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Molecular characterization of MHC class II in a nonmodel anuran species, the fire-bellied toad *Bombina bombina*

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Abstract While the anuran *Xenopus* comprises one of the best characterized nonmammalian taxa regarding the major histocompatibility complex (MHC), the organization of this gene complex has never been studied in other anurans, and information on amphibian MHC (other than *Xenopus*) is generally very scarce. Here, we describe the characterization of the first MHC class II B cDNA sequences from a nonmodel anuran species, the European fire-bellied toad (*Bombina bombina*). We isolated two transcript sequences differing substantially in amino acid composition and length within the β2 domain. To investigate the variability of the peptide binding region in this species, we sequenced a 158-bp large fragment from wild *B. bombina* (n=20) and identified eight distinct alleles. All substitutions but one were nonsynonymous, and many of the highly polymorphic sites corresponded with amino acid positions known to be involved in antigen binding. The level of variation we found in *B. bombina* was similar compared to that previously found in a comparable sample of a wild urodelan species, *Ambystoma tigrinum*, and to that found in *Xenopus laevis*. Based on the cDNA data and the individual's allelic diversity, we conclude that *Bombina* possesses at least two class II B loci. With our new β1 primers, we were able to generate sequences in other species of anurans. We provide here a first phylogenetic analysis of this gene in amphibians.

Keywords Beta chain · Amphibia · *Alytes obstetricans* · *Xenopus laevis* · *Rana temporaria*

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Introduction

Genes of the major histocompatibility complex (MHC) play an important role in adaptive immunity and are present in all jawed vertebrates; even agnathan vertebrates have recently been found to possess recombinatorial antigen receptors (e.g., Pancer et al. 2004). In humans, the extended MHC complex comprises over 421 loci, of which 252 are expressed; approximately 70 of these are potentially associated with immunity (Beck et al. 1999). Polymorphism is extremely high in the antigen binding sites (ABS) of MHC class I and II, and these are among the most dynamic coding regions in the genome (Kelley et al. 2005), with nucleotide diversity reaching twice the level of the genomic average in humans (Piertney and Oliver 2006). Variation in the MHC is thought to be maintained by balancing selection, i.e., heterozygote advantage and/or frequency-dependent selection in response to parasites and pathogens (reviewed in Penn and Potts 1998; Hedrick 2002). In addition, MHC-disassortative mating preferences (Landry et al. 2001; Penn 2002; Zelano and Edwards 2002; Milinski 2006), as well as prenatal selection in mammals (Ober 1999) contribute to maintaining extreme levels of polymorphism. Due to these mechanisms, MHC diversity is often high, even in species or populations were neutral markers indicate a loss of genetic variation due to random genetic drift (e.g., Aguilar et al. 2004; van Oosterhout et al.

2006). Therefore, the study of MHC gene variation is widely used to understand evolutionary processes, sexual selection, or local adaptation. Although the implications of MHC diversity for conservation genetics should not be overrated (Acevedo-Whitehouse and Cunningham 2006), associations between certain MHC alleles and disease resistance or susceptibility have been found in a number of species (Sommer 2005). Overall, this marker has been found often to be more informative than neutral markers to judge the evolutionary potential of a population (Piertney and Oliver 2006).

While MHC class I molecules are expressed on most cells and bind to short (up to 10 residues long) peptides of endogenous origin, the class II molecules, composed of an α and β chain forming a heterodimer, are expressed only on specialized antigen-presenting cells (such as B cells or macrophages). They bind to exogenously derived up to 22-amino-acid-residue-long peptides, e.g., from parasites, which they present to CD4+ T-helper cells (Reche and Reinherz 2003). Specifically, it is the α1 and β1 subdomain of class II that, together, form the ABS (Madden 1995). In humans, most of the variable sites are found in the β1 segments. The most variable locus coding for the β chain is DRB1. Here, over 271 alleles are known that differ in at least one amino acid (Reche and Reinherz 2003).

Due to their importance in immunity and fitness, MHC genes are among the most intensely studied regions in vertebrate genomes (Horton et al. 2004). However, research has focused mainly on mammals, birds, and fish. In reptiles, MHC structure and variation has so far only been studied in a few species (e.g., Edwards et al. 1995; Miller et al. 2005, 2006), but no complete genomic mapping exists for any reptilian species. In amphibians, MHC class II has been characterized in only two closely related species of urodela, the axolotl, *Ambystoma mexicana*, (Sammut et al. 1997, 1999; Laurens et al. 2001), and the tiger salamander, *Ambystoma tigrinum*, (Bos and DeWoody 2005), and in a single genus of anurans, the model taxon *Xenopus* (e.g., Kaufman et al. 1985; Sato et al. 1993; Flajnik and Kasahara 2001; Bos and Waldman 2006; Ohta et al. 2006); for *Xenopus tropicalis*, a complete mapping of the entire MHC exists (Ohta et al. 2006).

The organization of MHC in *Xenopus* was found to be the most evolutionary conserved of all vertebrates (Ohta et al. 2006); all clusters are linked and class I is located between the class II and III regions (Flajnik et al. 1999). In *Xenopus laevis*, three polymorphic class II loci were found (Sato et al. 1993), whereas Ohta et al. (2006) identified two class II B genes in *X.* (= *Silurana*) *tropicalis*. In the tiger salamander *A. tigrinum*, a single class II locus with two cDNA isoforms was characterized (Bos and DeWoody 2005). In the first – and so far only – study on allelic variation in the β 1 domain in a wild amphibian, Bos and DeWoody (2005) identified nine distinct alleles in 33 individuals, and the polymorphic sites corresponded to amino acids thought to be involved in peptide binding. In its sister species, *Ambystoma mexicanum*, no allelic variation was found (Laurens et al. 2001). The only anuran model species, *Xenopus*, is in, many respects, not a suitable representative of all anurans, as it diverged very early from other anuran taxa and is evolutionarily highly derived (Cannatella and De Sa 1993). We argue that knowledge on the MHC of other anuran lineages, especially on more basal ones such as our focus taxon *Bombina*, should provide a more complete picture of the organization of MHC in anurans.

