitutions in the antigen-binding codons compared with non–antigen-binding codons. All populations show PBR Dn/Ds ratios greater than 1 in the antigen-binding region and non-PBR Dn/Ds ratios close to 1. Hot Spot, the population with the largest sample size, has Dn and Ds values that are significantly different from each other (t = 3.27, $P < 0.002$) (Kumar, Tamura, and Nei 1993). Thus, positive diversifying selection is clearly acting on the inferred antigen-binding regions of the molecule particularly because Dn/Ds ratios are a conservative test of positive selection (Yang and Bielawski 2000).

Using all amino acid sequences (including duplicated alleles) from all four populations for comparison (fig. 1), the numbers of unique population-specific amino acid replacements for the two largest populations (Hot Spot and Gloucester) were compared across different structural locations in the class 2 DB molecule (figs. 4 and 5). Assignment of structural locations follows mammalian models (Brown et al. 1993; Klein and O'hUigin 1995): *B*-pleated sheet strand 1 (residues 9–

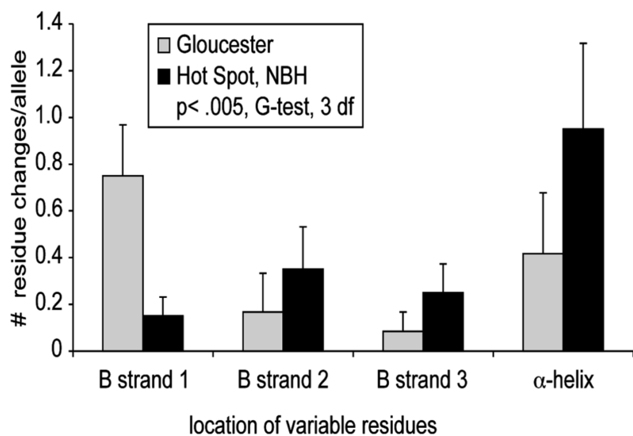


FIG. 4.—The distribution of population-specific amino acid changes per allele across the inferred B1 molecule for fish from two populations. See *Results* for further explanation. Changes are grouped on the x axis according to structural location, corresponding with the structural regions shown in figures 1 and 5.

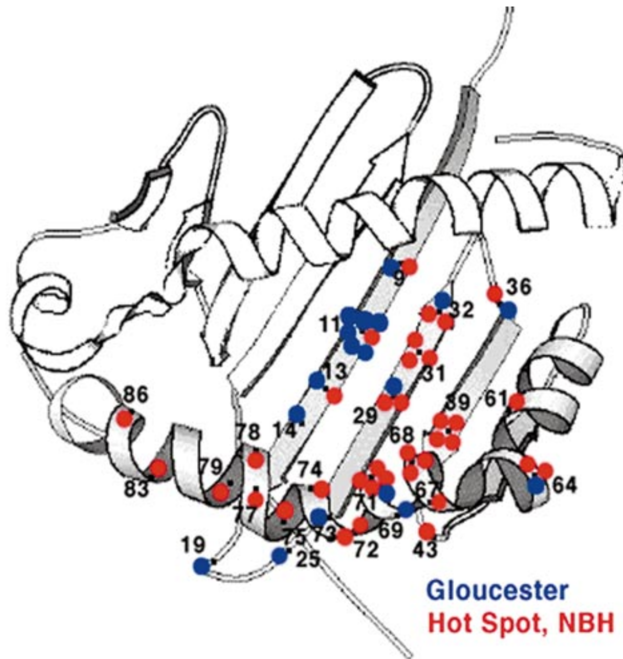


FIG. 5.—The *Mhc* class II B structural diagram with inferred locations of population-specific amino acid changes superimposed for Gloucester ($n = 12$) and Hot Spot ($n = 20$). Each colored dot represents a single population-specific amino acid change in a unique allele.

