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Paucity of Genetic Variation at an MHC Class I Gene in Massachusetts Populations of the Diamond-backed Terrapin (*Malaclemys terrapin*): A Cause for Concern?

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Abstract.—**The Diamond-backed Terrapin (*Malaclemys terrapin*), endemic to the brackish marshes of the eastern and Gulf of Mexico coasts of the United States, is a threatened species in Massachusetts with populations suffering drastic declines in the late 19th and early 20th centuries. To assess the potential effects of population bottlenecks on contemporary levels of genetic variation, we analyzed 219 bp of a major histocompatibility complex class I gene region (MHCI) by direct sequencing and single-strand conformational polymorphism analysis and six microsatellite loci from three locations around Cape Cod, Massachusetts. No variation was found at the MHCI, despite finding appreciable levels of variation within and among populations at the microsatellite loci. We discuss alternative explanations for these results, and we propose that the lack of variation at the MHCI may be due to the effects of selection rather than demographic changes in terrapin populations.**

A prevailing view in conservation genetics is the importance of preserving levels of genetic variation within populations of threatened and endangered species. Decreases in population size and changing demographics can result in a loss of genomic variation, potentially decreasing mean population fitness and viability (Hedrick, 2001; Allendorf and Luikart, 2007). This effect is particularly true for genes that are thought to be of adaptive importance where low levels of variation may have profound implications on the long-term adaptability of a species to changing environmental conditions (Moritz, 2002; Kohn et al., 2006; Gebremedhin et al., 2009).

Genes at the major histocompatibility complex (MHC) have become increasingly popular targets for studying levels of adaptive molecular variation in nonmodel organisms (Bernatchez and Landry, 2003; Hedrick, 2004; Sommer, 2005; Acevedo-Whitehouse and Cunningham, 2006; Piertney and Oliver, 2006). With an increasing awareness of the threat of emergent pathogens on wildlife populations (Daszak et al., 2000; Morens et al., 2004), the MHC loci have become important candidate gene regions for studying the affects of changing population sizes on levels of adaptive molecular variation in threatened and endangered species.

The Diamond-backed Terrapin (*Malaclemys terrapin*) is a brackish water–adapted terrapin with a wide distribution ranging from Cape Cod, Massachusetts, to Corpus Christi, Texas (Ernst et al., 1994). Because of a directed fishery dating from before the 1800s, excessive habitat loss, by-catch from crab fisheries, predation, and road mortalities, terrapin populations in Massachusetts are considered threatened and are highly regulated (e.g., Brennessel, 2006). Here, we report the results of a study estimating the level of variation at the MHC class I gene region (MHCI) within and among local Cape Cod populations of the Diamond-backed Terrapin by using direct sequencing and single-strand conformational polymorphism (SSCP) analysis. We compare the level of variation found at MHCI to estimated levels of variation at six microsatellite loci to infer the effects of population bottlenecks on levels of adaptive genetic variation.

Materials and Methods

Fifty-nine samples were collected from terrapins at three breeding sites in Massachusetts, the northern limits of the species (Fig. 1). Blood samples were collected from Wellfleet Harbor ($n = 22$); Sandy Neck, Barnstable ($n = 7$); and Sippican Harbor, Marion ($n = 22$) by syringe and preserved on Whatman FTA cards (Whatman Inc., Piscataway, NJ). An additional eight individuals from Sandy Neck were sampled using tail clips stored at −80°C. Whole genomic DNA was extracted using the QIAmp DNA Blood Mini-Extraction kit or the QIAmp DNAeasy kit (QIAGEN, Valencia, CA).

