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Molecular characterization of major histocompatibility complex class II alleles in wild tiger salamanders (*Ambystoma tigrinum*)

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Abstract Major histocompatibility complex (MHC) class II genes are usually among the most polymorphic in vertebrate genomes because of their critical role (antigen presentation) in immune response. Prior to this study, the MHC was poorly characterized in tiger salamanders (*Ambystoma tigrinum*), but the congeneric axolotl (*Ambystoma mexicanum*) is thought to have an unusual MHC. Most notably, axolotl class II genes lack allelic variation and possess a splice variant without a full peptide binding region (PBR). The axolotl is considered immunodeficient, but it is unclear how or to what extent MHC genetics and immunodeficiency are interrelated. To study the evolution of MHC genes in urodele amphibians, we describe for the first time an expressed polymorphic class II gene in wild tiger salamanders. We sequenced the PBR of a class II gene from wild *A. tigrinum* (n=33) and identified nine distinct alleles. Observed heterozygosity was 73%, and there were a total of 46 polymorphic sites, most of which correspond to amino acid positions that bind peptides. Patterns of nucleotide substitutions exhibit the signature of diversifying selection, but no recombination was detected. Not surprisingly, transspecies evolution of tiger salamander and axolotl class II alleles was apparent. We have no direct data on the immunodeficiency of tiger salamanders, but the levels of polymorphism in our study population should suffice to bind a variety of foreign peptides (unlike axolotls). Our tiger salamander data suggest that the monomorphism and immunodeficiencies associated with axolotl class II genes is a relict of their unique historical demography, not their phylogenetic legacy.

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Introduction

Major histocompatibility complex (MHC) class I and class II genes are found in all gnathostomes and encode structurally similar proteins. These proteins bind short peptide chains and present these antigenic peptides to T lymphocytes. Class I proteins are expressed in almost all cells and are involved in monitoring proteins chiefly drawn from the internal environment of the cell. Class II proteins are expressed mainly on specialized antigen-presenting cells and primarily function to bind peptides derived from extracellular processing pathways. In conjunction with T lymphocytes, MHC/peptide complexes play a central role in self/nonself-discrimination and activation of cell-mediated immunity (Abbas et al. 2000). This is accomplished by recognizing the origin of protein peptides as either native or foreign to the host. Because of their importance in immunity and fitness, MHC genes have been isolated in a variety of taxa (Kelley et al. 2005).

The molecular genetics of the MHC has been studied in two urodele amphibians, the tiger salamander (*Ambystoma tigrinum*) and the Mexican axolotl (*Ambystoma mexicanum*). Little work has been done on the tiger salamander, but Southern blots reveal a single class II locus with two cDNA isoforms (Laurens et al. 2001). The axolotl, on the other hand, has been widely used as a model organism in immunology. Multiple class I genes that collectively exhibit high levels of polymorphism have been isolated from the axolotl (Sammut et al. 1999, 1997). Functional class II sequences from this species have also been isolated, revealing a single locus that is almost completely monomorphic (Laurens et al. 2001). This gene has one predominant allele; another allele, which differs by only three nonsynonymous substitutions, was isolated just once (screening for polymorphisms relied largely on inbred laboratory stocks). Similar to tiger salamanders, axolotl class II molecules display alternate splicing of the β1 domain, re-

sulting in two isoforms: one full-length transcript and a truncated transcript that lacks part of the peptide binding region (PBR). In the axolotl, these two isoforms are estimated to be found in a 2:1 ratio of full-length/truncated transcripts (Laurens et al. 2001).

The unique features of the axolotl class II gene may be related to the lack of cellular immunity in this species. The axolotl has been characterized as having an unusually poor immune response, being labeled as immunodeficient. The underlying cause of the immunodeficiency is not clear, but several lines of evidence point to an improper T-cell response (Tournefier et al. 1998). The immunodeficiency could be related to MHC genetics in this species, where the class II gene has been labeled an "unrecognized MHC", implying that functional T cells in the axolotl do not interact effectively with class II molecules (Kaufman et al. 1995). Such a mechanism could explain the lack of variation at the class II locus by eliminating the balancing selection that typically drives MHC polymorphism. Transplant studies suggest that other salamanders also may exhibit a poor immune response (Kaufman et al. 1995), and thus, the general role (if any) of the salamander class II protein is unclear.

