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Identification and Mapping of Two Divergent, Unlinked Major Histocompatibility Complex Class II *B* Genes in Xiphophorus Fishes

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ABSTRACT

We have isolated two major histocompatibility complex (MHC) class II *B* genes from the inbred fish strain *Xiphophorus maculatus* Jp 163 A. We mapped one of these genes, designated here as *DXB*, to linkage group III, linked to a malic enzyme locus, also syntenic with human and mouse MHC. Comparison of genomic and cDNA clones shows the gene consists of six exons and five introns. The encoded β1 domain has three amino acids deleted and a cytoplasmic tail nine amino acids longer than in other teleost class II β chains, more similar to HLA-DRB, clawed frog Xela-F3, and nurse shark Gici-B. Key residues for disulfide bonds, glycosylation, and interaction with α chains are conserved. These same features are also present in a swordtail (*Xiphophorus helleri*) genomic *DXB* PCR clone. A second type of class II *B* clone was amplified by PCR from *X. maculatus* and found to be orthologous to class II genes identified in other fishes. This *DAB*-like gene is 63% identical to the *X. maculatus DXB* sequence in the conserved β2-encoding exon and was mapped to new unassigned linkage group LG U24. The *DXB* gene, then, represents an unlinked duplicated locus not previously identified in teleosts.

MAJOR histocompatibility complex (MHC) class II αβ heterodimers present peptides to T helper cells, providing a signal necessary for activation and subsequent initiation of a specific immune response to a foreign peptide or protein (Germain 1994). Since the first isolation of an *MHC* class II *B* gene in a teleost (Hashimoto *et al.* 1990) and an elasmobranch (Bartl and Weissman 1994), this gene has been identified in a number of teleosts (Klein *et al.* 1997). *MHC* and complement genes have been mapped in zebrafish (Bingulac-Popovic *et al.* 1997) and medaka (Kuroda *et al.* 1996), respectively. A teleost model system particularly suited to genomic mapping and linkage analysis is the assemblage of inbred strains of platyfish, *Xiphophorus maculatus.*

The platyfish and the swordtail, *Xiphophorus helleri*, (Teleostei: Poeciliidae) are livebearers native to streams of eastern Mexico and Central America. Hybrids of *X. maculatus* and *X. helleri* were originally noted to be tumor-susceptible by fish hobbyists, and further characterized as highly susceptible for development of malignant melanomas from pigment cells of the platyfish (Bellamy 1922; Gordon 1927; Kosswig 1928; Haussler 1928). To identify the genes responsible for the development of these tumors, inbreeding of platyfish was initiated in 1939. These platyfish strains were used in the first inbred fish histocompatibility studies (Kallman and Gordon 1957; Kallman 1958). These same strains, and others, were used in early attempts to estimate the number of histocompatibility loci in the fish *X. maculatus* (Kallman 1964). Inbred strains of platyfish have also been instrumental in the identification of melanoma-inducing loci in platyfish-swordtail hybrids (Vielkind *et al.* 1989; Wittbrodt *et al.* 1989; Adam *et al.* 1993; Nairn *et al.* 1996). Availability of inbred strains of *X. maculatus* and the fertile hybrids of interspecific crosses have led to the development of extensive Xiphophorus gene maps (Morizot *et al.* 1991) that are presently unavailable in most other fish species. Members of this genus are, therefore, particularly suitable for determining genetic linkage relationships for study of evolution of the *MHC* in teleosts. In this study, we report the identification of a novel *MHC* class II *B* locus in Xiphophorus as well as the more commonly characterized II *B* locus, and the genetic linkage mapping of these two loci. The Xiphophorus gene maps allow prediction of ancestral vertebrate genome organization (Morizot 1990, 1994) and could provide insight into evolution of vertebrate *MHC* gene complexes.

The genes we have cloned and mapped are designated as *MhcXima-DXB*01* and *MhcXihe-DXB*01*, in general accordance with the guidelines of Klein *et al.* (1990), where *Xima* and *Xihe* refer to *X. maculatus* and *X. helleri*, respectively, *D* to class II; *X* to a new and as yet uncharacterized family designation; *B* to the β chain-encoding gene; and **01* to the allelic form of the gene shown.

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*MhcXima-DAB*01* also represents a class II *B* gene, with the *A* referring to a relatively well-characterized family of teleost class II *B* genes.

MATERIALS AND METHODS

Fish: Platyfish (*X. maculatus*), strain Jp 163 A (inbred since capture from Rio Jamapa, Veracruz, Mexico in 1939), and the swordtail (*X. helleri*), Sarabia strain pedigree 6243 originally from Rio Sarabia, Veracruz, were obtained from the Xiphophorus Genetic Stock Center at Southwest Texas State University, San Marcos, TX.

Preparation of genomic DNA: Gill tissue of 10 specimens of *X. maculatus* Jp 163 A or *X. helleri* 6243 was removed, frozen in a dry ice/ethanol bath, ground into a powder in the presence of liquid nitrogen, and resuspended in lysis buffer [10 mm Tris-HCl, pH 7.5, 5 mm EDTA, 1% sodium dodecyl sulfate (SDS), 1 mg/ml protease K], incubated overnight at 54°, phenol/chloroform extracted, ethanol precipitated and resuspended in 10 mm Tris-HCl, pH 7.5, 1 mm EDTA.

Preparation of RNA: Spleens of 10 specimens of *X. maculatus* Jp 163 A were dissected, minced, and resuspended in TRIzol reagent. RNA was isolated according to the manufacturer's protocol (Life Technologies, Gaithersburg, MD).

PCR amplification and cloning of *Xima-DXB*: Genomic DNA of *X. maculatus* Jp 163 A and the oligonucleotide primers TM215 and TM216 (primers described in Walker and McConnell 1994) were used in the initial PCR amplification of a *Xima* class II *B* DNA fragment using previously described conditions (Walker and McConnell 1994). Briefly, primer TM215 corresponds to the amino acid sequence CSAYDFYP of a class II *B* gene in carp (Hashimoto *et al.* 1990) that includes the first cysteine of the β2 domain. The antisense primer TM216 corresponds to the amino acid sequence CMVEHASL, including the second cysteine of the β2 domain. The resultant 190-bp fragment was cloned into pCR I (Invitrogen, San Diego, CA) and sequenced. The initial database searches using NCBI BLASTN (Altschul *et al.* 1990) found the sequences to be similar to other vertebrate MHC class II genes. This DNA fragment was subsequently used for screening of a genomic library as described below.