A 219-bp fragment of the MHCI was amplified from 34 samples (13 Wellfleet, 4 Sandy Neck, and 17 Buzzard's Bay) by using the primers PSMHCIa2-f (5′-CAGCTGTATGGGTGT-GATCT-3′) and PSMHCIa2-r (5′-TTTAAGCCACTCGATGC-3′) designed from *Pelodicus sinensis* (GenBank accession AB022885). Polymerase chain reaction (PCR) was performed in 25-μl reactions by using 2.0 μl of DNA, 0.5 μM of each primer, and GoTaq Green Master Mix (Promega) under the following conditions: 2-min denaturing at 94°C; 35 cycles of 94°C denaturing, 56°C annealing, and 72°C extension for 30 sec each; 72°C elongation for 4 min. The resulting PCR products were cleaned (AMPure PCR Purification kit, Agencourt Bioscience, Beverly, MA) and directly sequenced in both directions using the DTCS Quickstart kit (Beckman Coulter, Fullerton, CA). The resulting sequencing products were cleaned by ethanol precipitation and analyzed on a CEQ8000 Genetic Analyzer (Beckman Coulter) following the manufacturer's recommendations. The resulting sequences were edited and aligned using Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, MI). An additional 25 individuals (9 from Wellfleet, 11 from Sandy Neck, and 5 from Sippican Harbor) were amplified as described above, cleaned using EXOSAP-IT (Invitrogen, Carlsbad, CA), and analyzed by SSCP. Cleaned PCR reactions were denatured in a formamide-NaOH solution at 95°C for 5 min, snap-cooled for 3 min, and separated on a GMA gel by using an Origins system (Elchrom Scientific AG, Cham, Switzerland) following the manufacturer's recommendations. The resulting fragment patterns were visualized using SYBER Green II. Two control samples of known sequence were run on each SSCP gel, and any samples that were not clearly resolved were rerun with appropriate controls. Ten samples that were sequenced previously for MHCI also were analyzed using SSCP to verify the relationship between SSCP fragment profile and DNA sequence.

The edited MHC sequences were checked for homology to MHC by using tblastn against all vertebrate nucleotide sequences in GenBank. A multiple sequence alignment of terrapin MHC sequences to other known MHCI sequences (Glaberman et al., 2008) was performed by first translating the

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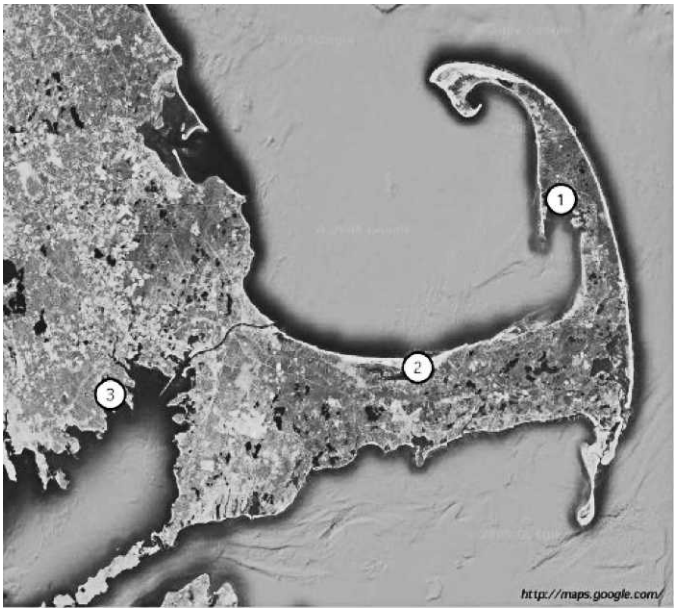

FIG. 1. Sampling location of *M. terrapin*. (1) Wellfleet Harbor, (2) Sandy Neck, Barnstable, and (3) Sippican Harbor, Marion (Buzzards Bay).

nucleotide data into amino acid data, aligning the amino acid data by using CLUSTALX, and then reverting the amino acid alignment back into nucleotide data by using the online version of TranslatorX (Abascal et al., 2010). We tested for evidence of selection on the terrapin MHCI based on the ratio of the number of nonsynonymous substitutions per nonsynonymous site to the number of synonymous substitutions per synonymous site (dN/dS) by using the program MEGA 4.1 (Kumar et al., 2008). Site-specific tests for selection were performed based on maximum likelihood estimates by using the online service Datamonkey (Kosakovsky and Frost, 2005). We used the fixed effects likelihood method incorporating the general reversible substitution model with the phylogenetic tree inferred using neighbor joining.