The European fire-bellied toad, *Bombina bombina*, is regarded as one of the most threatened species of amphibians in central Europe. Population numbers have declined drastically during the last few decades due to loss, pollution, or eutrophication of aquatic habitats. In many regions this species has become extinct. As part of a comprehensive population genetic study on Baltic populations of this species using neutral genetic markers, we were interested in determining MHC class II B variation. Here, we present the first description of MHC class II encoding genes of this nonmodel anuran species based on genomic and cDNA. We compare variable amino acid positions in *B. bombina* with those found in *X. laevis*, and we are presenting the first phylogenetic analysis of MHC class II B sequences derived from six anuran species, representing four anuran families (Roelants and Bossuyt 2005). Given the very limited current knowledge on anuran MHC organization, our study aims at providing a starting point for further analyses on MHC class II B function in populations of *B. bombina* and other anuran species with different demographic histories or exposure to parasites.

Materials and methods

Animals and sample preparation for DNA analysis

DNA was extracted using the Qiagen tissue kit (Hilden, Germany) from toe clips or buccal swabs from 20 *B. bombina* from seven Baltic populations from Denmark, Germany, Poland, and Latvia. Between one and five individuals per population were analyzed. To test for cross-species amplification, we also extracted DNA from a single individual each of *Bombina variegata*, *Bombina pachypus*, *Alytes obstetricans*, *Rana temporaria*, and *X. laevis*.

Total RNA was extracted from the tissue of one tadpole and one toadlet using the NucleoSpin® RNAII Kit (Macherey & Nagel, Düren, Germany) following the protocol for RNA purification from cultured cells and

tissue. Experimental procedures were performed as recommended by the manufacturer, except that the tissue was homogenized using a dispersing device in 1 ml of buffer RA1 prior to adding 10 µl mercaptoethanol. The isolated RNA was stored at −70°C prior to reverse transcriptase (RT) PCR and rapid amplification of cDNA ends (RACE) cDNA synthesis.

Primer design and genomic PCR amplification

We used published class II exon 2 sequences from *X. laevis* (XELMHCF3, XELMHCT4, XELMHCF8, XELMHCP6, Sato et al. 1993; D50035, Kobari et al. 1995) and from two urodelan species, the tiger salamander (*A. tigrinum*; DQ071905-7, DQ071909-12 Bos and DeWoody 2005) and the axolotl (*A. mexicana*; AF209117 Laurens et al. 2001), to design sets of PCR primers for *Bombina* (Table 1). Primer pair 4 successfully amplified the MHC target sequence in *X. laevis* and *R. temporaria*, but did not yield amplicons in *Bombina*. Sequence information from *Rana* was then incorporated into the *Xenopus–Ambystoma* alignment to design additional primers. Primer pair 3 yielded a fragment of the β 1 chain in *B. bombina*, and – combining all sequence information – two primer sets (1 and 2) were developed, which produced amplicons of 158 bp. For genomic amplifications, the PCR volume was 37.5 µl and contained 0.75 u Taq DNA polymerase, 0.2 µM of each deoxyribonucleotide triphosphate, 1× PCR buffer with 1.5 mM $MgCl_2$ (all Q Biogene), 200 µM of each primer, and 2 µl of DNA template. PCRs were performed using a Biometra T3000 thermocycler using the following conditions: an initial denaturation for 5 min at 94°C was followed by 50 cycles consisting of a denaturation step at 94°C for 75 s, an annealing step at 40°C for 75 s, and an extension at 72°C for 75 s. The thermal profile was concluded with a final extension at 72°C for 10 min.

RT PCR and RACE

We performed an mRNA analysis to evaluate the structure of MHC class II β chain transcripts. Total RNA was used to synthesize complementary DNA using the cDNA Synthesis Kit (Bioline, London, UK), following the manufacturer's instructions. Expression analysis was performed through RT PCR using primer pairs 2 and 3. Based on two nucleotide sequences obtained from these RT-PCR experiments, four additional primers were designed to perform 5′ RACE and 3′ RACE (Table 1). RACE experiments were performed using the BD SMART™ RACE cDNA amplification kit (Clontech, Mountain View, CA, USA), following the instructions of the manufacturer. RACE PCR was performed using the BD Advantage™ 2 PCR Kit (Clontech) using a primer specific annealing temperature of 65°C.

Cloning and sequencing

We cloned PCR, RT-PCR, and RACE-PCR products into the TOPO vector of the TOPO TA-cloning kit (Invitrogen, Carlsbad, CA, USA). Between 10 and 20 transformant colonies per individual were picked and amplified with T3/T7 primers. Amplicons approximating the expected size were cleaned using a PCR clean-up kit (Macherey & Nagel), sequenced in one direction with BigDye 3.1. chemistry (Applied Biosystems, Foster City, CA, USA) and analyzed on an ABI 3100 automated sequencer. Fewer transformant colonies (maximum of 12) were picked for the cross-species amplification experiment. To obtain full

Table 1 Primer name, sequences, and length of amplicon (bp)

Pair	Primer name	5′–3′ sequence	bp
1a	MHCIIBExon2-F	GTGYYACTWYMKGAACGGSACSGA	158
1b	MHCIIBExon2-R	TAGTTGTGTCTGCAGAMYGTRTCCA	
2a	BobomMHCIIEXON2F2	CTGASTGTCACTTTATAAACGGCACTGA	157
2b	BobomMHCIIEXON2R1	CCATAGTTGTRTTTACAGACTGTTTCCAC	
3a	MHC-AN-3F	GGGTCAGTGTTATTACCGGAACGG	58
3b	MHC-XEN-M1-R	TCCCACATCRCTGTCRAAGT	
4a	MHC-F	CCSCAGAKGATTWCGTGWMTCA	186
4b	MHC-5R	TGTCTGCAGACTGTYTCCACCHCAGCC	
5	MHC5RACE_1.rev	CCTCCTTGTTGTAGAAATATCTGTCC	–
6	MHC5RACE_2.rev	GGAGAGATATTTCTACAACAAGGAGG	–
7	MHC3-RACE_3.for	GGAGAGATATTTCTACAACAAGGAGG	–
8	MHC3RACE_4.for	CTACAACAAGGAGGAGTTTGTTTACTT	–
9	BobomMHCIIExon2endF1	TGGAACAGTAATAAGGACWTCATAGAGC	–
10	BobomMHCIIExon2endF2	GGTGGAAACAGTCTGTAAAYACAACTATGGT	–
11	BobomMHCIIExon3midF	GGAGGAGACGTCTAGYGTTGAATATTCT	–

sequences of the cloned 3′ RACE PCR products, three additional sequencing primers (#9–11) were used (Table 1).