The monomorphism of axolotl class II genes could be due to relaxed selection, phylogenetic constraints, or demographic history. Here, we use a comparative approach with tiger salamanders to differentiate among these alternate hypotheses. Unlike axolotls, whose natural geographic distribution is limited to a single lake in Mexico, tiger salamanders are widespread across North America (Petranka 1998). They differ subtly from the axolotl in many regards, but the most pronounced difference is obligate paedogenesis in the axolotl (Tompkins 1978) vs. facultative paedogenesis in the tiger salamander (Collins 1981), where individuals in most populations transform into terrestrial adult forms. Tiger salamanders are paraphyletic with respect to axolotls; in other words, contemporary taxonomy does not accurately reflect historical phylogeny (Shaffer and McKnight 1996). Nevertheless, the close phylogenetic relationship between these organisms means that there are many evolutionarily shared features between them, perhaps including detailed modes of immunosurveillance. By investigating class II polymorphism in wild tiger salamanders, we address potential mechanisms of immunity (i.e., the effective use of the class II/T-cell pathway) as well as the potential effects of alternative splicing on the *A. tigrinum* class II molecule.

Materials and methods

To assess gene diversity, *A. tigrinum* tissue samples (consisting of tail or toe clips) were collected from a single site in a large wetland complex at the Purdue Wildlife Area in central Indiana (USA). Genomic DNA was isolated from 33 individuals using a standard lysis procedure [extraction buffer: 20 mM Tris–Cl, 400 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecyl sulfate, and 5 μl proteinase K (10 mg/ml)]. Phenol-chloroform organic extractions were performed on the cell lysate followed by isopropanol precipitation and a 70% ethanol wash (Sambrook et al. 1989). DNA was resuspended in 100 μl TLE buffer (10 mM Tris–Cl, 0.1 mM EDTA).

We used class II sequences from the axolotl (Laurens et al. 2001) to design novel polymerase chain reaction (PCR) primers which amplify a 264-bp portion of the β1 domain in the class II gene. The primer names and sequences are MHC-B1F, 5′-GAGACCCCAGATGATTT CGTG-3′, and MHC-B1R, 5′-GAAGTCTTCCAAGATC CCGTA- 3′. PCRs were performed in a final volume of 25 μl and contained 1.25 U Taq DNA polymerase (New England BioLabs), 0.005 U Pfu DNA polymerase (Stratagene), 1× PCR buffer (10 mM Tris–Cl, 50 mM KCl, 0.5 μg bovine serum albumin), 1.5 mM MgCl, 0.2 mM each deoxyribonucleotide triphosphate (dNTP), and 0.25 μM each primer. The thermal profile was preceded by 2 min at 94°C and consisted of 34 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s. These cycles were followed by a final extension step at 72°C for 5 min. PCR products were ethanol precipitated and washed prior to T/A cloning.

We evaluated the expression of our class II amplicon using reverse transcriptase–PCR (RT-PCR). Total RNA was extracted from fresh spleen tissue of adult as well as larval tiger salamanders; RNA was also extracted from larval gill tissue. We used TRIzol and the SuperScript III First-Strand Synthesis System (Invitrogen) to perform cDNA synthesis using a poly dT_{20} primer according to the manufacturer's recommendations. The 264-bp fragment of the *Ambystoma DAB* locus was amplified from cDNA using the MHC-B1F/MHC-B1R primers described above. Furthermore, novel primers spanning the entire coding region of the class II *DAB* locus were designed from axolotl sequences in GenBank (AmtiDAB-8 forward primer 5′-GTAGTCCGGACCTCCTGTGA-3′ and AmtiDAB-985 reverse primer 5′-GCTGGTGATTCCAGATTCCT-3′). PCRs were performed in a final volume of 25 μl and contained 1.25 U Taq DNA polymerase and 1× PCR buffer (both from New England BioLabs), 0.2 mM each dNTP, 0.25 μM each primer, and 3 μl of cDNA template. The thermal profile was preceded by 2 min at 94°C and consisted of 35 cycles of 94°C for 15 s, 50°C for 15 s, and 72°C for 30 s These cycles were followed by a final extension step at 72°C for 5 min. PCR products were cleaned using Qiaquick PCR columns (Qiagen) prior to T/A cloning.