In addition, the same 59 individuals were analyzed at six microsatellite loci (GmuB08, GmuD28, GmuD51, GmuD55, GmuD87, and GmuD121) by using the primers described in King and Julian (2004). Each locus was amplified individually with only the forward primer fluorescently labeled using the WellRead dyes D2, D3, or D4 (Beckman Coulter). PCR was performed in 15-μl reactions by using 1.5 μl of a 1:10 dilution of the genomic DNA, 0.5 μM of each primer, and GoTaq Master Mix (Promega) under the following conditions: 2-min denaturing at 94°C; 42 cycles of 94°C denaturing for 45 sec, 56°C annealing for 45 sec; 72°C extension for 90 sec. The resulting fragments were separated on a CEQ8000 Genetic Analyzer (Beckman Coulter) following the manufacturer's recommendations, and fragment sizes were determined using a Fragment Analyzer. Genotyping errors and the presence of null alleles were assessed using MicroChecker 2.2.3 (Oosterhout et al., 2004). Estimates of allele frequencies, levels of heterozygosity, tests of Hardy–Weinberg equilibrium, and estimates of F_{st} were performed using GenePop version 3.4 (Raymond and Rousset, 1995). A Baysian approach was taken to estimate the number of populations in the data based on multilocus genotypes by using the program STRUCTURE (Pritchard et al., 2000). The default values for most parameters were used with sample location as a prior based on both the admixture and correlated allele frequency models. Three independent runs of 1,000,000 generations, with a burn-in at 50,000 generations were conducted for each value of K (the number of populations) from 1 to 3.

RESULTS

Thirty-four individuals from Wellfleet, Sandy Neck, and Sippican Harbor were sequenced for 219 bp of the MHCI. A tblastn search of a representative sequence (GenBank accession GQ495891) had a highest match to the *P. sinensis* (AB185243), with all top 100 hits corresponding to MHCI from other vertebrates. Alignment of the *M. terrapin* MHCI sequence to Galápagos Marine Iguana (*Amblyrhynchus cristatus*; EU604309) shows that the region amplified is homologous to the MHCI α-2 region. An amino acid alignment of the putative terrapin MHCI to other reptiles can be found in Figure 2. We found no evidence for heterozygosity or polymorphisms in the 34 individuals sequenced. The 25 additional samples analyzed using SSCP also showed no evidence for variation. All fragment patterns were invariant for all SSCP run samples and corresponded to the fragment pattern seen in the 10 sequenced samples.

The dN/dS ratio was significantly different from 1 when comparing terrapin MHCI to the other reptile MHCIs (Fig. 2). We found strong evidence for the effects of purifying selection (H_A: $dN < dS$; $P < 0.05$ for all pairwise comparisons with Green Iguanas (*Iguana iguana*), Galapagos Land Iguanas (*Conolophus subcristatus*), Galapagos Marine Iguanas, and *Pelodiscus* turtles; $P = 0.072$ for comparison with *Ameiva* lizards) but no evidence for positive election (H_o: $dN > dS$; $P \gg 0.05$ for all pairwise comparisons with other reptiles). The results from the site specific tests for selection are also summarized in Figure 2. Two sites showed limited evidence for positive selection, whereas 15 sites showed evidence for negative or purifying selection. Six of these sites showed evidence for selection specifically along the terrapin branch.