The *Xima-DXB* sequence, as determined from clones isolated from a genomic library (see below), made possible the design of *DXB*-specific primers TM341 (5′-ATCTCTGTTGC CAATCTAAGA-3′) and TM328 (5′-ATGTGTAAAAGGCTAA ATGAT-3′). These primers were designed using Oligo Primer Analysis software (National Biosciences, Plymouth, MN) and used for amplification of *Xihe-DXB* genomic and *Xima-DXB* cDNA. For isolation of the *Xima-DXB* cDNA, the Capfinder protocol (CLONTECH, Palo Alto, CA) was used as directed by the manufacturer to transcribe cDNA from *X. maculatus* splenic RNA. Thirty cycles of amplification (94° for 30 sec, 58° for 30 sec, 68° for 4 min) were performed using 1 μl of the high-fidelity KlenTaq polymerase enzyme mixture, 5 μl 10× KlenTaq reaction buffer, 10 mm dNTP mix, 0.4 μg each of TM341 and TM328, and 2% of the amplified cDNA mix as per manufacturer's directions (CLONTECH). The resulting PCR mixture was then electrophoresed on a 1% agarose gel, the appropriate sized band excised, and DNA isolated from the agarose using the QIAquick protocol (QIAGEN, Chatsworth, CA). The DNA was then used as substrate for the +1 cycle of the PCR+1 reaction (Boriello and Krauter 1990; Walker and McConnell 1994; Hardee *et al.* 1995) with primer TM342 (5′-GAG<u>AAGCTT</u>ATCTCTGTTGCCAATCTAAGA-3′) with a *Hin*dIII site (underlined), using Taq polymerase according to manufacturer's protocol (Life Technologies). The resultant 777-bp fragment was cloned into pGEM-T (Promega, Madison, WI) and sequenced. Plasmid restriction digests and DNA sequence data were used to confirm the identities of the *Xima-DXB* cDNA PCR+1 clone. Excision of the fragment from the vector with the primer *Hin*dIII site and the vector *Not*I site resulted in a 795-base pair (bp) fragment. PCR+1 amplification and cloning procedures for the *Xihe-DXB* genomic fragment were identical to that of the *DXB* cDNA clone, with 1 μg *X. helleri* 6243 genomic DNA as template and resulted in a 1759-bp genomic PCR+1 fragment.

PCR amplification and cloning of *Xima-DAB*: Amplification of the *Xima-DAB* cDNA was performed as described for *Xima-DXB* cDNA, but using RNA isolated from intestinal tissue as template. Primers used were TM396 (5′-GCTGGGCTGGCT GCTGGTCAT-3′) based on the leader sequence of the guppy (Sato *et al.* 1995), TM398 (5′-GAAGCAGGAGGAACCAGAA CC-3′) in the 3′ untranslated region of the guppy (Sato *et al.* 1995), and TM399 (5′-AGA<u>AAGCTT</u>GCTGGGCTGGCTGCT GGTCAT-3′) as the +1 primer with the underlined *Hin*dIII site. The program Oligo (National Biosciences) was used in the design of these primers.

Screening of genomic library and subcloning of positive plaques for *Xima-DXB*: A platyfish genomic library in lambda FIX II vector, prepared from *X. maculatus* Jp 163 A adult males, was obtained from Stratagene (La Jolla, CA). The complexity of the original library was 2×10^6 plaque-forming units (PFU); the titer of the amplified library used for screening was 2.0×10^{10} PFU/ml. Fifty nanograms of the MHC class II *DXB* gene fragment described above was radiolabeled with [α-^{32}P]dCTP with the RadPrime DNA Labeling System (Life Technologies) according to the manufacturer's protocol. Replicate nylon filters (MSI, Westboro, MA) containing DNA of approximately 6×10^5 genomic clones were screened with the radiolabeled platyfish probe after a 2-hr prehybridization in 5× Denhardt's, 6× standard sodium citrate (SSC), 0.5% SDS, and 50 μg/ml calf thymus DNA at 42° in a Hybridizer 600 oven (Stratagene). Hybridization was in the identical solution with 10^6 cpm/ml radiolabeled probe added, and incubated at 65° for 16 hr. Filters were washed two times at 25° in 5× SSC, 0.5% SDS for 15 min; two times at 37° in 1× SSC, 0.5% SDS at 37°; two times at 37° in 0.1× SSC, 1% SDS; and three times at 65° in 0.1× SSC, 1% SDS. The filters were then used to expose X-ray film (Fuji Photo Film Co., Ltd., Japan) with DuPont Cronex intensifying screens for 1–3 days at −70°. Fourteen primary plaques were positive, five of which remained positive through secondary and tertiary screenings. Two of the plaques were analyzed by restriction enzyme mapping. One plaque yielded a 6.5-kb *Hin*dIII-*Not*I DNA single hybridizing fragment that was subcloned into the *Hin*dIII and *Not*I sites of pCR II (Invitrogen, Carlsbad, CA). Primers designed from the sequenced 190-bp β2-encoding fragment were used to begin DNA sequence analysis of this 6.5-kb cloned genomic DNA. As this fragment was sequenced, new primers were designed until the complete sequence was determined.

DNA sequencing and analysis: Sequencing was performed using universal Forward and Reverse primers (DNA International, Lake Oswego, OR) on PCR-derived fragments, and with gene-specific primers designed with Oligo. Both strands were sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using the fluorescence-based PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's protocol. The results were analyzed on the Applied Biosystems Model 373A DNA Sequencing System. Exon-intron splicing at the only ambiguous sites (intron 5, due to the nonconsensus nature of the splice signals) is described as predicted by PROCRUSTES (Gelfand *et al.* 1996).