Data analysis

To analyze variability of MHC class II β loci in *B. bombina*, PCR products derived from genomic DNA using primer pair 1 were cloned as described above. Sequences were screened for homology with gene sequences of *B. bombina*, as derived from our RT-PCR and RACE analysis, as well as with published sequences of *X. laevis* using the BLAST algorithm (National Center for Biotechnology Information). Sequence alignments were performed using Sequencher vs. 4.6 (Genecodes, Ann Arbor, MI, USA). We considered those alleles that were found in at least three clones from a single individual or in at least two individuals as validated.

We calculated the number of nonsynonymous substitutions per nonsynonymous site (d_N) and the number of synonymous substitutions per synonymous site (d_S) using MEGA 3.1 (Kumar et al. 2004). These values (*p* distances) were corrected for multiple substitutions at the same site using the Jukes–Cantor correction (Jukes and Cantor 1969) and the modified Nei and Gojobori method (Nei and Kumar 2000), the latter allowing for rate differences in transitional and transversional changes. These calculations were performed for the entire fragment, for the 14 putative peptide binding sites (as identified in comparison to the crystalline structure of the human HLA-DR1 gene; Brown et al. 1993), and for 10 pocket residues involved in three peptide-binding pockets that were identified by Tong et al. (2006).

We calculated the Wu–Kabat (W–K) variability measure to assess amino acid variability and the distribution of polymorphic sites. Highly polymorphic sites are those for which the W–K score is larger than twice the mean value. These analyses were conducted for both *B. bombina* (our data) and – for comparison – *X. laevis* (our data and GenBank sequences).

We conducted a phylogenetic analysis of the relationships among the genomic sequences of the 158-bp-long fragments of MHC class II analyzed in our study and included all published sequences for *X. laevis* that were variable in this region. We used sequences from two urodelans, *A. mexicanum* (AF209115) and *A. tigrinum* (DQ071906), as an outgroup. Data were analyzed by unweighted maximum parsimony (MP; heuristic searches with tree bisection–reconnection branch-swapping and 100 random stepwise sequence-addition replicates) (Felsenstein 1981), neighbor-joining (NJ) (Saitou and Nei 1987), and Bayesian methods (Rannala and Yang 1996; Mau and Newton 1997; Larget and Simon 1999; Mau et al. 1999). MP and NJ searches were performed using PAUP 4.0 (Swofford 2003): Bayesian analysis was carried out using MRBAYES (Huelsenbeck and Ronquist 2001). We used MODELTEST (Posada and Crandall 1998) to select the optimal model of sequence evolution; the Akaike information criterion (Akaike 1974) implemented in MODELTEST selected a general time reversible model + gamma distribution (GTR + Γ; variable rates, shape parameter α= 1.005) as the best model of DNA substitutions to fit our data. NJ analysis was carried on GTR distances calculated with the parameters selected by MODELTEST. We used the same model of sequence evolution also in the Bayesian searches, allowing site-specific rate variation partitioned by codon positions. MRBAYES was run for two million generations (one cold and three heated Markov chains) with a sampling frequency of 100 generations. To determine the appropriate burn-in, we plotted the likelihood scores of sampled trees against generation time. Burn-in corresponded to the first 20% of sampled trees; therefore, posterior probabilities for each node were calculated based on the remaining 80% of sampled trees by building a 50% majority rule consensus tree. The robustness of the MP and NJ phylogenetic hypotheses was tested by 1,000 bootstrap replicates (Felsenstein 1985). We statistically compared the MP, NJ, and Bayesian trees under a likelihood approach by using the Shimodaira–Hasegawa test (Shimodaira and Hasegawa 1999, 2001) implemented in PAUP 4.0. These trees were found to be statistically indistinguishable (p= 0.12–0.38).

Results

Isolation and characterization of MHC II beta chain and analysis of transcripts

Degenerate primers (primer pairs 1 and 3) were applied to genomic DNA of *B. bombina* to amplify two fragments of 158 and 58 bp. Nucleotide sequences of both fragments were found to be homologous to MHC class II B genes in *X. laevis*. To characterize transcripts of these genes in *B. bombina*, primer pair 3 was used to perform RT-PCR from cDNA samples of two individuals (Bobom-Beta1-1, 2). Cloned RT-PCR products were sequenced to design primers for 5′ and 3′ RACE (primers 5–11). With these primers we obtained fragments Bobom-5RACE-1 and -2 (EF210766 and EF210767) with 5′ RACE and Bobom-3RACE-1–5 (EF210761–EF210765) with 3′ RACE (Fig. 1). Nucleotide sequences derived from these experiments were used to design the additional primer pair 2 targeting a 157-bp fragment of the antigen-binding region in RT-PCR experiments (Bobom-Beta1-1–7; EF210768–EF 210774, Fig. 1). All nucleotide sequences obtained from RACE and RT-PCR experiments were used to construct a contig sequence representing the putative coding region of a *B. bombina* MHC class II B transcript (Fig. 1).

BLAST search using the partial and contig nucleotide sequences and their corresponding amino acid sequences revealed high similarity to MHC class II B genes of a wide variety of vertebrates including *X. laevis*. A similar analysis was performed using the Conserved Domain Architecture Retrieval Tool (Geer et al. 2002), which determined an MHC class II domain and an immunoglobulin domain within the amino acid sequences. Both analyses led to the functional domain annotation shown in Fig. 1. This figure also indicates the putative genomic organization of *B. bombina* MHC class II loci in comparison to the one found in *X. laevis* (Kobari et al. 1995).

In the functional domains of the MHC class II B, conservative amino acid residues involved in structural features of the protein were identified following Kaufman et al. (1994) (Fig. 1). Within the protein domains β1 and β2, cystein residues were found at positions identical to those found in other vertebrates, allowing the formation of disulfide bonds. Within the putative ABS, the following residues seem to be functional: the glycosylation site (NGT, contig positions 60–62), residues responsible for building salt bonds (K and G/E; contig positions 113 and 117), and amino acids binding peptide main chain atoms (H/Y and N contig positions 122 and 123). Additional conservative and variable amino acid positions important for antigen binding within the β1 domain will be shown and discussed below in the context of genomic variability. Finally, the transmembrane domain shows the conserved amino acid residues glycine (contig positions 247, 250, 254, and 261) and lysine (contig position 243) at identical positions compared to other vertebrate MHC molecules. These residues are considered to interact with the α chain.

