RESEARCH PAPER | Agriculture | Volume : 3 | Issue : 9 | Sept 2013 | ISSN - 2249-555X

Identification and characterization of major histocompatibility complex class IIB alleles in three species of European ranid frogs

KEYWORDS: antigen binding site, Anura, Pelophylax, Rana, Ranidae

BÉLA MAROSI
Department of Parasitology, University of Agricultural Science and Veterinary Medicine, Manastur street nr. 3-5, RO-400372 Cluj-Napoca, Romania,

KAREN M. KIEMNEC-TYBURCZY
Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY 14853 USA

IOAN V. GHIRA
Faculty of Biology and Geology, Babes-Bolyai-University Cluj-Napoca, Clinicilor street 5-7, RO-400006 Cluj-Napoca, Romania

TIBOR SOS
Association for Bird and Nature Protection "Milvus Group", Targu Mures, 22 Crinului Street, RO-540343

OCTAVIAN POPESCU
Molecular Biology Center, Interdisciplinary Research Institute on Bio-Nano-Sciences, Babes-Bolyai University Cluj-Napoca, 42 Treboniu Laurian Street, RO-400271

ABSTRACT *Immune genes of the major histocompatibility complex (MHC) are some of the most polymorphic genes in the vertebrate genome. Due to their polymorphic nature, they are often used to assess the adaptive genetic variability present in natural populations. Here, we describe the first molecular characterization of 13 partial MHC class IIB sequences from three European ranid frogs. We expand the utility of previously published primers by using them to successfully amplify eight exon 2 alleles from Rana arvalis. We also designed a novel pair of primers that successfully amplified exon 2 from Pelophylax kurtmuelleri and Pelophylax lessonae. Our results indicate the presence of one or two class IIB loci in these three species. In R. arvalis, we found significant evidence of positive selection acting on antigen binding sites. Many European ranid populations are experiencing disease-related declines and our newly developed primers can be used for further population analyses of native frog species.*

Introduction

The major histocompatibility complex (MHC) is a relatively large region found in the genomes of all the jawed vertebrates. Most of the genes in this region are necessary for a functional immune response and are thus vital to an organism's survival. MHC class II proteins, specifically, are expressed on the surface of specialized antigen presenting cells and their primary function is to present extracellular antigens to helper T cells (Klein 1986). The mature MHC class II complex is composed of one alpha (A) and one beta (B) peptide chain; these are encoded by the MHC II A and B genes, respectively. The antigen binding site (ABS) of this heteromeric protein complex is encoded by the second exons of the A and B genes (Edwards *et al*. 2000). The amino acid residues in the ABS bind pathogen-derived peptides are often extremely polymorphic and show signatures of natural selection (Hughes and Yeager 1998).

Because MHC genes play such a key role in the vertebrate immune response, they are often used to assess the structure and status of wildlife populations. Interest in monitoring amphibian populations, in particular, is increasing because of the emergence of infectious diseases caused by *Batrachochytrium dendrobatidis* (*Bd*) (Berger *et al*. 1998) and ranavirus(es) (Granoff 1989). Many European ranid frogs are experiencing population declines due to these diseases (e.g., *Rana klepton esculenta*; Wood *et al*. (2009) and *Rana temporaria*; Teacher *et al*. (2010), but there are only a few studies that characterize MHC genes in European frogs (but see Hauswaldt *et al*. 2007; Zeissest and Beebee 2009). Evaluation of the genetic variation at MHC loci in endangered and threatened amphibian species may be useful for developing conservation management strategies that target populations that are highly threatened by infections and/or by the effects of inbreeding (Sommer 2005). The aim of the present study was to develop ranid-specific primers and to characterize the MHC class IIB exon 2 in three European ranid species: the Moor frog (*Rana arvalis)*, the Balkan Water Frog (*Pelophylax kurtmuelleri)* and the Pool Frog (*Pelophylax lessonae*).

Materials and Methods

Specimens and DNA extraction

We obtained ethanol-preserved tissues from *R. arvalis* (n=3), *P. kurtmuelleri* (n=2), *R. temporaria* (n=2) that were collected in Romania and archived at the Babes-Bolyai University Zoological Museum. We collected two *P. lessonae* adults in south Romania near Danube River. All individuals from all species were collected from different populations. Genomic DNA was extracted from toe clips of all frogs using the Nucleospin®Tissue Kit following the standard protocol (Macherey Nagel, Düren, Germany). Since *P. kurtmuelleri* is difficult to distinguish morphologically from *R. ridibunda*, we isolated approximately 590 bps of the 16S RNA mitochondrial gene using 16Sar and 16Sbr primers (Palumbi 1996) and compared it to other sequences available in GenBank to confirm our individuals were *P. kurtmuelleri*.

