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Evidence for balancing selection at the *DAB* locus in the axolotl, *Ambystoma mexicanum*

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Summary

The axolotl (*Ambystoma mexicanum*) has been characterized as immunodeficient, and the absence of major histocompatibility complex (MHC) class II polymorphism has been cited as a possible explanation. Here we present evidence for considerable allelic polymorphism at the MHC class II *DAB* locus for a sample of wild-caught axolotls. Evidence that these sequences are the product of balancing selection for disease resistance is discussed.

Introduction

Among the best examples of genetic variation maintained by selection is that of extreme polymorphism at class I and II histocompatibility genes in vertebrates. In tetrapod vertebrates examined so far, these genes occur together in the genome in a gene dense region called the major histocompatibility complex (MHC) (Kelley *et al*., 2005). The proteins encoded by class I and II MHC loci are responsible for presentation of foreign antigens to T cells, a key step in recognition of the presence of infection and generation of an adaptive immune response. Studies of molecular sequence polymorphism at class I and II molecules demonstrate strong diversifying selection on sites affecting antigen binding (Hughes & Nei, 1989). Nevertheless, how functional differences in antigen binding are translated into selection capable of maintaining many alternative alleles in populations, hereafter collectively referred to as balancing selection, remains controversial. Potential explanations include spatial and temporal variation in host pathogens (Hedrick, 2002), overdominant and frequency-dependent selection (Hughes & Yeager, 1998).

The genetics of the MHC has been investigated in two urodele amphibians, the axolotl *Ambystoma mexicanum* and *Ambystoma tigrinum*. The immunogenetics of the axolotl in particular has received more attention, as a model organism for the study of the immune system in amphibians. The axolotl has been characterized as immunodeficient due to slow humoral and cytotoxic immune responses (Tournefier *et al*., 1998). It has been proposed that these observations are due to impaired antigenic presentation by class I and II molecules, and a lack of polymorphism at the one class II gene characterized in this species, *DAB*, has been cited in support of this idea (Tournefier *et al*., 1998; Sammut *et al*., 1999; Laurens *et al*., 2001). A parallel study in the closely related *A. tigrinum* found significant molecular and allelic polymorphism at the *DAB* locus (Bos & DeWoody, 2005), indicating that this locus is indeed the target of balancing selection. However, the evidence that *A. mexicanum* is deficient in *DAB* polymorphism is weak; almost all individuals so far examined are from laboratory stocks removed from the wild many decades ago. The ability to sample wild populations more recently has been restricted due to its status as a critically endangered species. As part of a study of the conservation status of wild axolotls, we characterized *DAB* polymorphism in a sample of wild-caught *A. mexicanum*.

Materials and methods

Wild *A. mexicanum* individuals were captured by the use of throw nets. A small piece of the external gill tissue was removed and preserved in alcohol prior to release of the animal. DNA was extracted using the Qiagen DNAeasy kit (Qiagen, Hilden, Germany), following the manufacturer's recommendations for alcohol preserved samples.

Polymerase chain reaction (PCR) amplification of *DAB* sequences in *A. mexicanum* was carried out using locus-specific primers used to amplify *DAB* sequences in *A. tigrinum* (Bos & DeWoody, 2005). Analysis and characterization of *DAB* sequence polymorphism were performed using single-strand conformation polymorphism (SSCP) (Jaeckel *et al*., 1998). PCR samples identified as homozygotes were sequenced directly in both directions using the amplification primers. Alternative alleles identified in putative heterozygotes were excised from the SSCP gel, reamplified and directly sequenced.