DNA sequence alignments and construction of dendrograms: DNA sequence analysis, fragment assembly, homologous se-

quence overlays, and amino acid translations were generated using the DNAsis (Hitachi Software Engineering Co., Limited, 1991) sequence analysis as well as the Genetics Computer Group (GCG) (Devereux *et al.* 1984) programs. Searches for sequences similar to Xiphophorus class II *B* genes were performed and preliminarily aligned using the NCBI Blast E-mail server (Altschul *et al.* 1990). The final nucleotide sequence alignments were performed using the PILEUP and PRETTY programs of the GCG. Pairwise distances were calculated for exon 3 (class II β2-domain encoding) using the p-distance algorithm and the neighbor-joining method of Saitou and Nei (1987). Calculations and dendrogram construction were completed using the Molecular Evolutionary Genetics Analysis (MEGA) programs (Kumar *et al.* 1993).

Gene mapping: Interspecific hybrids used to produce backcrosses were made by artificial insemination (Clark 1950), using *Xiphophorus clemenciae* from the Rio Sarabia, Oaxaca, Mexico, maintained in closed colony since capture in 1968; *Xiphophorus milleri*, collected in tributaries to Lago Catemaco, Veracruz, Mexico in 1982; *X. helleri*, collected in the Rio Sarabia in 1963; and *X. maculatus* strain Jp 163 A, inbred by brother-sister matings for 89 generations since collection in the Rio Jamapa, Veracruz, Mexico in 1939. Backcrosses were produced by Klaus Kallman at the New York Aquarium Osborn Laboratories of Marine Sciences, Brooklyn, NY and at the Xiphophorus Genetic Stock Center at Southwest Texas State University, San Marcos, TX.

Brain and eye, skeletal muscle, testis, and liver tissues were prepared and used for starch gel electrophoresis and histochemical staining following methods of Morizot and Schmidt (1990). Genomic DNA was extracted from gill, testis, spleen, and/or kidney tissues using protocols of Harless *et al.* (1990). Preliminary digestions with a variety of restriction endonucleases identified interspecific polymorphisms at *DXB* cut with *Bam*HI and at *DAB* digested with *Pst*I. Genomic DNA from appropriate backcross individuals was digested according to manufacturers' directions, electrophoresed through 0.8% agarose gels, and blotted onto hybridization membranes by methods of Harless *et al.* (1990, 1991). Probes for *DXB* (190-bp fragment in the β2-encoding domain of *X. maculatus* cDNA) and *DAB* (full-length 795-bp *X. maculatus* cDNA) were ^{32}P-radiolabeled by nick translation and/or random priming and hybridized to membranes, washed at high stringency, and autoradiographed to visualize hybridizing fragments (Walter *et al.* 1993). Genotypes of each backcross hybrid individual were scored, usually based upon codominant inheritance models, for all allozyme, DNA RFLP, and arbitrarily primed PCR (AP-PCR; Kazianis *et al.* 1996) polymorphisms.

Each polymorphic locus was assessed for agreement with the expected 1 homozygote:1 heterozygote backcross segregation; loci significantly ($P \leq 0.05$) deviating from Mendelian expectations were excluded from linkage analyses. Pairwise tests for deviation from 1 parental:1 recombinant independent assortment expectations were performed using MAPMAKER software (Lander and Green 1987) with LOD > 3.0 or $\chi^2_{1\ d.f.} > 13.8$ ($P < 0.001$) used as the criterion for presumption of genetic linkage. Map positions were compared to existing gene map assignments summarized in Morizot *et al.* (1991, 1993, 1998) and Kazianis *et al.* (1996), which references also should be consulted for gene nomenclature and mapping methodology.

RESULTS AND DISCUSSION

Nucleotide sequence of *Xima* and *Xihe DXB* genes: The *X. maculatus*, strain Jp 163 A, genomic library was screened with the PCR-derived β2-encoding fragment as described in materials and methods. A 6.5-kb *Hin*dIII-*Not*I fragment from a positive plaque was subcloned and sequenced with gene-specific primers and found to contain the complete coding region of a *Xima* class II *B* gene. The sequence (Figure 1) includes the 5′ untranslated region (UTR), six exons, five introns, and the 3′ UTR to the polyadenylation signal. The exon-intron boundaries were determined from comparisons with a cDNA clone (PCR-amplified from *X. maculatus* RNA with primers designed to the 5′ and 3′ ends of the genomic sequence) that was identical in coding regions. Proposed transcription start sites (underlined in Figure 1 in the region upstream of the START codon and listed in order) of the 5′ UTR including S box, pyrimidine-rich region, X box, X2 box, Y box, and CCAAT box are marked (Benoist and Mathis 1990; Prestridge 1991; Glimcher and Kara 1992; Singal and Qiu 1995). Exon 1 encodes the first 20 amino acids of the leader peptide followed by intron 1, which is 137 bp long (data of introns not shown). Exon 2 encodes 2 additional amino acids of the leader and 86 of the β1 domain. Intron 2 is 116 bp and exon 3 encodes the complete β2 domain (94 amino acids). A third intron of 369 bp follows. The 9 amino acids of the connecting peptide, the 23 amino acids of the transmembrane region and the first 5 amino acids of the cytoplasmic tail are encoded in exon 4. Intron 4 is 133 bp. Exons 5 and 6, together encoding the remaining 19 amino acids of the cytoplasmic tail, are separated by a fifth intron. Exon 6 proceeds to the TAG stop codon, followed by 3′ UTR. This exon-intron structure of the *DXB* locus is more similar to the class II *B* genes found in amphibian (Kobari *et al.* 1995), chicken (Xu *et al.* 1989), and human (Kappes *et al.* 1984) than to the class II *B* genes characterized in zebrafish (Sültmann *et al.* 1994) and cichlid (Ono *et al.* 1993). This exon-intron organization, in combination with the longer cytoplasmic tail encoded by DXB (see below), raises the interesting question of whether a *DXB*-like locus originally led to the tetrapod lineage of MHC class II *B* genes.