PCR amplification

We used a degenerate primer pair (MHC-F and MHC-5R) developed by Hauswaldt *et al*. (2007) to amplify a 235 bp fragment from *R. arvalis* and *R. temporaria* genomic DNA. The PCR was carried out with the GoTaq® Flexi DNA Polymerase (Promega, Madison, WI) and PCR conditions are available from the corresponding author by request. Using the published sequences from other *Rana* (Kiemnec-Tyburczy *et al*. 2010; Zeisset and Beebee 2009), we developed a new set of degenerate primers (RanaF 5′ -CAGTGTTATTACCGGAACGGGACG-3′ and RanaR2: 5′ -TTTSMGSTCTATGGCTGYAGG-3′) that we used to amplify exon 2 from *P. kurtmuelleri* and *P. lessonae*. The samples were run on 2.5%

Volume : 3 | Issue : 9 | Sept 2013 | ISSN - 2249-555X

agarose gel and PCR products were extracted from gel using the Nucleospin® Extract Kit (Macherey Nagel). Purified PCR products were ligated into the pTZ57R/T vector using the InsTAclone™ PCR Cloning Kit (Fermentas, Cluj-Napoca, Romania). XL1 blue *Escherichia coli* were used for transformation. Plasmid DNA was extracted from 10 positive colonies by GeneJET™ Plasmid Miniprep Kit (Fermetas). After restriction enzyme testing of the size of each vector insert, five positive clones from each individual were sent to Macrogen Inc., (Seoul, Korea) for sequencing.

Data analysis

We used BioEdit v7.0.9 (Hall 1999) to edit and trim our sequences. For alignment of the sequences, (nucleotide and amino acid) we used MEGA version 5.0 (Tamura *et al*. 2011). To estimate the evolutionary divergence between sequences the average pairwise nucleotide distances (Kimura 2-parameter model) and Poisson correction were used.

To test for positive selection on the putative ABS of *R. arvalis* and *P. lessonae* separately we used a one-tailed Z-test of selection calculated in MEGA. We determined the ABS residues for our frog sequences according to the model of Tong *et al*. (2006). The average codon-based evolutionary divergence was analyzed separately for the ABS and non-ABS; the rate of synonymous substitutions (d_S) and rate of nonsynonymous (d_N) substitutions and the differences between synonymous and nonsynonymous distances (d_N - d_S) were estimated using the Nei-Gojobori method with Jukes-cantor correction in MEGA 5.0. The variances were computed using the bootstrap method (1000 replicates). To test for positive selection acting on the entire alignment of all four species (including both ABS and non-ABS), we performed PARRIS (Scheffler *et al*. 2006).

A phylogenetic tree of anuran MHC IIB DNA sequences was constructed from an alignment of 152 bp that were present in multiple anuran sequences (Fig. 1). To determine which model of molecular evolution best the fit data we used FindModel (available at http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html). Evolutionary relationships were inferred using maximum likelihood based on Kimura 2-parameter plus Gamma model with 1000 bootstrap replicates in MEGA.

Results

We isolated a total of 13 MHC class II beta exon 2 alleles from the genomic DNA of the three focal frog species we sampled (see Fig. 1 for GenBank accession nos.). The translated amino acid sequences of all alleles showed significant amino acid similarity to other frog sequences accessioned in GenBank (BLAST e-values less than $2.4*e^{-48}$). Using the Hauswaldt *et al*. (2007) primer pair, we amplified eight alleles (186 bp) from three *R. arvalis* individuals. We also isolated two additional alleles from two *R. temporaria* that were longer and not identical to the ones characterized by Zeisset and Beebee (2009).

Using our newly developed primer pair (RanaF and RanaR2), we obtained four 196 bp alleles from two *P. lessonae* and one allele from two *P. kurtmuelleri*. We found that the divergence in the deduced amino acid sequences of our sequences varied among species. Amino acid divergence ranged from 0.017 (± 0.012) - 0.053 (± 0.031) and from 0.00-0.016 (± 0.015) within *R. arvalis* and *P. lessonae* individuals, respectively. In *R. arvalis*, out of the 61 amino acids in the alignment, there were 12 variable amino acid positions among the eight alleles. Eight of these variable sites were predicted to be in the ABS based on the model of Tong *et al*. (2006). In addition, d_N was significantly higher than d_S(Table 1). In contrast, d_N was not higher than d_S in sites outside the ABS. In *P. lessonae*, we found 30 variable amino acid sites, with seven of them occurring in the ABS and 23 in the non–ABS region. We found no significant evidence of positive selection in the ABS and or non-ABS in *P. lessonae* (Table 1). The PARRIS analysis of positive selection did not detect any evidence of positive selection acting on the alignment as a whole.