In our transcript analysis, two alleles were found which differed substantially in amino acid composition and slightly in length regarding their immunglobulin (β 2) domain (Fig. 1). Based on BLAST searches, the shorter variant (Bobom-3RACE-4, 5) corresponds in length to the respective sequence in *X. laevis* and *X. tropicalis* (Sato et al. 1993; Ohta et al. 2006), as well as in the human HLA-DRB locus (Brown et al. 1993). The longer variant (Bobom-3RACE-1, 2, 3) carries an insertion of two amino acids, putatively histidine and threonine (contig positions 157 and 158). This insertion is outside of the loop formed by cysteine-mediated disulfide bridges and is located outside the conserved region involved in CD4+ binding according to Kaufman et al. (1994) (contig positions 178–192). To further analyze a possible impact of this insertion, amino acid sequences of the long variant were aligned to human HLA-DR1 used for crystal structure analysis (Brown et al. 1993; Stern et al. 1994: mmdbID: 764; Wang et al. 2001: mmdbID: 17360). The insertion is positioned within a beta sheet, suggesting no detrimental effect on the secondary structure. Furthermore, a direct interaction of the inserted amino acids with bound CD4+ seems unlikely.

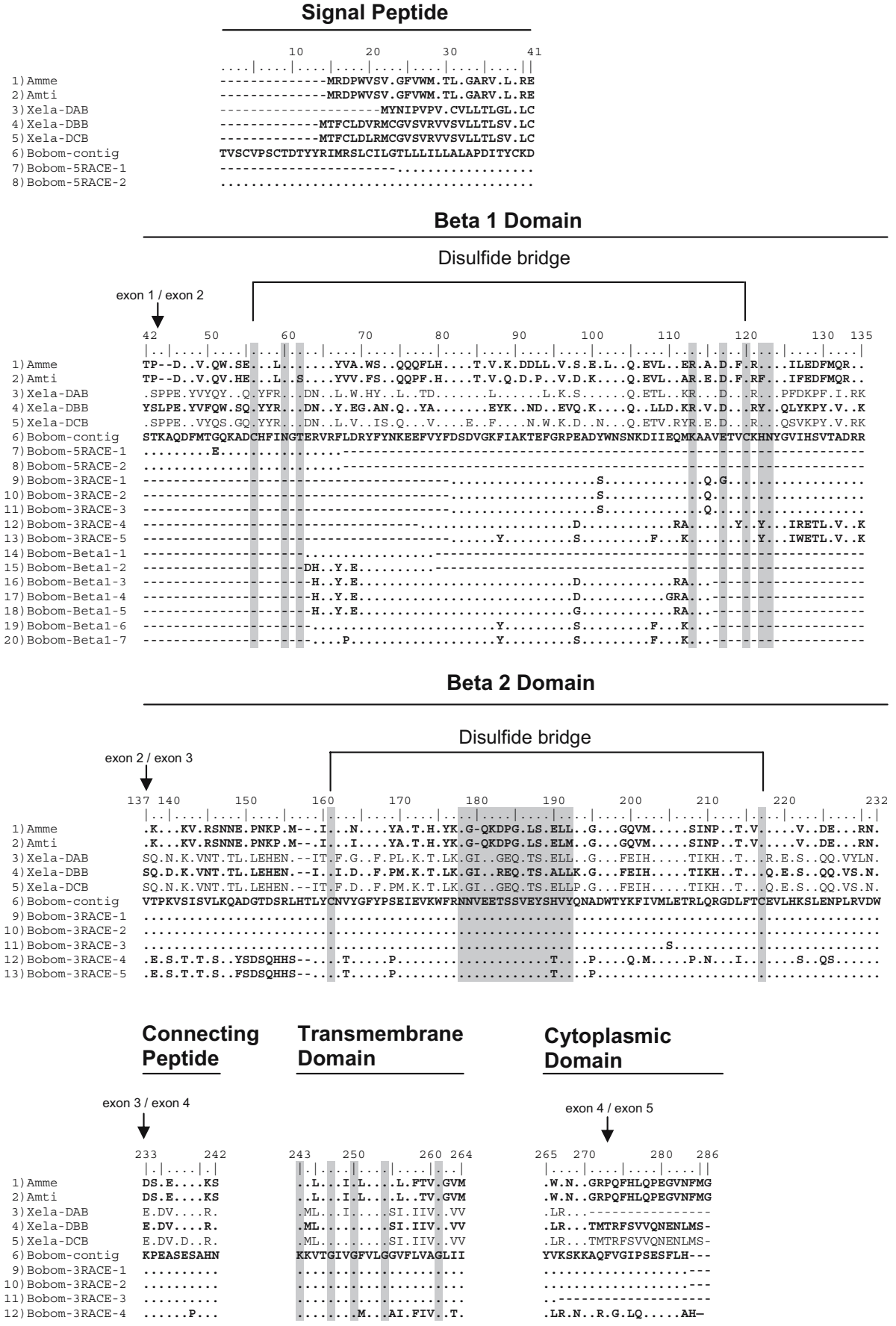
Genomic variation of MHC class II beta chain in *B. bombina*

To assess variation in wild populations of *B. bombina*, we analyzed 20 animals representing seven European populations and sequenced between 10 and 20 clones (mean= 14.1) per amplicon, yielding a total of 252 sequences. Of these, 205 (81%) could be matched with our *B. bombina* consensus MHC class II sequence. Sequences from 12 clones, of which 11 were unique, were removed from further analyses for the following reasons: three sequences could not be aligned with any of the existing alleles, one contained a stop-codon, and eight sequences were considered artifacts presumably resulting from PCR jumping. Of the remaining 193 sequences, 26 occurred in fewer than three clones of a single individual (Table 2). These alleles differed by only one or two bases from verified alleles. They were omitted, rendering 167 verified sequences according to our criteria. The second nucleotide position coded for the first codon and was identified as residue 23 of the complete β1 chain.

According to our criteria, eight alleles were identified (Fig. 2; EF210739–EF210743). Among those, two were found to be expressed. Allele Bobom-D was identical to a transcript amplified by RT-PCR (Fig. 1; Bobom-Beta 1-6). Furthermore, the 3′ RACE PCR revealed fragments of transcripts that were highly similar to allele Bobom-D (Fig. 1; Bobom-3RACE-5) and Bobom-C (Fig. 1; Bobom-3RACE-1, Bobom-3RACE-2).