16), strand 2 (25–34), strand 3 (36–40), and the *a*-helix (52–88). Thus, for all alleles shown in figure 1, any amino acid substitution found exclusively in one population, in comparison with all four other populations, was counted each time that particular substitution occurred within the population, either in the same or a different allele. The number of population-specific substitutions is divided by the total number of alleles sampled per population to account for unequal sample sizes between populations. Figure 4 shows a significant difference between the two populations in the molecular location of population-specific amino acid replacements (G with William's correction = 13.444, df = 3, $P < 0.005$) (Sokal and Rohlf 1995, p. 736). Gloucester-specific residues are concentrated on the first strand of the *B*-pleated sheet, whereas Hot Spot–specific residues are concentrated in the *a*-helix portion of the molecule. Dividing the *a*-helix section into two categories (residues 52–76 and 77–88) does not alter the result (corrected $G = 13.801$, df = 4, $P < 0.01$). In addition, removal of unusual, extremely divergent alleles (three in Hot Spot and one in Gloucester; G10c1, H4c5, and two copies of H6c4) from the analysis does not alter the results of a significant difference between Hot Spot and Gloucester in patterns of amino acid replacement (corrected $G = 15.195$, df = 3, $P < 0.005$). Figure 5 shows these population-specific changes plotted onto the inferred structural *Mhc* class II model.

Discussion

Fundulus heteroclitus alleles isolated in this study show the hallmark features of vertebrate *Mhc* at class II B group loci involved in antigen recognition and also show habitat-specific patterns that may reflect a selective response to environmental stress. In contrast, sequence from the same populations at the hypervariable section I of the mitochondrial genome control region locus shows no pattern of differentiation between populations. At the *Mhc* locus, allelic diversity is high both in numbers of alleles within populations and in genetic distances between alleles. In addition, three different signals of strong selection acting on *Mhc* were found: (1) elevated rates of amino acid replacement in the PBR; (2) slow coalescence of *Mhc* alleles across the North-South cline known for this species, consistent with the action of balancing selection; and (3) population-specific localization of amino acid substitutions in different functional regions of the peptide-binding cleft. Antigen-binding codons inferred from the human DRB1 model show a strong signal of positive selection relative to non–antigen-binding codons.

The Superfund site population (Hot Spot) shows a significantly elevated rate of nonsynonymous substitutions in the PBR. A similar pattern of elevated Dn/Ds in other populations indicates that the inferred antigen-binding sites are under strong positive selection outside the contaminated site as well as is expected for this *Mhc* locus in vertebrates in general. This is consistent with a response by each population to local selection, affecting the antigen-binding region. Nonsynonymous values in the PBR are elevated in comparison with synonymous PBR sites, showing that silent changes occur significantly less frequently than residue-changing substitutions at the antigen-binding residues in each of these particular environments. Non-PBR sites show similar levels of substitution between synonymous and nonsynonymous sites, thereby indicating a lack of positive selection at these residues, and non-PBR rates of change match the PBR synonymous rates. Thus, the signal of positive selection is detectable at inferred peptide-binding codons, as predicted by the human *Mhc* functional model. More limited sampling of salmonids (Miller, Withler, and Beacham 1997; Binz et al. 2001) has also shown a positive signal of selection, again suggesting that structural inferences from the human model to *Fundulus* are appropriate. Thus, differences in Dn/Ds ratios between PBR and non-PBR sites and differences at antigen-binding sites in amino acid replacement patterns between populations may be related to differences in pathogen pressures between environments.

An alternative explanation for population-level differences in replacement patterns, introgression of southern *Fundulus* genotypes or more generally phylogeographic sorting, was excluded by sampling mitochondrial genotypes. The *Mhc* PBR substitution patterns found in this study might have reflected genetic differences between the two clades or subspecies of *F. heteroclitus*, i.e., southern and northern fish (Bernardi, Sordino, and Powers 1993; Powers and Schulte 1998). Such a finding would represent an unusually far northern population of southern fish because the geographic break for these two clades is located in New Jersey. In any case, the control region mitochondrial data show that fish sampled from Massachusetts for this study all rep-

resent the northern form of *F. heteroclitus*. Thus, differences between Hot Spot and Gloucester populations cannot be attributed to an anomalous occurrence of southern genotypes at either location.