The phylogenetic analysis indicated common alleles between species (Fig. 1). The *R. arvalis* alleles formed one well-defined clade, but two alleles of *P. lessonae* grouped together with the *P. kurtmuelleri* sequence with very high support. Notably, the two other *P. lessonae* alleles formed a separate clade that was more closely related to the New World ranid species. Surprisingly, the *R. temporaria* alleles we amplified grouped together with MHC sequences from New World *Rana* sequences, although the node separating *R. temporaria* and New World ranids from the *R. arvalis* sequences had low support.

Discussion

In our study, we present the first molecular characterization of the MHC class IIB exon 2 sequences from three European ranid species: *R. arvalis*, *P. kurtmuelleri* and *P. lessonae*. Although we sampled a fairly small number of individuals, the number of alleles we isolated identified was relatively high and we found evidence of positive selection acting on the ABS in *R. arvalis*, a pattern that has been found in other anuran MHC characterization studies (e.g., Zeisset and Beebee 2009). Although we did not detect positive selection in *P. lessonae* or on the alignment of all sequences as a group, we suspect this result occurred due to small sample size, rather than a true lack of historical selection. As more data is gathered from these species, more comprehensive tests of selection can be used to better assess the effects of selection.

We infer that the *R. arvalis* genome has least two MHC class IIB loci because we recovered four unique alleles from one individual. We also predict that *P. lessonae* has at least two loci, because the number of amino acid differences between two distinct groups of alleles isolated from this species is much larger than those between sequences isolated from other species. The amino acid distances between the two individuals were high (0.654) while distances between alleles within individuals were low (0.000 and 0.020). The presence of two loci may also explain why the four *P. lessonae* alleles did not group together in the phylogenetic tree.

European frogs are predominately threatened by two major emerging infectious agents, *Bd* and ranavirus (reviewed in Duffus and Cunningham 2010). Two recent studies, in particular, have highlighted the utility of MHC molecular markers in assessing population response to disease. Teacher *et al*. (2009) found an association between MHC class I alleles and ranavirus infection status in *R. temporaria*. In addition, May *et al*. (2011) found that MHC class II genotype frequencies in wild toad population may have experienced directional selection in response to *Bd* introduction. These types of studies can inform management and conservation and our study provides a resource that can be used to perform these types of studies on other ranid species.

Acknowledgements

We thank Duma Dániel, Bartha Csaba and Bartha László for help with specimen collection and Jakab Endre and Chira Sergiu for assistance with molecular protocols.

Table 1. Estimates of nonsynonymous (d_N) and synonymous (d_S) substitution rates of the MHC class II exon 2 sequences from two ranid frog species

Sites	N	d_c	d_N	d_N-d_c	P
R. arvalis ABS	8	0.015 ± 0.014	0.154 ±0.009	0.139 ± 0.043	0.003
R. arvalis Non-ABS	8	0.032 ± 0.016	0.01±0.005	-0.022 ± 0.017	1.0
P. lessonae ABS	4	0.324 ± 0.280	0.431 ±0.214	0.107 ± 0.333	0.375
P. lessonae Non-ABS	4	0.334 ± 0.121	0.199 ± 0.041	-0.135 ± 0.134	1.0

Volume : 3 | Issue : 9 | Sept 2013 | ISSN - 2249-555X

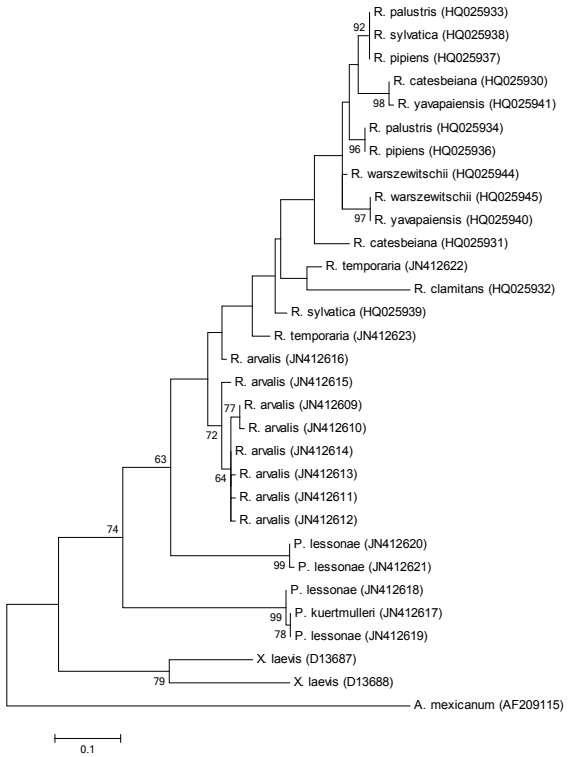

Figure 1.Text under figures

Figure 1. Phylogenetic relationships of the MHC class IIB exon 2 sequences in frogs generated with maximum likelihood (see text for details). *Ambystoma mexicanum* was used as an outgroup and bootstrap values (1000 replicates) are shown for nodes that received greater than 60% support. GenBank accession numbers are given after the sequence name.