We cloned PCR product into the pGEM-T vector (Promega) in 8-μl reactions following manufacturer's recommendations and typical cloning procedures (Sambrook et al. 1989). Recombinant pGEM plasmids were transformed into competent DH5α cells, which were plated on Luria–Bertani media. Prior to sequencing, we used vector primers to amplify the cloned MHC molecules from single bacterial clones. For these amplifications, the thermal profile consisted of 2 min at 94°C followed by 30 cycles

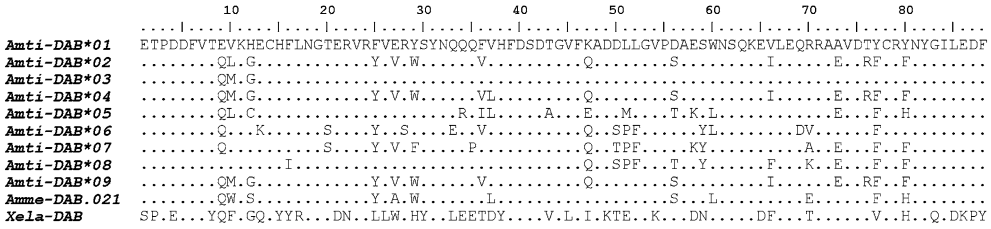

Fig. 1 Amino acid sequence of tiger salamander (*Amti*), axolotl (*Amme*) and African clawed frog (*Xela*) MHC class II alleles. The sequenced section of the gene includes nearly the entire length of the β1 domain. This domain codes for the PBR and typically contains polymorphic sites that determine peptide specificity

of 30 s at 94°C, 30 s at 42°C, and 30 s at 72°C. We amplified 8 to 12 clones from each individual salamander; templates were sequenced using BigDye v3.1 (ABI) chemistry and vector primers. Sequences were edited and aligned in Sequencher v3.0 (GeneCodes), and sequence alignment was straightforward. Each new allele was bi-directionally sequenced multiple times to confirm its authenticity.

DNA and protein sequences were analyzed using several statistical methods. We tested for departures from Hardy–Weinberg equilibrium in GENEPOP (Guo and Thompson 1992). To gauge genetic diversity, we used DnaSP to characterize polymorphism at the DNA level (Rozas and Rozas 1999). To measure amino acid variability and distribution of polymorphic sites, the Wu–Kabat (W–K) variability measure was calculated (Wu and Kabat 1970). In the W–K analysis, highly polymorphic sites were defined as those having greater than twice the mean W–K score.

We also calculated phylogenetic-based ratios of synonymous and nonsynonymous substitutions to infer levels of natural selection (Yang et al. 2000). The program PAML was used to calculate substitution rates using models designated M0, M3, M7, and M8 Yang (2000). The likelihood scores from these models were compared using the Akaike information criterion (AIC; Akaike 1974), and the model with that smallest relative AIC score was selected as the best approximation to these data (Burnham and Anderson 2002). Natural selection was inferred when the best-fit model specified a nonsynonymous to synonymous rate ratio higher than 1 (i.e., $d_N/d_S>1$). Identification of individual codon sites that are under natural selection was carried out using an empirical Bayesian procedure (Yang 2000). We tested for the signature of recombination using the Recombination Detection Program (RDP) (Martin and Rybicki 2000). The phylogenetic relationships among the sequences were determined using model-based approaches (Bos and Posada 2005). The best-fit model was selected using the AIC criterion and likelihood scores were automatically generated and compared using MODELTEST (Posada and Crandall 1998).

Results

We used the MHC-B1F and MHC-B1R primers to sequence 264 bp of the β1 domain in 33 wild tiger salamanders and discovered nine unique alleles in these data (Fig. 1; GenBank accession numbers DQ071905–DQ071913). Several lines of evidence indicate these sequences are allelic (i.e., derived from a single locus), most notably (1) the lack of departures from Hardy–Weinberg equilibrium (see below) and (2) a maximum of two alleles in any one individual despite having sequenced as many as a dozen clones from an individual. Furthermore, only a single classical class II locus is found in the axolotl (Laurens et al. 2001).