Allele sharing among distant populations and allelic lineage sharing across species boundaries are characteristic features of some *Mhc* loci (Klein et al. 1998) and are thought to result from balancing selection (Hughes, Yeager, and Carrington 1996). MHC evolution may occur over both very long (transspecific) and much shorter time scales (e.g., local variation in protective alleles for severe malaria in Africa) depending on population genetic parameters (Parham and Ohta 1996; McNicholl et al. 2000). An overwhelming phylogeographic *Mhc* signal is not predicted for species such as *Fundulus* with high levels of genetic variability and large population sizes, where positive selection for antigen recognition acts on *Mhc* genotypes. Instead, *Mhc* variation may reflect the pattern of antigenic stressors in the local environment (Edwards and Hedrick 1998; Hughes 1999, p. 63; Hedrick and Kim 2000, p. 205).

Other population-level *Mhc* studies on fish (Miller, Withler, and Beacham 1997) have found a phylogeographic signal related to patterns of diversification at other loci; however, this work involved relatively limited *Mhc* (and other genetic) variation in comparison with that in *Fundulus*. For example, in a comparison between two populations, the three salmonid class II DB exon 2 alleles showed a mean pairwise nucleotide difference of 3% (Miller, Withler, and Beacham 1997) in contrast to mean differences of 15%–18% in interpopulation comparisons of *Fundulus* alleles. The limited variation found in salmonids is most likely a result of population bottlenecking (Miller, Withler, and Beacham 1997) that would affect *Mhc* as well as other loci if population numbers are constricted sufficiently. In contrast, *Fundulus* population numbers are large, often with 10,000 or more individuals per estuary (Abraham 1985, 23 p.). Lack of historical bottlenecks in *Fundulus* is evident in the high genetic diversity at *Mhc* and other loci both in numbers of alleles per population and in levels of genetic divergence between *Mhc* alleles within a population. Indeed, levels of genetic variation at other *Fundulus* loci are high along the East Coast (Ropson, Brown, and Powers 1990) including New Bedford Harbor (McMillan et al. 2000; Roark, Guttman, and Nacci 2000; McMillan et al. 2001). In addition, *Fundulus* populations are not affected by the harvesting and aquacultural practices common to many salmonid populations that may reduce genetic diversity in commercial fish populations.

Despite much motif or epitope sharing between populations, e.g., HATAF, HIWN, LQQM/LQSM, NER in the *a*-helix area (fig. 1), unique patterns of substitution between the two populations are outstanding (figs. 1, 4, and 5). Differences found in this study between *a*-helix and *B*-pleated sheet substitutions relate to the location of pockets in the *Mhc*-binding cleft, where antigen anchor residues are thought to bind (Brown et al. 1993; Stern et al. 1994). In the Gloucester population, unique replacements occur mostly at position 11, an important residue in binding pocket 6. In contrast, in Hot Spot, replacements are concentrated around pocket 7, which includes the area associated with residues 61, 47, 67, 28, and 71 in the human class II structural model. Although antigen-binding pockets may shift somewhat between species and alleles, the functionally significant pattern of amino acid replacement differences between Gloucester and Hot Spot populations is clear statistically (fig. 4) and has a functional difference between strand 1 of the *B*-pleated sheet (pocket 6) and the *a*-helix (pocket 7).