The significant difference in organization of the *DXB* locus from the *DAB* organization of other advanced teleost fishes leads to some additional observations. Ono *et al.* (1993) originally described the presence of an additional intron that splits the coding region of the β2 domain for *DAB*-like gene of cichlids (Percomorpha). A subsequent survey among teleost fishes by Figueroa *et al.* (1995) also found this intron in several other percomorph species and in *Melanotaenia trifasciata* of the Atherinomorpha, sister clade to the Percomorpha (Nelson 1994), but the intron was absent in more basal teleost clades (*i.e.*, Ostariophysi and Protacanthopterygii). Our data clearly indicate that this intron is absent in the *DXB* gene of Xiphophorus (Poeciliidae), also a member of the Atherinomorpha. Thus the exon-intron structure of the *DXB* gene is more similar to the *DAB* of primitive teleosts, than to the *DAB* gene of advanced teleosts,

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5'UNTRANSLATED OR INTERGENIC REGION
                                                                                  50
Xima-DXB*01  AAATCAATGT TTTTAAGAGA TACTGTAAAC AATGGCTACT CTGGCATCAC CATGTTGAGA TACCAACTTC
                                                 100
             TCCAGCTGTG CTGTGACCTC AGGAGTGACA TTAATTCAGC TCCTGATTGT TTGTTGCTCA ATCTTTCTGC
                    150                                                          200
             TCTACCCTCT GTAAGTTTTC TTTTTATTAT TCATAACAAT TTTATTTTGC CAAAGATGGT CTCTGTTCTT
                                                            250
             AGGTCCCATG TACAGCATCC CATAATGTTT TGCATACTAC TCATAAACAA AATGTTTTAC ACCACATTAG
                                  300                                                        350
             ATGTCATTTT TAAATTTATG AGGCCTAAAT TATAGTTTAC CTCAGGATTT CATGGGGTGG GCACTTGATG
                                                                                  400
             TTTTTTAAAT GACTACATAA GAAACTGAGA CTGTTGTGGA GAGCTGGACC ACTGCCTTAA CACACATCTT
                                                 450
             TCAATATATG TTTGGAAAAT AAATGCAAAT AAATATTTTA AAAAATTATA AATACTGTCT GTATTTTTTC
                    500                                                          550
             ACCCTTGCTT AATAAACAAA AAACTGGTGT GAGTGTTAAG CTGTAAGTCA TGTTTCTATG GTTTGAAGTG
                                                            600
             ATGTTACTTG TACTACGAAG GATGATGATG AAGAATGAAA CAGTTAAAAG AATAAAAAGA AACTTACTCA
                                  650                                                        700
             AACTGGTAGA AACACAGAGA GAGAGAGAAT ATGAAACAAA TGAGTGACAT GAGGAAAGGC AACAGGAGAG
                                                                                  750
             TCAAACACTT GATTATTTCT TACATTTATT CTTTGTACTA CTAAAACTGC ATGTTAGACA GATTATTTGA
                                                 800
             CATTAACAGA ATTGGAGAAC AAAAACTATT TATGGATAAG CAAGCGCGCC ACGCCTGCAC TGAAAACAGT
                    850                                                          900       **
             AACATTGGTG GATTTGTTTC TGTACTTCCT CATTGTTGTT GATCAGAACC AGTTAGCTCT CACTCGGAAT
             ********** *********
Xima-DXB*01  CTCTGTTGCC AATCTAAGAG G
Xihe-DXB*01    -------- ---------- -
             EXON 1                   950
             LEADER
              M   A   Q   A   Q   G   C   S   V   F   L   V  F/L  F   L   A
Xima-DXB*01  ATG GCT CAG GCT CAG GGC TGC TCT GTT TTC CTG GTG TTT TTT CTG GCG
Xihe-DXB*01  --- --- --- --- --- --- --- --- --- --- --- --- C-- --- --- ---

              F   S   P   G
Xima-DXB*01  TTT TCA CCA GGA G
Xihe-DXB*01  --- --- --- --- -

             INTRON I
Xima-DXB*01  GT ...... AG
Xihe-DXB*01  -- ...... --


             EXON 2
                      BETA-1 DOMAIN      1150
              G   A   F   Y   L   T   V   L   E   R   C   Q  F/S  S   S   T   D   G   H
Xima-DXB*01   GT GCT TTC TAT TTG ACT GTG CTG GAA CGG TGC CAG TTT AGC TCA ACT GAC GGT CAT
Xihe-DXB*01   -- --- --- --- --- --- --- --- --- --- --- --- -C- --- --- --- --- --- ---
                                 1200
              D   A   V   L   L   D   Q  I/V  Y   F   N   K   I   L   E  G/V Q/E  Y   N
Xima-DXB*01  GAT GCC GTG TTA CTG GAT CAG ATT TAC TTC AAC AAA ATA CTG GAA GGA CAA TAC AAC
Xihe-DXB*01  --- --- --- --- --- --- --- G-- --- --- --- --- --- --- --- -T- G-- --- ---
                      1250
              S   T   V   G   K   A   I   G   Y   T   E   K  V/A  E   A   L  V/A  I  F/I
Xima-DXB*01  AGC ACT GTA GGA AAA GCG ATT GGG TAC ACA GAA AAA GTT GAA GCA CTT GTC ATT TTT
Xihe-DXB*01  --- --- --- --- --- --- --- --- --- --- --- --- -C- --- --- --- -C- --- A--
             1300                                                                1350
              L    N   N   N  T/P  G   F   I   T   H   E   I   W   K   T   N   L   C   K
Xima-DXB*01  CTA  AAC AAC AAT ACT GGG TTT ATT ACT CAC GAG ATA TGG AAA ACA AAC CTC TGC AAA
Xihe-DXB*01  ---  --- --T --- C-- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

              R   N  T/A  P   L   A   Q   K   L   L   T   P
Xima-DXB*01  AGA AAT ACT CCA CTG GCA CAA AAA CTA CTA ACA CCA G
Xihe-DXB*01  --- --- G-- --- --- --- --- --- --- --- --T --- -