The eight alleles described here differed by at least one and at most 18 nucleotides (mean=10.64±SD 7.02); all alleles differed also by at least one and at most 13 amino acids (mean =7.39±SD 4.63) (Fig. 2). In our 158-bp fragment, we found 27 segregating sites, of which only one contained a synonymous change (position 83 in Bobom-C). All other variants resulted in a change of amino acid. On average, two randomly chosen alleles differed at 6.7% of their sites from each other. Between one and four alleles were found per individual (mean 2.75±SD 1.02) (Fig. 3a). The most common allele (Bobom-A) was found in all but one individual and in 60% of the verified sequences (Fig. 3b). The second most common allele (Bobom-B) was found in 11 individuals and 14% of the sequences. Four alleles differed from allele A by only one nucleotide each, and two of these (A3 and A4) were only found in two individuals.

Of the 52 codons, 16 were found variable, and eight of these (57%) belonged to the 14 predicted to be involved in antigen recognition (Brown et al. 1993). An even better agreement was found when comparing our polymorphic sites to the pocket residues identified by Tong et al. (2006). All of their 10 pocket residuals occurring in our segment are variable in *B. bombina*. The average W–K variability value for all amino acid positions was 3.67, and seven


Signal Peptide
Beta 1 Domain
Disulfide bridge
exon 1 / exon 2
Beta 2 Domain
Disulfide bridge
exon 2 / exon 3
Connecting Peptide
Transmembrane Domain
Cytoplasmic Domain
exon 3 / exon 4
exon 4 / exon 5
1) Amme
2) Amti
3) Xela-DAB
4) Xela-DBB
5) Xela-DCB
6) Bobom-contig
7) Bobom-5RACE-1
8) Bobom-5RACE-2
9) Bobom-3RACE-1
10) Bobom-3RACE-2
11) Bobom-3RACE-3
12) Bobom-3RACE-4
13) Bobom-3RACE-5
14) Bobom-Beta1-1
15) Bobom-Beta1-2
16) Bobom-Beta1-3
17) Bobom-Beta1-4
18) Bobom-Beta1-5
19) Bobom-Beta1-6
20) Bobom-Beta1-7

◀ **Fig. 1** Amino acid alignment of the MHC class II β chain sequences of *B. bombina* [contig derived from RT-PCR (Bobom-Beta1-1-7), 5′ RACE (Bobom-5RACE-1, 2), and 3′ RACE (Bobom-3RACE-1–5)] and three other amphibians: *X. laevis* [Xela-DCB: D13685, Xela-DBB: D13684 (Sato et al. 1993), and Xela-DAB: D50039 (Kobari et al. 1995)], *A. mexicanum* [Amme: AF209115 (Laurens et al. 2001)], and *A. tigrinum* [Amti: DQ125480 (Bos and DeWoody 2005)]. Exon borders within the transcript as found in *X. laevis* (Kobari et al. 1995) are labeled with an *arrow*, and exon numbers are indicated. Conserved residues are *shaded* according to Kaufman et al. (1994). With respect to the observed length variation of the Beta II domain, sequences Bobom-3RACE-4, 5 represent the short variant and sequences Bobom-3RACE-1, 2, 3 the long variant

highly polymorphic sites were identified (Fig. 4). Most diversity was found at position 31 with four different amino acids. Among these seven sites, five residues are known to be involved in peptide binding (Brown et al. 1993; Tong et al. 2006). In *Xenopus*, eight highly variable sites were identified, of which five were in agreement with those found in *B. bombina*. The remaining sites (8, 10, and 15) were among those residues recognized by Brown et al. (1993) to be interacting with the peptide.

The rates of nonsynonymous (d_N) and synonymous (d_S) substitutions were estimated for ABS and non-ABS residues (Table 3). We found the ratio to be more than twice as high for ABS compared to non-ABS in *B. bombina*. In *Xenopus*, we also found much higher d_N/d_S ratios in the ABS residues. A formal statistical evaluation of this difference was precluded by the low absolute frequency of synonymous substitutions. Nonetheless, we consider such skewed d_N/d_S ratios indicative of positive selection. For both species, the signal of selection is stronger if one considers the pocket residue sites according to Tong et al. (2006).

Cross-species amplification and phylogenetic analysis

Apart from *B. bombina*, we were able to successfully amplify MHC class II β 2 in *B. variegata*, *B. pachypus*, *A. obstetricans*, and *X. laevis* with primer pair 2. Between three and eight clones were sequenced per animal and all sequences are presented (EF210744–EF210756).

Among the 52 residues of the 25 newly generated sequences combined with eight obtained from GenBank, eight were conserved and six of these were among the conserved sites identified by Kaufman et al. (1994). When considering anurans only, we identified 11 conserved sites (Fig. 2). Residue 46, identified by Kaufman et al. (1994) as highly conserved for leucine, contains the closely related isoleucine in all discoglossid anurans, and leucine in all others, except for XELMHCF8 containing valine at that position. Many of the ABS identified by Brown et al. (1993) and Tong et al. (2006) were found to be variable. The two highly polymorphic sites (residues 1 and 31) in *B. bombina* that had not been designated ABS were also variable in the other amphibians studied here. Comparing sequences of *Bombina* with those of other anurans, the most striking differences in chemical properties of amino acids are as follows: (1) all species of *Bombina* contain the positively charged lysine at site 12, but all other amphibians carry either glutamine or leucine. (2) *Bombina* is the only genus carrying lysine at site 24, while all other species either have a polar but uncharged side chain (serine) or hydrophobic side chains (leucine, phenylalanine or valine). (3) Only alleles of *B. bombina* (Bobom-A and derivates) have the large aromatic amino acid tryptophan at site 32, at which all others are fixed for the very small glycine. Glycine is supposed to be highly conserved at this position according to Kaufman et al. (1994).