Population differences in amino acid replacement patterns seen in the comparison of Gloucester and Hot Spot populations suggest that the inferred antigen-binding region is responding to habitat-specific selective pressures in Gloucester and in New Bedford Harbor. Apparently the selective regime affecting antigen-binding abilities at these two geographic locations is altering the allelic composition at different functional locations within the molecule. This suggests that the different geographic locations have different antigens or suites of antigens that are interacting with *Mhc* receptors or that *Mhc* receptors are functioning differently in the two environments. Environmental differences between the New Bedford Harbor Superfund site and a relatively healthy marsh environment are extreme. Selective pressures at the New Bedford site are related to the harsh chemical environment and unusual suites of pathogens found in association with the Superfund site. Indeed, antigen differences between the two sites are clear on a gross level because New Bedford Harbor Hot Spot fish have a number of unusual helminth parasites not found in Gloucester or other *Fundulus* populations at all, or in some cases, at the high frequencies seen in Hot Spot (see *Materials and Methods* for further parasite description). Although the ecological basis for these local abnormal patterns of parasitism is unknown, in experimental infections in the laboratory with a ubiquitous bacterial pathogen *Vibrio carchariae*, *Fundulus* from New Bedford Harbor show survival rates equal to or greater than those for fish from clean reference populations (Nacci et al. 2001).

Genetic adaptation to DLCs has been demonstrated in *Fundulus* from New Bedford Harbor and other toxic waste sites in comparison with control sites using assays of embryonic tolerance to PCB laboratory challenges that have shown strong heritable differences in survival rates (Nacci et al. 1999, 2002*a*) (and see *Study Sites* in *Materials and Methods*). Genetic adaptation to DLCs contaminating New Bedford Harbor since the 1940s supports the likelihood of accompanying immunogenetic adaptation to severe environmental stress. Thus, the extraordinary DLC concentrations in the Superfund area may be having selective effects on the *Mhc* allelic repetoires directly as well as through indirect pathways related to differential parasite loads.

Analysis of primate and other *Mhc* class 2 DB data has led to vigorous discussion on the possibility that the *B*-pleated sheet and *a*-helix experience different kinds of evolutionary change, with the *B*-pleated sheet accumulating changes in a conventional manner by single

mutations, whereas the *a*-helix may be subject to substantial levels of gene conversion, moving blocks of amino acids between alleles, e.g., mice (Wakeland et al. 1990) and primates (Gaur and Nepom 1996; Takahata and Satta 1998). If this is true for the *Fundulus* populations studied in this work, then interpopulation differences in the locations of unique replacements may also reflect population differences in the mechanism of evolutionary change occurring at this locus. Different intensities of selection as well as differences in specific selective agents in the two environments may actually be responsible for the production of changes in different parts of the molecule. The geographic location experiencing a more recent shift in selective pressure, the Superfund site, has unique amino acid substitutions concentrated in the *a*-helix section, where recombinational mechanisms may permit particularly rapid generation of new alleles.

Functional analysis, somewhat similar to the approach used in this study, has been applied to specific human pathologies and to patterns of amino acid replacement in humans as a whole by cataloging functional motif changes in *Mhc* (Ou, Mitchell, and Tingle 1998). Allelic correlations with particular diseases may often involve more than a single amino acid replacement at any particular residue, making detection of allelic linkages, as opposed to linkages by functional categories, statistically challenging (Apple and Erlich 1996). The strategy used in this study has an advantage over standard allelic frequency methods in its ability to detect a pattern of disease association involving more than one or a few alleles by grouping functionally similar changes together in analysis.

As demonstrated in this study, *Mhc* offers an additional opportunity to relate functional analysis at the genetic level to environmental stresses and allelic composition at the population level in wildlife. Each population experiencing a different suite of antigenic stressors may show novel patterns of amino acid replacement in the antigen-binding cleft. The *Mhc* sequence data provide the information to calculate Dn/Ds ratios, a measure of the relative strength of positive selection, and specific patterns of replacement may be used to make structural comparisons on the basis of the human crystallographic *Mhc* models. Further studies are underway with *Fundulus* to compare immunogenetic profiles in field populations and laboratory challenges to test whether particular stressors affect wildlife immune systems in diagnostic ways. By taking full advantage of Dn/Ds measures and positional information on amino acid replacement patterns in different environments, it may be possible to understand how this enigmatic polymorphic genetic recognition system maintains such extraordinary levels of diversity in wild populations.

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