The new *DAB* sequences were aligned to published *A. mexicanum* and *A. tigrinum DAB* sequences. Amino acid sites participating in antigen binding were identified

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          1         11        21        31        41        51        61        71        81
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AT01      ETSDDFVTEV KHECHFLNGT ERVRFVERYS YNQQQFVHFD SDTGVFKADD LLGVPDAESW NSQKEVLEQR RAAVDTYCRY NYGIYEDF
AT02      ..P.....QL .G........ ....Y.V.W. .....V.... ......Q... .....S.... .....I.... ..E..RF..F ....L...
AT03      ..P.....QM .G........ .......... .......... .......... .......... .......... .......... ....L...
AT04      ..P.....QM .G........ ....Y.V.W. .....VL... ......Q... .....S.... .....I.... ..E..RF..F ....L...
AT05      ..P.....QL .C........ .......... ...R.IL... ..A...E... M....T.K.L .......... ..E...F..H ....L...
AT06      ..P.....Q. ..K......S ....Y..S.. ..E..V.... ......Q..S PF......YL ........DV ......F... ....L...
AT07      ..P.....Q. .........S ....Y.V.F. ....P..... ......Q..T PF.....KY. .........A ..E...F..F ....F...
AT08      .......... .....I.... .......... .......... ......Q..S PF...T..Y. .....F...K ..E...F..F ........
AT09      ..P.....QM .G........ ....Y.V.W. .....V.... ......Q... .....S.... .....I.... ..E..RF..F ....L...
AM5       ..P.....QW .S........ ....Y.V.W. ......L... .......... .....S...L .........E ......F..H K...L...
AM6       ..P.....QW .S........ ....Y.A.W. ......L... .......... .....S...L .........E ......F..H ....L...
AM3       ..P.....QM .G........ ....Y.V... ...L...... .....YE..T PF...T..Y. .....F...E ......V..H ....L...
AM7       ..P.....QM .G........ ....Y.V... ...L...... .....YE..T PF...T..Y. .........K ..E...V..H ....L...
AM10      ..P.....QM .G........ ......V.C. .....VL... .......... .....T.... .........A ......V..F ....L...
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Figure 1. Alignment of *Ambystoma DAB* sequences. Key to sequence abbreviations: AT, *Ambystoma tigrinum*, AM, *Ambystoma mexicanum*. *Indicates putative antigen-binding site. Sites providing significant evidence for diversifying selection ($\omega > 1$), identified using OMEGAMAP, are in boldface.

assuming homology to those sites identified by three-dimensional crystallography of the human *DRB*-01 allele (Brown *et al*., 1993). Evidence for recombination among *Ambystoma* sequences was assessed by estimating the correlation between pairwise linkage disequilibrium with physical distance, implemented in the program LDHAT (McVean *et al*., 2002). Evidence for diversifying (positive) selection acting at the molecular sequence level was assessed using the programs CODEML (Yang, 1997) and OMEGAMAP (Wilson & McVean, 2006). Positive selection acting to change a coding sequence over time is expected to result in a larger number of non-synonymous substitutions per non-synonymous site (dN) relative to the number of synonymous substitutions per synonymous site (dS); accordingly, positive selection is indicated by a dN/dS ratio significantly greater than 1. OMEGAMAP generates means and credible intervals for dN/dS (ω) and recombination ($\rho = 4Nr$) for each codon, where N and r represent the effective population size and the per codon rate of recombination, respectively. Furthermore, the method provides posterior probabilities for ω assuming particular prior probability distributions for ω and ρ. Estimates of ω and ρ were obtained assuming either uniform or exponential prior distributions. OMEGAMAP explores parameter space using Markov Chain Monte Carlo (MCMC) sampling. To assess convergence for a given set of prior distributions, results of independent runs using different starting parameters were compared. Independent MCMC chains were run for 250 000 iterations, and the first 5000 were discarded prior to analysis.

Results and discussion

Sequence polymorphism and heterozygosity

We identified five different *DAB* alleles (GenBank accession no. EF585228–32) in our sample of nine wild *A. mexicanum* individuals, which included one sequence identical to the one published axolotl *DAB* sequence (Fig. 1). No more than two sequences were identified in any one individual, suggesting that these sequences are allelic. This inference is consistent with a Southern blot analysis in *A. mexicanum*, which indicated the presence of a single *DAB* locus (Laurens *et al*., 2001). Three individuals were heterozygous. Expected heterozygosity for the *A. mexicanum* sample ($H_e = 0.53$) is about two-thirds that estimated in a larger sample of *DAB* polymorphism in the closely related *A. tigrinum* (Bos & DeWoody, 2005).