             INTRON 2
Xima-DXB*01  GT ...... AG
Xihe-DXB*01  -- ...... --
```

Figure 1.—Genomic nucleotide sequences of platyfish *MhcXima-DXB*01* and swordtail *MhcXihe-DXB*01.* Dashes indicate identity of *Xihe* with *Xima.* Dots represent intron sequences. Asterisks denote the position of the initial primers used to amplify swordtail genomic DNA and also platyfish cDNA to obtain the coding regions. The proposed positions of the S box, pyrimidine-rich region, X box with X2 box, Y box, and CAAT box (in order from 5′ to 3′) are underlined; protein domains, exons, and introns are in bold; and the deduced amino acid sequence is printed above the codons. The *Xima DXB*01* and *Xihe-DXB*01* genomic sequences (complete with introns) have been submitted to the GenBank database and assigned accession nos. AF040762 and AF040763, respectively.

including other atherinomorph fishes. This leads to two alternative evolutionary hypotheses. In the first, the *DXB* and *DAB* genes were produced by a gene duplication event after the acquisition of the sixth intron, but this intron was subsequently lost in the *DXB* gene of Xiphophorus. Alternatively, the gene duplication event giving rise to the *DXB* gene occurred before the evolution of the sixth intron, very early in the diversification of tele-

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             EXON 3
             BETA-2 DOMAIN
                                                                   1550
              V   E   P   Y   V   Q   L   R   L   E   K   A   E   Y   S   Q   H   Q
Xima-DXB*01   TG GAG CCC TAT GTT CAG TTG AGG TTA GAG AAG GCA GAG TAC AGT CAA CAT CAG
Xihe-DXB*01   -- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                                                               1600
             Q   M   L   I   C   S   A   Y   D   F   Y   P   K   Q   I   R   V  T/M
Xima-DXB*01  CAG ATG CTC ATC TGC AGC GCG TAC GAC TTC TAT CCA AAG CAA ATC AGG GTG ACG
Xihe-DXB*01  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -T-
                                     1650
             W   L   R   D   G   K   E  A/V  T   S   D   V   T   S   T   D   E   L
Xima-DXB*01  TGG CTG AGA GAC GGA AAG GAG GCC ACA TCT GAT GTG ACA TCC ACG GAC GAG CTG
Xihe-DXB*01  --- --- --- --- --- --- --- -T- --- --- --- --- --- --- --T --- --- ---
                                                  1700
             P   N   G   N   W   L   Y   Q   I   H   T   Y   L   E   F   T   P   K
Xima-DXB*01  CCC AAT GGG AAC TGG CTT TAT CAG ATC CAC ACC TAC CTG GAG TTC ACA CCT AAA
Xihe-DXB*01  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                                             1750
             P   G   E   K   I   T   C   M   V   E   H   A   S   L   K   K   P   N
Xima-DXB*01  CCT GGA GAG AAA ATC ACC TGC ATG GTG GAG CAT GCA AGC CTC AAG AAA CCA AAT
Xihe-DXB*01  --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

             L   Y   D   W
Xima-DXB*01  CTT TAT GAC TGG G
Xihe-DXB*01  --- --- --- --- -

             INTRON 3
Xima-DXB*01  GT ...... AG
Xihe-DXB*01  -- ...... --

             EXON 4                                    2200
             CONNECTING PEPTIDE                  TRANSMEMBRANE REGION
              E   P   E   P   D   S   K   W   S   K   I   V   V   G   S   A   G   L
Xima-DXB*01   AG CCG GAG CCG GAT TCA AAG TGG AGT AAG ATT GTT GTT GGC TCA GCA GGG CTG
Xihe-DXB*01   -- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
                                                 2250
                                                                 CYTOPLASMIC TAIL
             L   L   G   L  V/M  F   S   I   A   G   F   I   Y   Y   K   T   T   S   N
Xima-DXB*01  CTG CTC GGT TTG GTG TTT TCA ATT GCT GGC TTC ATC TAT TAC AAG ACA ACA TCA AAT G
Xihe-DXB*01  --- --- --- --- A-- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -

             INTRON 4
Xima-DXB*01  GT ...... AG
Xihe-DXB*01  -- ...... --

             EXON 5
              G   R   V   V   V   P   T   T   E
Xima-DXB*01   GA CGG GTG GTG GTG CCT ACA ACA GAG G
Xihe-DXB*01   -- --- --- --- --- --- --- --- --- -

             INTRON 5
Xima-DXB*01  TG ...... GG
Xihe-DXB*01  -- ...... --

             EXON 6
              D   V   C   P   E   E   T   L   *
Xima-DXB*01   AT GTA TGT CCA GAA GAA ACC CTT TAG
Xihe-DXB*01   -- --- --- --- --- --- --- --- ---

             3' UNTRANSLATED REGION
                 2550                                                        2600
Xima-DXB*01  GCTGCAGAAA TGTTGAGAAG CCCCACACAC ATCTATCAAC CACTGGAAAC TGGTCTTAAT ATTTGAGAGA
Xihe-DXB*01  ---------- ---------- ---------- ---------- -G-------- ---------- ----------
                                                        2650
                                             ********** ********** *
Xima-DXB*01  CTACAAGCCT TCACATGCCT GAAAGTTTTT ATCATTTAGC CTTTTACACA TTTCTGCAGA CAAAAAATAT
Xihe-DXB*01  ---------- ---T------ ---.------ ---------- ----------
                                 2700                                             2750
Xima-DXB*01  TGCAGCATAT TTATTTCATA CTTGTACTTA ACACACTATT GGTAAAACTG CAAATAAATA AACCCTGTCT

Xima-DXB*01  TTGG
```

Figure 1.—*Continued.*

ost fishes. The latter hypothesis is supported by the phylogenetic analysis of nucleotide changes for the class II *B* genes (see below). If so, the investigation of evolution of the *DXB* gene adds an additional independent line of inquiry to the important insights already provided by the study of MHC genes in elucidating phylogenetic patterns (Klein *et al.* 1997).