All six loci showed appreciable levels of variation within and among populations comparable to Hauswaldt and Glenn (2005) and Hart (2005) (Table 1). Mostly, the populations were found to be at Hardy–Weinberg equilibrium except Wellfleet and Sandy Neck at GmuD87 and Sippican Harbor at GmuD28. There was no evidence for null alleles or other genotyping artifacts based on MicroChecker, and the levels of divergence among populations were similar to that described by Hauswaldt and Glenn (2005) and Hart (2005) (Table 1). Estimates of F_{st} (Table 2) show a substantial level of divergence between the Cape Cod Bay (Wellfleet and Sandy Neck) and Buzzard's Bay samples (Sippican Harbor), with a lower level of divergence between Wellfleet and Sandy Neck. The results from the Baysian analysis for population structure (Fig. 3) are consistent with the F_{st} results, suggesting that these data are optimally structured into two clusters (posterior probabilities: $K = 1$, $Pr(X|K) \ll 0.01$; $K = 2$, $Pr(X|K) > 0.999$; $K = 3$, $Pr(X|K) \ll 0.01$), a Cape Cod Bay population consisting of Wellfleet and Sandy Neck and a Buzzards Bay population consisting of Sippican Harbor. The Sandy Neck locale is somewhat intermediate as evidenced by a proportion of individuals from Sandy Neck having a high probability of falling into the Sippican cluster (Table 3; 3 of 15 individuals have an assignment probability of <0.6 to the Cape Cod Bay population). This result may be due to relatively recent migration between Buzzard's Bay and Sandy Neck.

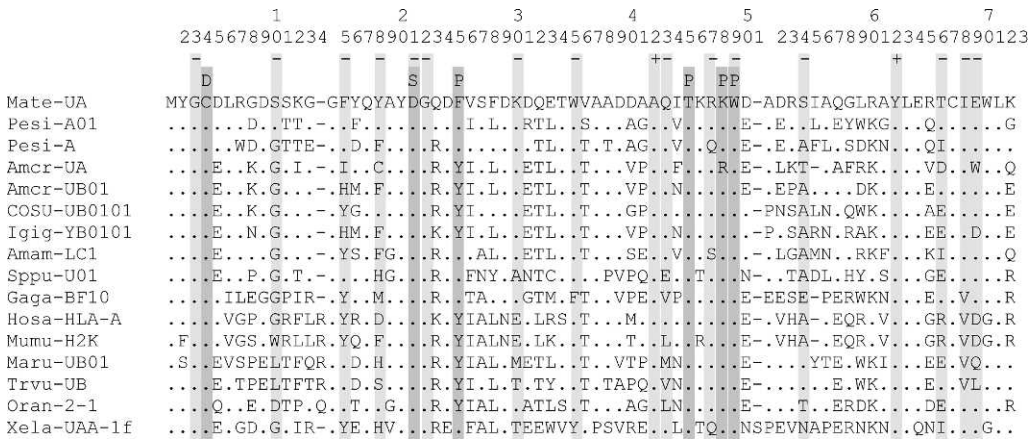

FIG. 2. Amino acid alignment of MHCI α-2 domain. Conserved sites are marked by a D (disulfide bridge forming cysteine), S (salt bridge forming residue), or P (conserved peptide-binding residue of antigen N- and C-terminal binding site). After Glaberman et al. (2008). Results for site specific tests for selection also are shown with a dash (-) marking sites that show evidence for negative selection and a plus (+) for positive selection. The probabilities resulting from the maximum likelihood test for each site for reptiles only are as follows: positive selection (42, $P = 0.081$; 62, $P = 0.040$); negative selection (3, $P = 0.015$; 10, $P = 0.049$; 15, $P = 0.018$; 18, $P = 0.0004$; 21, $P = 0.058$; 22, $P = 0.098$; 30, $P = 0.055$; 35, $P = 0.009$; 43, $P = 0.001$; 47, $P = 0.016$; 49, $P = 0.028$; 54, $P = 0.053$; 66, $P = 0.067$; 68, $P = 0.040$; and 69 $P = 0.010$). Sites 15, 21, 30, 42, 47, and 66 were along the branch leading to terrapins. Mate, *Malaclemys terrapin*; Pesi, *Pelodiscus sinensis*; Amcr, *Amblyrhynchus cristatus*; Cosu, *Conolophus subcristatus*; Igig, *Iguana iguana*; Amam, *Ameiva ameiva*; Sppu, *Sphenodon punctatus*; Gaga, *Gallus gallus*; Hosa, *Homo sapiens*; Mumu, *Mus musculus*; Maru, *Macropus rufogriseus*; Trvu, *Trichosurus vulpecula*; Oran, *Ornithorhynchus anatinus*; Xela, *Xenopus laevis*.