The most common allele in the *A. mexicanum* sample (AM6, see Fig. 1) was also the one previously published for this species. We recovered one allele that differed from this sequence by a single substitution (AM5). Other alleles are more similar to *DAB* sequences recovered from *A. tigrinum* (Fig. 1). This suggests that at least some allelic polymorphism in *A. mexicanum* was inherited from a common ancestor of both species, as is commonly inferred for MHC loci under balancing selection. We therefore analysed *Ambystoma* sequences together in assessing evidence for balancing selection and recombination in the history of these sequences.

DAB sequence diversity and evidence for balancing selection

Phylogenetic tests for diversifying selection such as those implemented in CODEML (Yang, 1997) are sensitive to the effects of recombination, which increases the frequency of falsely positive results (Shriner *et al*., 2003). Because balancing selection maintains selectively distinct alleles for long periods of time, we expect that appreciable recombination may have occurred in the history of sampled *DAB* sequences. There is in fact a significant correlation between amount of pairwise linkage disequilibrium and distance between sites in the *Ambystoma* data as whole ($r = -0.19$, $P < 0.005$) and the *A. mexicanum* sequences in particular ($r = -0.23$, $P = 0.01$). This correlation is consistent with the effects of intragenic recombination (and/or gene conversion). We therefore assessed the evidence for positive selection using OMEGAMAP, which jointly estimates both the dN/dS ratio and the amount of recombination for each codon using a Bayesian methodology, generating posterior probabilities of positive selection for

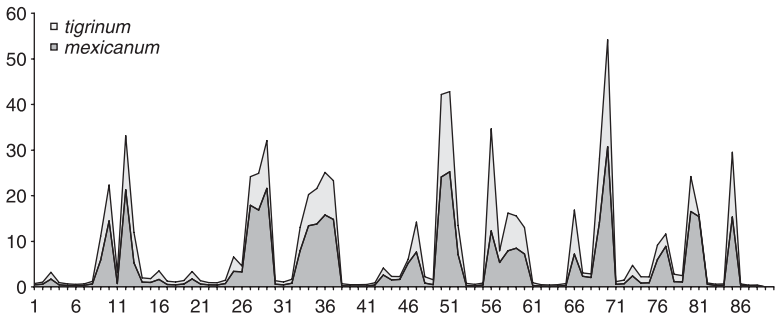

Figure 2. Site estimates of ω (dN/dS) for *DAB* sequences from *Ambystoma tigrinum* and *Ambystoma mexicanum* estimated using OMEGAMAP, controlling for recombination (see Methods).

each site. Both the *Ambystoma* data set as a whole and the *A. mexicanum* sequences in particular show significant evidence for diversifying selection, controlling for the effects of recombination (Fig. 2). The sites identified as under diversifying selection in *A. mexicanum* correspond well with those sites expected to participate in antigen binding (Fig. 1). In general, the results using CODEML and OMEGAMAP were highly comparable (data not shown). The more conservative procedure implemented in OMEGAMAP treats recombination as nuisance parameter. We note that if recombinant sequences are maintained by balancing selection, then this procedure will be overly conservative.