Expression analyses

Our sequences could, in theory, be from a single unexpressed locus (e.g., a pseudogene). However, several lines of indirect evidence indicate that the class II locus we assayed is expressed. First, a BLAST search of the novel *A. tigrinum* sequences reveals closest similarity to other expresses class II sequences in the GenBank database. Second, each allele contained an open reading frame (ORF). Third, residues that are evolutionarily conserved and are known to bind the peptide main chain are conserved in these data (Brown et al. 1993; Kaufman et al. 1994). Finally, positions that form salt bonds (72R, 76D), glycosylation sites (19N, 21T/S), and disulfide bonds (45G, 54G) are conserved in these salamander class II sequences.

In addition to the indirect evidence, our RT-PCR data provide direct evidence for the expression of the β1 MHC-B1F/MHC-B1R amplicon. First, cDNA synthesis was robust when using RNA from adult spleen, larval spleen, and larval gill tissues. Second, all β1 domain cDNA clone sequences perfectly matched known alleles originally isolated from genomic DNA. Finally, we used the DAB primers and cDNA templates to isolate the complete coding region of the *Ambystoma* class II gene. Two amplicons were sequenced; a full-length transcript (1,047 nt) that included an ORF throughout the coding region of the gene as well as a truncated transcript (763 nt) that lacked most of the β1 domain (see supplementary material). Both transcripts were isolated from adult spleen, larval spleen, and larval gill tissue. The full-length *A. tigrinum* transcripts bore 96–98% similarity to each other (30 segregating sites), and each had approximately 90% similarity to the *A. mexicanum DAB* locus (Laurens et al. 2001). Thus, the direct as well as the indirect evidence suggest the sequences reported herein represent a functional class II

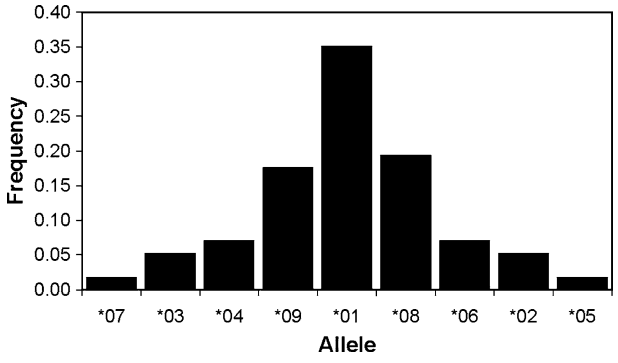

Fig. 2 Allele frequency data of the *Amti-DAB* locus from a wild population of *n*=33 tiger salamanders

locus that is expressed and binds peptides in classical immunologic function. This expressed class II locus is orthologous to that of axolotls (Laurens et al. 2001).

Molecular population genetics

The nine *A. tigrinum* alleles isolated from wild tiger salamanders exhibit patterns of variation expected for a classical MHC gene. Nine out of the 33 (27%) salamanders were homozygous; heterozygosity was 73%, consistent with high levels of microsatellite diversity in this population (Williams and DeWoody 2004; R. Williams, unpublished data). MHC *DAB* allele frequencies ranged from a high of 0.35 to a low of 0.02 (Fig. 2). Genotype frequencies were consistent with Hardy–Weinberg equilibrium. Overall, nucleotide sequences contained 46 segregating sites, with a mean difference of 19.6 nucleotides among alleles. Thus, on average, a randomly chosen allele was identical with another randomly chosen allele at 92.6% of the sites. In comparison, tiger salamander alleles shared an average of 91.8 and 62.4% nucleotide identity with axolotl and *Xenopus laevis* class II sequences, respectively. Given the near identity between axolotl and tiger salamander alleles, it is important to note that there has never been any axolotl tissue, DNA, or PCR products in our newly constructed laboratory; thus, we can rule out transspecies PCR contamination.

Only five synonymous substitutions were observed among the nine *A. tigrinum*, alleles compared to 41 amino acid replacement differences, for an observed d_N/d_S ratio of 8.2:1. Thus, amino acid sequence variation was high as well. The average W–K variability value for all amino acid positions was 1.97, and 11 highly polymorphic sites were identified (Fig. 3). These sites were spread across the exon and have a strong correspondence with sites that are highly polymorphic and bind peptides in humans. Variation at these sites suggests that these tiger salamander class II alleles can bind different sets of peptides.