REFERENCE

BERGER L., R. SPEARE, P. DASZAK, D. E. GREEN, A. A. CUNNINGHAM, C. L. GOGGIN, R. SLOCOMBE, M. A. RAGAN, A. D. HYATT, K. R. MCDONALD et al. 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proceedings of the National Academy of Sciences USA, 95: 9031–9036. | DUFFUS A. L., A. A. CUNNINGHAM 2010. Major disease threats to European amphibians. Journal of Herpetology, 20:117–127. | EDWARDS S.V., J. NUSSER AND J. GASPER 2000. Characterization and evolution of major histocompatibility complex (MHC) genes in non-model organisms, with examples from birds. In: BAKER A. J. (ed.): Molecular methods in ecology. Blackwell Science Ltd, Oxford, 168–207. | GRANOFF A. 1989. Viruses of Amphibia: an historical perspective. In: AHNE W., E. KURSTAK (eds.): Viruses of Lower Vertebrates. Springer Verlag, Berlin, 3-12. | HALL T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analyses program for Windows 95/98/NT. Nucleic Acids Symposium Series, 41: 95-98. | HAUSWALDT J. S., H. STUCKAS, S. PFAUTSCH AND R. TIEDEMANN 2007. Molecular characterization of MHC class II in a nonmodel anuran species, the fire-bellied toad Bombina bombina. Immunogenetics, 59: 479-491. | HUGHES A. L., M. YEAGER 1998. Natural selection at major histocompatibility complex loci of vertebrates. Annual Review of Genetics, 32: 415–435. | KIEMNEC-TYBURCZY K. M., J. Q. RICHMOND, A. E. SAVAGE AND K. R. ZAMUDIO 2010. Selection, trans-species polymorphism, and locus identification of major histocompatibility complex class IIβ alleles of New World ranid frogs. Immunogenetics, 62: 741–51. | KLEIN J. 1986. Natural history of the major histocompatibility complex. Wiley, New York. | MAY S., I. ZEISSET AND T. J. BEEBEE 2011. Larval fitness and immunogenetic diversity in chytrid-infected and uninfected natterjack toad (Bufo calamita) populations. Conservtion Genetics, 12: 805–811. | PALUMBI S. R. 1996. Nucleic acids II: the polymerase chain reaction. In: HILLS D. M., C. MORITZ AND B. MABLE (eds.): Molecular Systematic. Sinauer & Associates Inc, Sunderland, 205-247. | SCHEFFLER K., D. MARTIN AND C. SEOIGHE 2006. Robust inference of positive selection from recombining coding sequences. Bioinformatics, 22: 2493–2499. | SOMMER S. 2005. The importance of immune gene variability (MHC) in evolutionary ecology and conservation. Frontiers in Zoology, 2: 16–34. | TAMURA K., D. PETERSON, N. PETERSON, G. STECHER, M. NEI AND S. KUMAR 2011. MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution, 28: 2731-2739. | TEACHER A. G., A. A. CUNNINGHAM AND T. W. GARNER 2010. Assessing the long-term impact of ranavirus infection in wild common frog populations. Animal Conservation, 13: 514–522. | TEACHER A. G., T. W. GARNER AND R. A. NICHOLS 2009. Evidence for directional selection at a novel major histocompatibility class I marker in wild common frogs (Rana temporaria) exposed to a viral pathogen (Ranavirus). PLoS One, 4: e4616. | TONG J., J. BRAMSON, D. KANDUC, S. CHOW, A. A. SINHA AND S. H. RANGANATHAN 2006. Modeling the bound conformation of Pemphigus Vulgaris-associated peptides to MHC Class II DR and DQ alleles. Immunome Research, 2 (1): 1745-7580. | WOOD L. R., R. A. GRIFFITHS AND L. SCHLEY 2009. Amphibian chytridiomycosis in Luxembourg. Bulletin de la Société des Naturalistes Luxembourgeois, 110: 109–114. | ZEISSET I., J. C. BEEBEE 2009. Molecular characterization of major histocompatibility complex class II alleles in the common frog, Rana temporaria. Molecular Ecology Resources, 9: 738–745.