DISCUSSION

We were unable to detect any variation at the MHCI based on direct sequencing and SSCP analysis of 59 individuals derived from three Massachusetts populations, suggesting that *M. terrapin* populations in this region are genetically depauperate at this potentially important immune locus. However, an analysis of six microsatellite regions showed substantial levels of variation within and among these three terrapin populations, with sufficient variation to suggest that these three locales may represent two distinct populations, one population in Cape Cod Bay and the other population south of Cape Cod in Buzzard's Bay.

A possible explanation for the observed lack of variation at the MHCI is that the region amplified was from a nonclassical MHCI, a region usually characterized by low levels of nucleotide variation (e.g., Glaberman et al., 2008), although several studies have shown the MHCI α-2 region to be variable in other reptiles (Madsen et al., 2000; Glaberman and Caccone, 2008; Miller et al., 2010). An alignment of *M. terrapin* MHCI with other MHCI α-2 domains clearly shows that the *M. terrapin* MHCI shares several key conserved residues with reptiles and other species (Fig. 2), suggesting the region sequenced may be a classical MHCI (Kaufman et al., 1994). However, Glaberman et al. (2008) suggest that sharing of conserved sites may not be sufficient evidence for determining whether an MHC region is classical or nonclassical. Repeated attempts to amplify MHCII regions or other MHCI regions proved unsuccessful (McCafferty et al., unpubl. data), and we have yet to assess tissue expression patterns, evidence that would go far in resolving whether we are looking at a nonclassical MHCI. Therefore, we cannot say for certain whether the MHCI sequences presented here are classical or nonclassical. However, this distinction may not be a particularly important distinction because nonclassical MHCI loci also may act as part of the innate immune system; they only function in ways that differ from classical loci (Glaberman and Caccone, 2008). Evidence for conserved binding sites and purifying selection argue that the region we are studying is an adaptive gene region and that it may be involved in antigen binding, although perhaps in a manner that differs from classical MHCI.

Another explanation for the lack of variation at the MHCI is that purifying selection acted recently on this gene region, with a lack of variation at synonymous sites due to linkage effects (selective sweep). To test this possibility, we compared the dN/dS ratio to other reptile MHCIs and found significant evidence for purifying selection. Site-specific tests also suggest purifying selection at several sites. Unfortunately, we were not able to compare our results with other MHC gene regions in *M. terrapin*, and little is known concerning levels of variation at MHC in Testudines in general. As far as we are aware, this is the first population study of any turtle MHC gene region.

TABLE 1. Variation at six microsatellite loci from Massachusetts populations of the Diamond-backed Terrapin. N_a, number of alleles; H_o, observed heterozygosity; and H_e, expected heterozygosity.

Locus	N_a	H_o	H_e
Wellfleet (22)			
GmuD28	7	0.792	0.766
GmuB08	3	0.417	0.398
GmuD87*	8	0.818	0.685
GmuD51	8	0.826	0.734
GmuD55	3	0.542	0.624
GmuD121	4	0.333	0.327
Sandy Neck (15)			
GmuD28	6	0.500	0.592
GmuB08	4	0.500	0.679
GmuD87**	7	0.933	0.691
GmuD51	12	0.900	0.880
GmuD55	3	0.400	0.451
GmuD121	4	0.600	0.516
Sippican (22)			
GmuD28***	7	0.636	0.752
GmuB08	4	0.636	0.611
GmuD87	9	0.905	0.842
GmuD51	12	0.762	0.858
GmuD55	7	0.591	0.638
GmuD121	5	0.727	0.617

*, $0.05 > P > 0.01$; **, $0.01 > P > 0.001$; ***, $P < 0.001$; Hardy–Weinberg test.