According to our phylogenetic analysis, all sequences derived from anurans form a monophyletic group (Fig. 5). Within the amphibians, two strongly supported clusters could be identified: one containing all sequences of *X. laevis* (derived from GenBank and our study) and the other consisting of the MHC alleles of Discoglossoidea. In the latter, the separation of *A. obstetricans* from all *Bombina* is strongly supported. Within *Bombina*, the two sequences obtained from *B. variegata* form a strongly supported group that is basal to its congeners. Whereas sequences from *B. pachypus* form a monophyletic group, their placement within *B. bombina* is not supported. Beta chain sequences of *R. temporaria*, representing the

Table 2 MHC class II exon 2 alleles found in *B. bombina*

Bobom-exon 2 allele #	No. of individuals in which allele was found	No. of clones sequenced with exact match	No. of clones with 1 and/or 2 bp mismatch
A	19	100	13/4
A1	3	13	0/2
A2	3	3	–/–
A3	2	2	–/–
A4	2	2	–/–
B	11	23	2/–
C	8	15	5/–
D	7	9	–/–
Total	–	167	20/6

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                                10        20        30        40        50
                    ....|....|....|....|....|....|....|....|....|....|....|..
Bobom-A             HVRLLERWFYNKEETVYFDSDVGKYIAKTELWRPDADYWNSNKDLIEQRKAQ
Bobom-A1            R.....................................................
Bobom-A2            ..........................R..........................
Bobom-A3            ..............................P.......................
Bobom-A4            ...................................R...................
Bobom-B             R..F.D.Y.....D..............NG..............RA..A
Bobom-C             R..F.Q.Y.....D.........F.....FG..E..S......I...M...
Bobom-D             R..F.D.Y.....F..............FG..S.........F...K..A
Bovar-3A            R..Y.V.Y.....F.........F.....FG............I..RQ..E
Bovar-6C            R..Y.V.Y.....F.........F..E..FG............I..RQ..E
Bopac-3F            .....D.........F.........F...A.FG.........D..F......A
Bopac-2F-(4)        .....D.........F.........F.....FG.........D..F......A
Alobs-H7-(4)        R..Y.Q.Y...Q..I.........E.VP....G....E...K...I....R.A
Alobs-E             R..Y.Q.Y...Q..I.........E.VPR...G....E...K...I....R.A
Alobs-F7            R..Y.Q.Y...Q..I.........E.VPR..FG....E...K...I....R.A
Alobs-A7            R..Y.Q.Y...Q..I.........E.VP...FG....E...K...I....R.A
Alobs-C7            R..Y.Q.Y...Q..I.........E.VP...FG....E...K...I....R.A
Ratem-3A            D....K.FM..Q..F......K.F..P...YG...........P.ILG.AR.G
Xelae-C1            N..Y.Q.FS..Q..I.........LF......GK.......NQ.EFL..KL.E
Xela-DCB (D13695.1) N....V.YIS.Q..F.....E..FF...N.WGK....N...Q.ETV.RYR.E
Xelae-E1-(2)        N..F.A.LIS.Q..F.........LF......GK.S......Q..FL..TR.A
XELMHCP6 (D13687.1) N..F.A.YIN.Q..YA.......SF...NDFG.VQ......Q.EILD.HR.E
Xelae-A1            N..F.A.LIS.Q..F.........F....NDFGEVQ..HL..Q.EIL...R.E
Xelae-D1            N..F.AHLIS.Q..F.........L....NDFGEVQ..HL..Q.EIL...RTE
Xelae-F1            N..F.W.HY..L...D.......LF......GK.SV....GQ..FL..TR.A
Xelae-H1            N..F.W.HY..L...D.......LF......GK.......NQ.EFL..KL.E
Xelae-G1            N..F.W.HY..L...D.......LF......GK.S......Q..FL..TR.A
Xela-DAB (D50036.1) N....W.HY..L...D.......LF......GK.S......Q.ETL..KR.A
XELMHCT4 (D13688.1) N....W.HY..L...D.......LF......GK....N...Q..FL..TR.A
Xela-DBB (D13686.1) N..Y..GYAN.Q..YA.......E.K..NDFGEVQ.K....Q...LD.KR.V
Amme (AF209115)     R..YVA..S..QQQFLH....T.VFK.DDL.GV.S.ESL..Q.EVL..ER.A
Amti (DQ071906.1)   R..YVV..S..QQQV.H....T.VFQ.DDL.GV.S.ES...Q.EIL...R.E
                         * * +    *+        *         *#   *+    + #+ **   +
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Fig. 2 Amino acid alignment of MHC class II beta chain. Bobom (*B. bombina*), Bovar (*B. variegata*), Bopac (*B. pachypus*), Alobs (*A. obstetricans*), Ratem (*R. temporaria*), and Xelae (*X. laevis*); additional GenBank sequences from *X. laevis*, *A. mexicanum*, and *A. tigrinum*. *Numbers in parentheses* indicate multiple occurrence of an allele. Sites involved in peptide binding are marked with *asterisks* (identified by both Brown et al. 1993 and Tong et al. 2006), *plus signs* (identified by Brown et al. 1993 only), or *sharp signs* (identified by Tong et al. 2006 only). *Shaded* residues were identified as evolutionarily conserved by Kaufman et al. (1994)

Ranidae, had been obtained with four different primer pairs; however, all clones obtained (n=10) yielded the identical sequence. The placement of *R. temporaria* in this tree is not resolved.

None of the sequences of *X. laevis* generated in this study were identical to the six identified in other studies and deposited in GenBank. In this tree, the previously defined alleles separate into two large clusters, one containing the genes Xela-DAB and the other Xela-DBB and Xela-DCB (Kobari et al. 1995). Three of our *Xenopus* sequences (Xelae-F1–H1) cluster tightly with those from the Xela-DAB gene, and two are most similar to the one defined as Xela-DBB (D13684.1). None of our sequences clusters closely with Xela-DCB (D13685.1).

Discussion

This study comprises the first characterization of MHC class II B gene loci in *B. bombina*, a nonmodel anuran species (Fig. 1). The analysis of transcripts allowed the partial description of the *B. bombina* MHC class II B

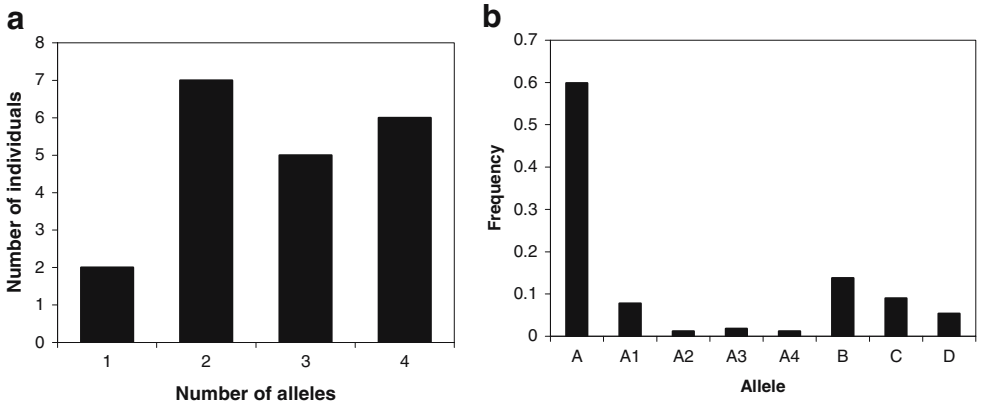

Fig. 3 Number of alleles found per individual (**a**) and frequency of alleles among 167 clones derived from 20 fire-bellied toads (**b**)