The genomic sequence of *Xima-DXB*01* has conserved features with other teleost class II *B* genes previously studied (Ono *et al.* 1993; Sültmann *et al.* 1994; Van Erp *et al.* 1996). Compared to human MHC class II introns (Klein 1986), those of teleosts are relatively short. All but one exon-intron boundary has canonical splice signals (Shapiro and Senepathy 1987; Senepathy *et al.*

1990). Intron 5 in Xiphophorus, splitting exons 5 and 6 encoding the cytoplasmic tail region, has not been reported in other teleosts. Intron 5 in Xiphophorus has nonconsensus splice signals both at the 5′ donor and 3′ acceptor site. Even though these nonconsensus splice sites appear to be rare, they nevertheless have been reported in 0.7% of 7500 mammalian splice sites examined by Senepathy *et al.* (1990). Van Erp *et al.* (1996) reported a nonconsensus splice signal in the *Cyca-DAB3*01* and *Cyca-DAB4*01* genomic sequences, as did Dixon *et al.* (1996) in *Barbus intermedius intermedius* and *Barbus bocagei.* It has been suggested that nonconsensus splice sites may be important in slowing the upregulation of the expressed gene (Haviland *et al.* 1991). Incomplete splicing may compromise the stability of the mRNA, thus providing a means of gene regulation (Glimcher and Kara 1992).

To test for the presence of the *DXB* locus in a species closely related to *X. maculatus*, swordtail (*X. helleri*) genomic DNA was isolated and a *Xihe-DXB* PCR fragment was amplified. The resulting fragment was cloned and sequenced, revealing a complete MHC class II *B* gene including start and stop codons (Figure 1). The two sequences are very similar, differing in only 25 nucleotides throughout the compared sequence, plus an insertion of 4 nucleotides in intron 2 (data not shown). Eleven of the nucleotide differences between the two *DXB* sequences are clustered in exon 2, the β1 encoding domain. Nine of these nucleotide changes result in nonsynonymous amino acid substitutions indicative of a functionally encoded peptide binding region (Hughes and Hughes 1995), though these are not two sequences from the same species. In contrast, only three nucleotide differences occur in exon 3, resulting in two amino acid changes. *Xihe-DXB*01* has an identical intron 5 with the same noncanonical exon-intron splice sites as the platyfish.

The promoter region at the 5′ end of the *Xima-DXB*01* sequence shows similar features to those previously identified (reviewed in Benoist and Mathis 1990; Glimcher and Kara 1992; Mach *et al.* 1996). Considerable allelic polymorphism in this region has been reported in the literature and may account for differences in gene expression (Singal and Qiu 1995). The CCAAT box is present, but a typical TATA box cannot be found in the expected region of the promoter (−180 to +10). Sültmann *et al.* (1994) also reported the lack of a TATA box in the zebrafish *Dare-DAB* and *Dare-DEB* genes. It has been suggested that the TATA box may not be needed for proper transcription initiation in those genes that are tissue-specific, where other transcription factor binding sites such as X box, X2 box and Y box are involved (Glimcher and Kara 1992). A sequence suggestive of an octamer motif (GATTTGTT) is immediately adjacent to the Y box (CATTGGTG). Initially described in immunoglobulin genes, this element appears to be involved in gene expression (Glimcher and Kara 1992).

Sequence comparison of *Xima-DXB* and *-DAB*: The nucleotide and amino acid sequences of *Xima-DXB*01* and *-DAB*01* are compared in Figure 2. The two sequences are 43 and 63% identical in the β1- and β2-coding regions, respectively, and 27 and 55% identical in the β1 and β2 amino acid sequences. Thus β1 sequence identity between these two genes is low, but more informative is the low level of 55% identity for β2. This is at the lower limit of the 54–85% identity found between the protein chains coded by known class II loci, excepting DM (Cho *et al.* 1991; Kelly *et al.* 1991), which is 31–39% identical to the other respective class II β2 domains. Neither *Xima* gene demonstrated sequence patterns indicative of a *DM*-like locus, although similar functions could be carried out without sequence homology. The longer cytoplasmic tail of *Xima-DXB* could be involved in either specific trafficking or in MHC class II signaling to the interior of the cell. The three codon deletions in the β1-encoding region and the one codon deletion in the CP-encoding region of *Xima-DXB* relative to *Xima-DAB* also indicate *DXB* is a significantly divergent locus. The nucleotide and amino acid sequence variability coding for the β1 and β2 domains is readily apparent in Figure 3. Surprisingly, the longest string of consecutive identical nucleotides in the cDNAs of these two loci in *Xima* is 16. The extensive diversity between these two genes may also affect preferential pairing of the encoded β chains with as yet uncharacterized α chains from different class II *A* loci in Xiphophorus. Differences in glycosylation sites and the length of the cytoplasmic tail are discussed below.

Sequence alignment of selected teleost class II β chains: An amino acid (aa) sequence alignment of Xima-DXB*01 and Xima-DAB*01 with those of other representative teleost class II B chains found as related with an NCBI BLAST search, is shown in Figure 3. The Xima-DXB*01 leader sequence/β1 boundary was determined with the SignalP computer program (Nielsen *et al.* 1997). The hydrophobicity of the leader peptide, variability between the two Xiphophorus species in the β1 domain, overall consensus residues of the β2 domain, connecting peptide, transmembrane and cytoplasmic region are all similar to those of the previously identified organisms. Key residues such as those involved in disulfide bonds, glycosylation, and interaction with the α chain are also seen in the Xima-DXB sequence. Differences in DXB compared to the other class II chains shown include 3-aa deletions (positions 65, 80, and 81) in β1 and a 1-aa deletion (position 186) in the connecting peptide. Atlantic salmon cDNA clones 144 and 22 (Hordvik *et al.* 1993) and zebrafish pseudogene genomic sequences *DCB* and *DBB* (Sültmann *et al.* 1994) show codon deletions in the β1 domain that differ from those of DXB.