Unobserved substitutions and their rates were estimated for the nucleotide data using various codon models; those that allowed $d_N > d_S$ were a better fit to these data. The best-fitting substitution model according to the AIC was M8 (Table 1). In this model, the majority of codon sites are under purifying selection with a $d_N/d_S<1$. Codon sites that are identified as having $d_N/d_S>1$ with $P<0.001$ correspond well with highly polymorphic codon sites identified in the W–K analysis. In fact, each highly polymorphic site is under selection, but some selected sites are not classified as highly polymorphic.

Recombination is often important in the maintenance of variation at MHC genes (Jakobsen et al. 1998). However, there was no evidence for interallelic genetic exchange among these alleles (data not shown). Therefore, these data are amenable to phylogenetic analysis, which was conducted using maximum likelihood. Using the AIC to

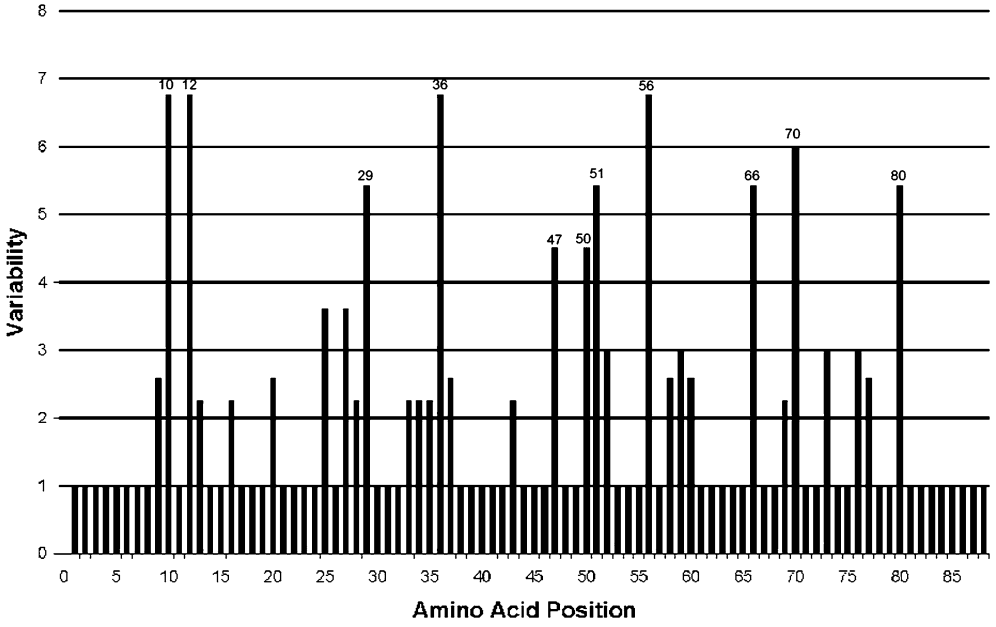

Fig. 3 W–K variability plot of *A. tigrinum* MHC class II alleles. The average W–K value is 1.97, and those sites with a W–K score ≥4 are highly polymorphic. The 11 amino acids that are highly polymorphic are labeled with their position number

Table 1 Likelihood scores and δAIC scores of various codon models used to estimate substitution rates

Various models make assumptions regarding d_N/d_S ratios (ω) and whether they can be higher than 1. The ω values in M0 and M7 are average values over all rate ratio categories, and parameter annotation follows Yang et al. (2000)
[a]Indicates model constraint $\omega \leq 1$

Model	d_N/d_S[a]	Likelihood	δAIC	Parameters		
M8	N	−700.44	Best	p_0=0.595	p=0.041	q=2.596
				p_1=0.405	ω=2.94	
M3	N	−700.10	1.32	p_0=0.584	p_1=0.399	p_2=0.017
				ω_0=0.000	ω_1=2.671	ω_2=17.651
M7	Y	−705.82	5.58	p=0.200	q=0.031	ω=0.350
M0	Y	−723.15	39.42	ω=0.956		

distinguish among models, the F81 nucleotide model (Felsenstein 1981) with invariable sites and a gamma distribution for rate variation among sites (Yang 1994) was selected to approximate the evolution of nucleotide substitutions. The reconstructed gene tree is seen in Fig. 4, where the axolotl class II sequence (*Amme-DAB.021*) is nested within the *A. tigrinum* sequences, closely related to *Amti-DAB*02*, **04*, and **09*. This gene tree is consistent with the idea that *A. mexicanum* is derived from *A. tigrinum* (Shaffer 1984).