Conclusions

Collectively, our results are consistent with balancing selection acting to maintain selectively distinct alleles in the population for prolonged periods relative to expectation for selectively neutral alleles. While similar results are routinely reported for homologous class II genes in other taxa, in this case such results are of interest because *A. mexicanum* has been supposed to be immunodeficient (Tournefier *et al*., 1998), as well as nearly monomorphic at the *DAB* locus (Laurens *et al*., 2001). Our results clearly overturn the supposition regarding lack of *DAB* polymorphism in *A. mexicanum*. Evidence for balancing selection summarized above also casts doubt on the more general proposition that this species is immunodeficient, although the small sample size limits the strength of this conclusion. The most definitive evidence for recent balancing selection would be to show that the current allele frequencies are too even to be explained by a process of genetic drift alone (Slatkin, 1994, 1996; Slatkin & Muirhead, 2000). However, the sample analysed here is too small to allow for a robust test of this hypothesis.

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References

Bos, D.H. & DeWoody, J.A. (2005) Molecular characterization of major histocompatibility complex class II alleles in wild tiger salamanders (*Ambystoma tigrinum*). *Immunogenetics*, **57**, 775.

Brown, J.H., Jardetzky, T.S., Gorga, J.C., Stern, L.J., Urban, R.G., Strominger, J.L. & Wiley, D.C. (1993) Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1 [see comments]. *Nature*, **364**, 33.

Hedrick, P.W. (2002) Pathogen resistance and genetic variation at MHC loci. *Evolution. International Journal of Organic Evolution*, **56**, 1902.

Hughes, A.L. & Nei, M. (1989) Nucleotide substitution at major histocompatibility complex class II: Evidence for overdominant selection. *Proceedings of the National Academy of Sciences of the USA*, **86**, 958.

Hughes, A.L. & Yeager, M. (1998) Natural selection at major histocompatibility complex loci of vertebrates. *Annual Review of Genetics*, **32**, 415.

Jaeckel, S., Epplen, J.T., Kauth, M., Miterski, B., Tschentscher, F. & Epplen, C. (1998) Polymerase chain reaction-single strand conformation polymorphism or how to detect reliably and efficiently each sequence variation in many samples and many genes. *Electrophoresis*, **19**, 3055.

Kelley, J., Walter, L. & Trowsdale, J. (2005) Comparative genomics of major histocompatibility complexes. *Immunogenetics*, **56**, 683.

Laurens, V., Chapusot, C., del Rosario Ordonez, M., Bentrari, F., Padros, M.R. & Tournefier, A. (2001) Axolotl MHC class II beta chain: predominance of one allele and alternative splicing of the beta1 domain. *European Journal of Immunology*, **31**, 506.

McVean, G.A.T., Awadalla, P. & Fearnhead, P. (2002) A coalescent-based method for detecting recombination from gene sequences. *Genetics*, **160**, 1231.

Sammut, B., Du Pasquier, L., Ducoroy, P., Laurens, V., Marcuz, A. & Tournefier, A. (1999) Axolotl MHC architecture and polymorphism. *European Journal of Immunology*, **29**, 2897.

Shriner, D., Nickle, D.C., Jensen, M.A. & Mullins, J.I. (2003) Potential impact of recombination on sitewise approaches for detecting positive natural selection. *Genetics Research*, **81**, 115.

Slatkin, M. (1994) An exact test for neutrality based on the Ewens sampling distribution. *Genetics Research*, **64**, 71.

Slatkin, M. (1996) A correction to the exact test based on the Ewens. *Genetics Research*, **68**, 259.

Slatkin, M. & Muirhead, C.A. (2000) A method for estimating the intensity of overdominant selection from the distribution of allele frequencies. *Genetics*, **156**, 2119.

Tournefier, A., Laurens, V., Chapusot, C., Ducoroy, P., Padros, M.R., Salvadori, F. & Sammut, B. (1998) Structure of MHC class I and class II cDNAs and possible immunodeficiency linked to class II expression in the Mexican axolotl. *Immunological Review*, **166**, 259.

Wilson, D.J. & McVean, G. (2006) Estimating diversifying selection and functional constraint in the presence of recombination. *Genetics*, **172**, 1411.

Yang, Z. (1997) PAML: a program package for phylogenetic analysis by maximum likelihood. *Computer Applications in Biosciences*, **13**, 555.