The teleost MHC class II β sequences shown all have

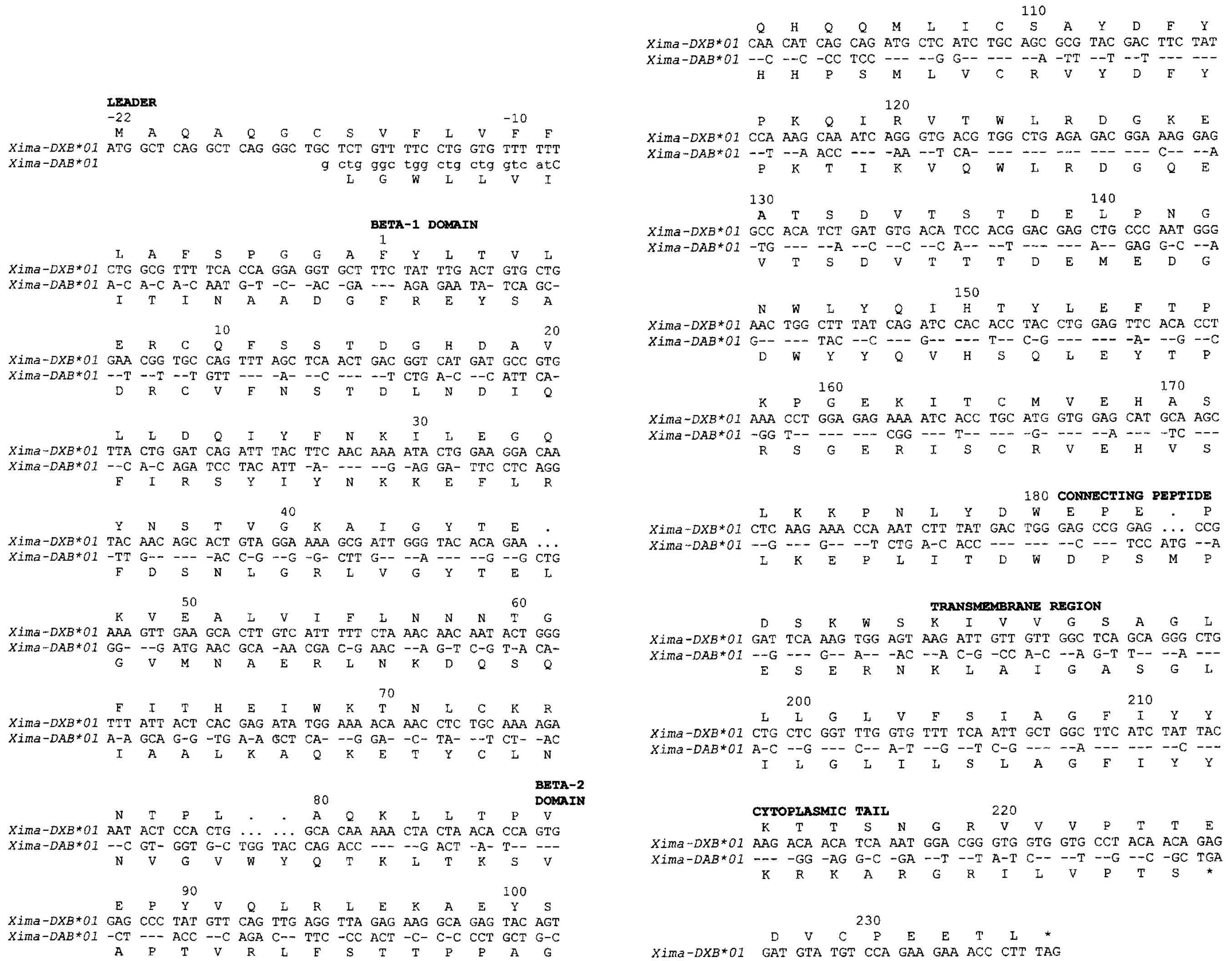

Figure 2.—Nucleotide and putative amino acid sequence comparisons of *Xima-DXB*01* and *Xima-DAB*01* cDNA clones. The *Xima-DXB*01* and *Xima-DAB*01* cDNA sequences have been submitted to the GenBank database and assigned accession nos. AF040761 and AF040760, respectively.

a glycosylation signal sequence at positions 12–14 or 36–38 (Figure 3), including Xima-DXB, with the single exception of the guppy sequence Pore-DB-4-28, which lacks any such site. The Xima-DAB sequence, clearly related to the guppy sequence at 92% identity, does have a glycosylation signal sequence, at consensus positions 12–14.

The 22-aa cytoplasmic tail of Xima-DXB is surprisingly long, but similar in length to the human HLA-DRB (Tieber *et al.* 1986), the clawed frog Xela-F3 (Sato *et al.* 1993), and the nurse shark Gici-β (Bartl and Weissman 1994). This could have functional significance as discussed above.

Comparison of the Xima sequences to zebrafish (Dare-DAB4*01), among others, is shown in Figure 3. The genes coding for the zebrafish class II β sequences have been particularly well characterized at the genomic level (Sültmann *et al.* 1994). Within the *Dare-DAB1* family of related loci, coded amino acid sequence identity of DAB2*01, DAB3*01, and DAB4*01 to DAB1*01 β2 domain is 84–89%, though evidence indicated only one locus was transcribed. Comparison of Dare-DAB β2 sequence to the presumed pseudogenes of distantly related loci Dare-DBB, -DCB, -DDB, and -DEB revealed 63–71% identity. This contrasts with the more distantly related Xima-DXB *vs.* -DAB at 55% identity in β2, particularly when considering that both the *Xima* loci are transcribed with all features necessary for expression.

Mapping of MHC class II B loci: Linkage analyses for *DXB* and *DAB* are presented in Tables 1 and 2, respectively. Unfortunately, polymorphisms for both genes were not detected in the same cross type, so that direct linkage tests could not be performed. However, each locus was assigned to a multipoint linkage group with high confidence.