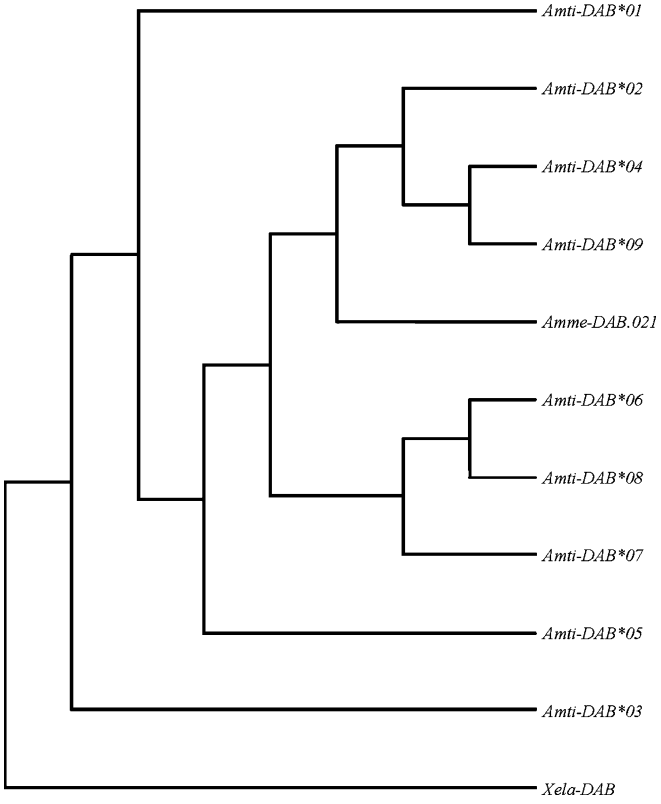

Fig. 4 Neighbor-joining estimate of evolutionary relationships among amphibian class II alleles (branch lengths not shown to scale); alternative models of nucleotide substitution produced trees with similar topologies. Urodele amphibian sequences are monophyletic, and the axolotl sequence (*Amme*) is nested within tiger salamander (*Amti*) alleles. As an out-group, we included in this analysis an *X. laevis* class II sequence (*Xela-DAB*). The paraphyly in this gene tree reflects that of the taxonomy (Shaffer 1984)

Discussion

This research describes the first reported polymorphic MHC class II locus in a urodele amphibian. We have identified allelic sequences from a single expressed class II locus in *A. tigrinum*, and these sequences bear the well-known conserved residues common to class II genes in many evolutionarily diverse organisms (Kaufman et al. 1994). Our relatively large sample from a single wild population of *A. tigrinum* shows polymorphism comparable to the average number of observed differences among *HLA-DRB* alleles in humans. This is in sharp contrast with the axolotl, in which only two very similar alleles have been found (Laurens et al. 2001). Heterozygosity was high in *A. tigrinum*, exceeding 70%. These high levels of genetic diversity in a wild population are consistent with some form(s) of balancing selection (e.g., natural selection or sexual selection).

The pattern of substitution rates provides strong evidence for natural selection acting on the class II locus of *A. tigrinum*. Amino acid sites that are under selection are those same sites that are highly polymorphic and are inferred to interact with the peptide (Brown et al. 1993). This implies that class II proteins are functioning to bind and present a large array of foreign peptides to T cells. Further, an effective class II/peptide/T-cell receptor interaction is necessary for natural selection to operate on these class II molecules and promote polymorphism. This contrasts with the class II molecules of the axolotl, where selection is apparently ineffective at maintaining allelic diversity and polymorphism. Natural selection is effective only if $N_e(s) \geq 1$, where s is the selective coefficient and N_e is the effective population size (Kimura and Ohta 1969). In axolotls, s may be smaller than in tiger salamanders due to poor MHC/T-cell interaction. Furthermore, even when s is large and positive, mutant alleles may drift to extinction simply because new mutants are effectively neutral in small populations. Axolotls evolved in a single Mexican lake and thus probably have a relatively small long-term N_e, and N_e is further reduced in inbred laboratory populations. In our large population of wild tiger salamanders, balancing selection appears to maintain genetic diversity, but in the axolotl population, demography likely has a stronger influence on molecular genetic variability than immunology or phylogeny.