TABLE 2. Pairwise F_{st} values among the Wellfleet (WFT), Sandy Neck (SNC), and Sippican Harbor (SH) sampling locations. Below diagonal elements are estimates of F_{st}, the diagonal elements are the within population mean heterozygosity ± SE.

	WFT	SNC	SH
WFT	0.621 ± 0.090		
SNC	0.032	0.639 ± 0.092	
SH	0.117	0.101	0.710 ± 0.047

TABLE 3. Proportion of membership of each predefined population in each of the two clusters. Values are the averages across all runs where K = 2.

	Cluster 1	Cluster 2
Wellfleet	0.97	0.03
Sandy Neck	0.779	0.221
Sippican Harbor	0.025	0.975

Our results implicate the role of selection in the lack of variation observed at MHCI. However, recent population bottlenecks or small effective population size also may have acted to reduce the overall level of variation in the terrapin genome. If this were the case, then we would expect reductions in levels of variation genome-wide, including at microsatellite loci. However, our microsatellite results show appreciable levels of genetic variation consistent with Hauswaldt and Glenn (2005) and Hart (2005). In fact, levels of microsatellite variation were sufficiently large within and among locales such that we were able to distinguish two populations of terrapins in Massachusetts with limited gene flow.

Based on these observations, we propose that the observed lack of variation found at the MHCI in *M. terrapin* is not due to recent population bottlenecks or demographic changes but is the result of natural selection acting some time in the recent past. The presence of substantial levels of variation at the microsatellite loci suggests that the lack of variation at MHCI may not be reflective of the genome in general. However, this conclusion may not necessarily be the case. It is well known that microsatellite variation is driven by a very different mutational process than nucleotide variation (Ellegren, 2004) and therefore may not reflect overall levels of variation at genomic regions other than simple sequence repeats. If this were the case, then the lack of variation at MHCI may reflect overall low levels of genomic variation in terrapins (e.g., Avise et al., 1992; Lamb and Avise, 1992; Parham et al., 2008) and may not be due to the effects of selection alone. To assess this possibility requires studying other genomic regions unlinked to the MHC; we are currently addressing this possibility by using random portions of the genome anchored by retrotransposons.

Irrespective of the cause, it is reasonable to ask whether this lack of variation at a potentially important immune locus is a concern for the long-term viability of these populations. Although examples can be found where low levels of MHC variation correlate with reduced population fitness (e.g., Hedrick, 2001; Siddle et al., 2007), there is also evidence for species with low variation at MHC that apparently remain viable over the long term (Ellegren et al., 1993; Mikko et al., 1999; Weber et al., 2004; Babik et al., 2009). Determining why there is limited variation at this potentially adaptive gene region is important in understanding the factors driving levels of genetic variation in Diamond-backed Terrapins and may give direction to the development of a sound conservation strategy for these threatened populations.

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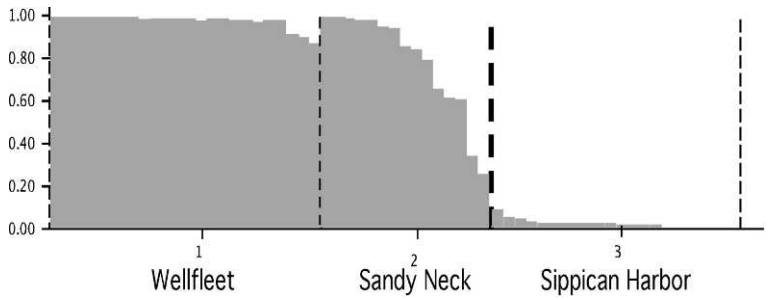

FIG. 3. Plot of individual assignment probabilities to Population 1 (Cape Cod Bay including Wellfleet and Sandy Neck) and Population 2 (Buzzard's Bay, Sippican Harbor). The grey histogram for each individual sampled shows the probability of assignment to Population 1.

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