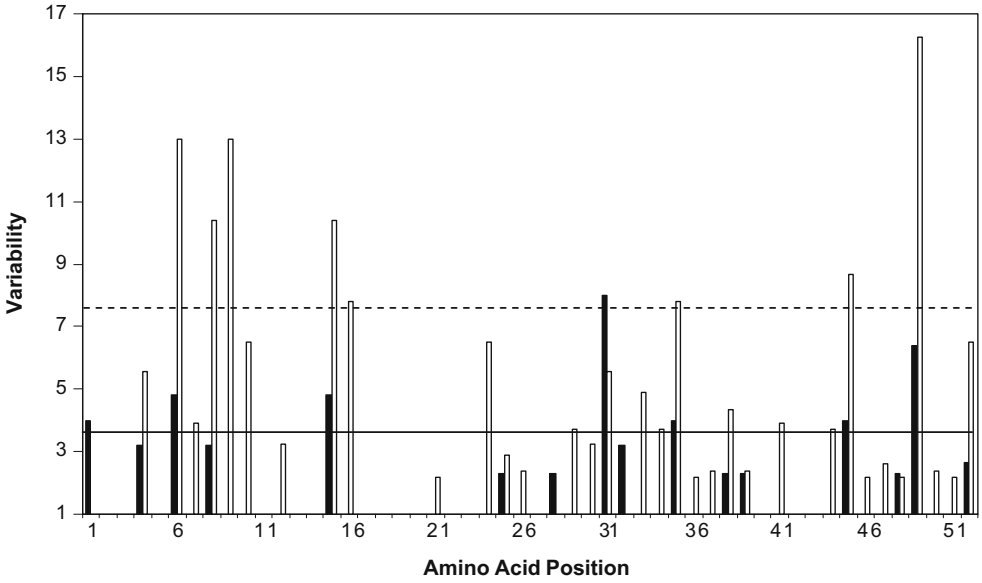

Fig. 4 W–K variability plot of *B. bombina* (*black bars*) and *X. laevis* (*white bars*) MHC class II β alleles. Highly polymorphic sites are those with W–K scores ≥3.6 (*B. bombina*, *solid line*) or ≥7.5 (*X. laevis*, *dashed line*)

protein through identification of six functional domains and conservative sites within them, considered being important for functional and structural properties (Kaufman et al. 1994). In addition, the nucleotide and the corresponding amino acid sequence variability of a large fragment of the β1 domain was estimated through analysis of genomic DNA of 20 wild European fire-bellied toad specimens.

As a result of this analysis, eight alleles have been found that were considered to be functional. This was because two of them (Bobom-C and Bobom-D) were found in the expression analysis. The remaining six alleles show characteristically structural features, such as conserved and variable sites involved in antigen binding (Figs. 2 and 5). However, because expression of these alleles has not been experimentally proven, it cannot be entirely excluded that they constitute pseudogenes. We estimated that two randomly chosen alleles in *B. bombina* were – on average – identical at 93.3% of the sites. In their study on *A. tigrinum*, Bos and DeWoody (2005) found nine alleles in 33 salamanders; here, two randomly chosen alleles were identical at 92.6% of the sites. These values appear similar; however, it is important to note that their estimation was based on salamanders from a single population, as well as alleles from a single locus.

Several lines of evidence point to the fact that *B. bombina* does possess at least two expressed loci of MHC class II B. First, at the genomic level, we found, at most, four alleles in one individual, and microsatellite data suggest *B. bombina* to be diploid (Stuckas and Tiedemann 2006; Hauswaldt et al. 2007); therefore, at least two loci must be present. Furthermore, we found a length variant in the β2 domain in two sequences derived from mRNA. Amino acid similarities allowed assigning allele Bobom-D as the longer variant, while the shorter variant is Bobom-C. A comparison of these length variants with the crystal structure of human HLA-DR1 suggests that the amino acid

Table 3 The estimated rates (±SD) of nonsynonymous (d_N) and synonymous (d_S) substitutions for the entire fragment, for the ABS and non-ABS according to Brown et al. (1993) (a) and Tong et al. (2006) (b)

Positions	*n*	d_N	d_S
Bombina bombina			
All	158	0.085±0.021	0.009±0.006
ABS (a)	42	0.192±0.049	0.013±0.0134
Non-ABS (a)	116	0.05±0.019	0.009±0.008
ABS (b)	30	0.315±0.046	0.019±0.019
Non-ABS (b)	128	0.042±0.016	0.007±0.007
Xenopus laevis			
All	158	0.197±0.028	0.116±0.030
ABS (a)	42	0.327±0.057	0.109±0.053
Non-ABS (a)	116	0.149±0.032	0.119±0.036
ABS (b)	30	0.421±0.061	0.106±0.051
Non-ABS (b)	128	0.145±0.027	0.116±0.034