The pattern of axolotl class II monomorphism differs from the variability reported for the class I genes. This discrepancy may seem difficult to reconcile with the importance of population demographics that we advocate. However, we note that only a single class II locus has been identified, while as many as 16 class I genes exist (Laurens et al. 2001; Sammut et al. 1999). The variability noted for class I genes is collective, accrued over many loci. Based on the numbers of class I cDNA sequences obtained from each axolotl individual (19–21) (Sammut et al. 1999), many individual class I loci must also display low levels of allelic diversity. Thus, the class I molecules may have escaped the effects of a population bottleneck through polylocism, whereas such an avenue for escape was not available to the class II molecules. Of course, differences in class I and class II polymorphism could also reflect different levels of selection or simply the stochastic nature of genetic drift causing loss of alleles during population declines.

Although the axolotl originated from a population of *A. tigrinum*, they do not share all functional properties in their class II genes. Certain features of the axolotl MHC are unique to that organism. In light of changes in the immune system of frogs during metamorphosis (Salter-Cid and Flajnik 1995), it is plausible to infer changes in immune function as related to axolotl speciation or as a consequence of hormonal shifts required by paedogenesis and repression of metamorphosis.

One aspect of MHC genetics that is shared by laboratory populations of *A. mexicanum* and *A. tigrinum* is that both have splice variants that lack the β1 domain, resulting in class II molecules without a complete functional PBR (Laurens et al. 2001). The presence of a sizeable population of class II molecules lacking a functional PBR on the surface of a cell could have numerous effects on MHC/T-cell interaction, not the least of which relates to dosage dependence of the T-cell response and functional MHC concentration (Matis et al. 1983). Therefore, it has been suggested that this splice variant has contributed to the monomorphism of the class II gene in *A. mexicanum* (Laurens et al. 2001). Because we find class II polymorphisms in *A. tigrinum*, it is unlikely that transcripts which lack the PBR have an overriding effect resulting in class II monomorphism. Nevertheless, it is very plausible that the presence of nonfunctional splice variants has other effects on immunobiology of ambystomatid salamanders.

There is no evidence for interallelic recombination at the *A. tigrinum* class II locus, although such recombination has been documented in the class II genes of other species (Jakobsen et al. 1998). A phylogenetic tree of *A. tigrinum* MHC alleles (Fig. 4), along with axolotl and *X. laevis* sequences, shows that *A. tigrinum* and axolotl sequences are intermingled. Furthermore, phylogenetic analyses of electrophoretic proteins has revealed that *A. mexicanum* is nested within the species *A. tigrinum* at large (Shaffer 1984). Such paraphyletic patterns could result if the axolotl was recently derived from local populations of *A. tigrinum*. Electrophoretic proteins are not generally considered to be under balancing selection, so it is likely that the trans-species nature of MHC polymorphisms is also influenced by incomplete lineage sorting (Avise 2000) in the short time since genetic separation into species-specific groups.

The urodele model organism has been compared with the patterns of MHC variation in the anuran model organism used in immunology, the African clawed frog (*X. laevis*) (Tournefier et al. 1998). In *X. laevis*, there are three class II loci that are polymorphic and one class Ia locus that has displayed some of the highest levels of MHC polymorphism yet seen (Flajnik et al. 1999; Sato et al. 1993). Previous analyses pointed out the contrasting high levels of polymorphism of the single class Ia locus in *X. laevis* with monomorphism of the single class II locus in *A. mexicanum* (Laurens et al. 2001). We find substantial polymorphism in terms of allelic as well as nucleotide diversity at the class II locus in *A. tigrinum*. While polymorphism is considerably lower than at the class Ia locus of *X. laevis*, it is comparable to the class II loci of that species and likely provides sufficient variation to bind a variety of foreign peptides.

Here, we have described a polymorphic class II locus in *A. tigrinum*, a close relative of the axolotl. These two species share many aspects of biology, including genetic aspects of their MHC class II immunology, but they differ drastically with respect to the polymorphism of their class II genes. This important difference helps clarify the basis of patterns of MHC variation in these two species and provides information with which to compare to the well-established axolotl model. *A. tigrinum* can be a valuable species in which to compare and conduct further research on functional immunology, immunodeficiency, and impact of splice isoforms on the immune response.

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