n number of base pairs

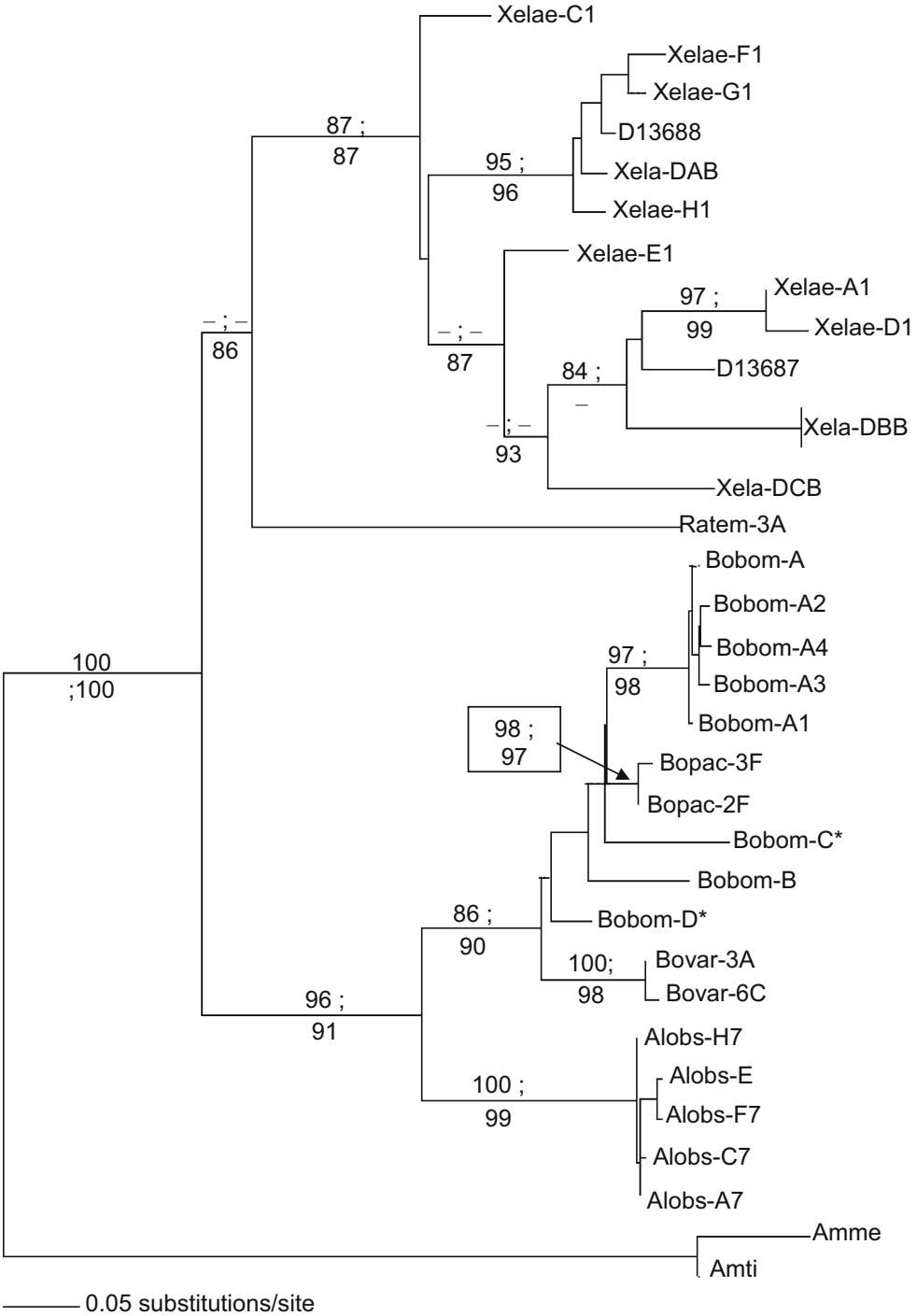

Fig. 5 NJ phylogram based on DNA sequences of a 158-bp segment of the β chain of MHC class II exon2 using the GTR. Bootstrap supports based on MP, maximum likelihood, and 50% majority rule Bayesian analysis are shown. For parsimony and likelihood analysis, only bootstraps >75 are shown, and for the Bayesian analysis, those >85. Alleles in *B. bombina* for which expression has been verified are indicated by a *star*. All GenBank sequences are as in Fig. 2

indel is unlikely to affect the function (e.g., CD4+ binding). Functional length variants of MHC class II B molecules have also been reported in other species, e.g., the red-necked wallaby (Schneider et al. 1991).

Clearly, future studies have to analyze the MHC class II B loci in more detail, allowing the exact assignment of all alleles. This also includes analyzing the 5′ region of the transcripts, which has not been feasible for the nucleotide fragments derived from 5′ RACE PCR. The necessity for comprehensive investigations of MHC loci in *B. bombina* is also motivated by another observation. In *X. laevis*, three class II loci have been identified (Kobari et al. 1995). As *Bombina* belongs to a basal lineage within the anurans (Roelants and Bossuyt 2005), further research will have to ascertain whether *B. bombina* indeed only has two loci and whether the possession of three functional MHC loci – as in *X. laevis* – is ancient or derived within anurans. This question is particularly interesting taking into account that

the tiger salamander (*A. tigrinum*), representing the taxon Urodela within the Amphibia, posses only a single MHC class II B locus (Bos and DeWoody 2005).

We found strong evidence for positive selection acting on *B. bombina* MHC class II β1. A detailed analysis shows that this selection acts particularly on amino acid residues involved in antigen binding (Brown et al. 1993; Tong et al. 2006). These sites are much more variable than those not involved in this function. This is both consistent with the expectation under heterozygote advantage and under frequency-dependent selection induced by varying antigens. Within the ABS, we also observed the lack of variability at those sites known to be evolutionarily conservative (Kaufman et al. 1994), indicating stabilizing selection on those ABS not interacting with antigens. The presence of positive selection on a gene locus with a high degree of polymorphisms maintained within populations is best explained by balancing selection leading to variety of functional alleles within a population. This is considered as having an adaptive value for wild populations, and the degree of MHC class II B variability has often being used for the assessment of the genetic state of a population in a conservation context (Hedrick 2003).

The present study is among the first ones that describe the MHC class II B locus for amphibian species in general and for anuran species (apart from *Xenopus* sp.) in particular. Therefore, we also provide a first description and functional characterization of MHC class II B β1 domain fragments in the Discoglossid species *B. variegata*, *B. pachypus*, and *A. obstetricans*, as well as in the Ranid species *R. temporaria*. Although the functional activity of some alleles has to be verified by detailed analysis of structural properties and transcriptional activity of the corresponding gene loci, alleles described for these species show features characteristic for this domain. We found great similarity among homologous evolutionarily conserved (Kaufman et al. 1994) and hypervariable sites (Brown et al. 1993; Tong et al. 2006) in *B. bombina*, other amphibians, and other vertebrates, indicating the structural conservation of the ABS. Furthermore, reconstruction of a gene tree using all new and previously described MHC class II B alleles in amphibians reflects the current view of the phylogenetic relationship (San Mauro et al. 2004; Roelants and Bossuyt 2005). As most sequences were not verified via expression analysis, we cannot rule out the possibility that single sequences in this tree constitute PCR artifacts and/or pseudogenes. Xelae-C1, for example, appears ambiguous, as it does not cluster closely with any defined *X. laevis* allele. Nevertheless, all sequences derived from anurans resembled a monophyletic group with respect to the urodelan outgroup. Additionally, three of the families: Pipidae, Bominatoridae, and Discoglossidae were clearly resolved. Although our data should still be considered preliminary, they already indicate a translocus evolutionary coherence, potentially explained by concerted evolution, as has been postulated for other vertebrates (Nei and Rooney 2005).

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