Assignment of DXB to Xiphophorus linkage group (LG) III: Table 1 documents linkage of *DXB* and *GAPD1* with ~18% recombination. Unfortunately, crosses involving

```
                    LEADER               BETA-1 DOMAIN
                                           1         10         20          30
Consensus           MMS SFLCLSLLF- -SLFSTADGF -EYVVDRCVF SSTDL-DIEY IDSYYFNKVE
Xima-DXB*01         -aq aqg-svf-vf fla--pgga- ylt-le--q- ----gh-avl l-qi----il
Cyca-DAB4*01         lk llifhpi-ml saftg----y y--tmye--y -ts-ys-mv- lv-ls--q-v
Dare-DAB4*01            lkpf-vi-ml st-tg----y yd-ikqq-fy -ts-ys-mv- la--s----v
Onmy-DAB*01        ms-p iafyic-tll w-i--gt--y f-q--rq-ry --k--hg--f ----v---a-
Sasa-C-157           -- i.f-v--t-v l-i--gt--y f-q--rq-ry --k--qg--f ----v---a-
Auha-M-231            - ---------i .--.------ ls-s----q- n--e-k---- -r-v-y--l-
Cyfr-T-141            - ---------i .--.---g-- mm-s----n- n--e-q---f -r----r---
Mosa-C-1            -a- ---sf----i t--.y-g--- ln-a-n---- n---pkn--- -y-h-y--l-
Pore-DB-4-28        -ap -a-gw.--vi itvh-.-g-- r-fa------ --pe-k--qf -r--cy--l-
Xima-DAB*01               -gw.llvi itina.---- r--sa----- n----n--qf -r--iy--k-
Icpu-DAB*01          -- kl-kil-ivl pav-h--h-n flsqp-w-iw -ke--s-m-- -kplii--ik
                                                                      BETA-2
                                                                      DOMAIN
                    40         50         60         70         80          90
Consensus    --RFNSSVGK YVGYTELGVK NAEYWNKDPA -LA--KAQKE TYCKHNIGID YSAILDKSVE
Xima-DXB*01  egqy--t--- ai----kvea lvifl-nntg fith.eiw-t nl--r-tpl. .aqk-ltp--
Cyca-DAB4*01 dvqc---av- c-----e--- y--nf----s v-qdl-tsvd ---rs-aqla d-svr--a-q
Dare-DAB4*01 dtq------- f-----q-li f--nf---q- y-hql---vd -f-r--aqiw d--vr--a-l
Onmy-DAB*01  hv----t--r ------h-l- ---a--s-ag i-gqeq-el- r---psaai- -------t--
Sasa-C-157   yi----t--- f--------- ---a--s-a- v--vergel- rf----adlh --t----t--
Auha-M-231   if--s--l-- f-----y--- q-d-r-n-k- i-ssm----- ---hn---v- -----s---q
Cyfr-T-141   ft--d----- ---------r ---e----kg n--amn---g ---l----vw n-n--s--ak
Mosa-C-1     ia--s----e ------f--- q-k---s--s e--rrs---- -v-q---n-- -qv-------
Pore-DB-4-28 fi--d-nl-- ---------- ---r----ts li-am---r- ---ln-v-n- -qna-----a
Xima-DAB*01  fl--d-nl-r l--------m ---rl---qs qi-al----- ---ln-v-vw -qtk-t---a
Icpu-DAB*01  yley--t--- v-------i- --drf----- fmqgl--eld sv--n-v-ny --g--s-t--

                    100        110        120        130        140         150
Consensus    P-VKLHSVKP PSGRHPAMLV CSAYDFYPKK IKVSWLRDGK EVTSDVTSTE EMADGDWYYQ
Xima-DXB*01  -y-q-rle-a eysq-qq--i ---------q -r-t------ -a-------d -lpn-n-l--
Cyca-DAB4*01 -kit-r-arq ag-sr--v-m ----e----- ---------- -m-------m ---n-n-f--
Dare-DAB4*01 -e-tik--rq ae-----v-l ----e----- --m------- ---------m ----------
Onmy-DAB*01  -h-r-s--t- ---------m ---------q -r-t-----r --k------- -l-n------
Sasa-C-157   -h-r-s--a- ---------m ---------p -r-t-----r --k------- -l-n------
Auha-M-231   -s-----m-- a-sh------ --v-----se ---------- -i-------- ----------
Cyfr-T-141   -y-----tit a-sq------ --v---f-s- ---------q ---------- ----------
Mosa-C-1     -s-v----a- -a-k--s--- --vc--f--h -r-------q ---------d -l--a--f--
Pore-DB-4-28 -t-----rt- -a-h--s--- -rv------t ---h-----q -------t-d --e-------
Xima-DAB*01  -t-r-f-ttp -a-h--s--- -rv------t ---q-----q -------t-d --e-------
Icpu-DAB*01  -q--vkl--k sd-t---t-m ----s---pa -s-t---n-- -ikgg----- ----------
                                            CONNECTING TRANSMEMBRANE
                                            PEPTIDE   REGION
                    160        170        180        190        200         210
Consensus    IHSHLEYTPR SGEKISCMVE HASLKEPLVT DWDPSMPESE RNKIAIGASG LVLGLILSLA
Xima-DXB*01  --ty--f--k p----t---- -----k-nly --e-e.-d-k ws--vv-sa- -l---vf-i-
Cyca-DAB4*01 ---e-----k ---------- ---fsk-mi- -----l--g- ---------- ----i-iaa-
Dare-DAB4*01 ---------k -----q-l-- ----tq--tk --n-his--d ---f------ ----i-iai-
Onmy-DAB*01  ---------k ---------- -i--t--mmy h----l--a- ---------- ----t--a--
Sasa-C-157   ---------- ---------- -i--t--m-y h----l--a- ---------- ----a--a--
Auha-M-231   t--------- -------v-- --------k- ---------- --nv------ -i--------
Cyfr-T-141   ---------- -------v-- --------k- ---------- --nv------ -i--------
Mosa-C-1     ---------- -------v-- ---------- ---------- ---------- -i--------
Pore-DB-4-28 v--t------ ---r---r-- -pt------- --e----d-d ---l------ -i--------
Xima-DAB*01  v--q------ ---r---r-- -v------i- ---------- ---l------ -i--------
Icpu-DAB*01  v------m-e ---e---v-q ---ftk-mny k--s----pd ks-------- ----iv--a-
                CYTOPLASMIC TAIL
                    220        230
Consensus    GFIYYKKKSR GRILVPTN-- --------
Xima-DXB*01  ------tt-n --vv---ted vcpeetl*
Cyca-DAB4*01 -l-------a ------n... ........
Dare-DAB4*01 -l-------t ------n... ........
Onmy-DAB*01  -l-------s -vl....... ........
Sasa-C-157   -l-------s -vl....... ........
Auha-M-231   ------r-a- ------s-.. ........
Cyfr-T-141   ------r-a- --------.. ........
Mosa-C-1     ------r-a- ------s-.. ........
Pore-DB-4-28 ------r-a- -------s.. ........
Xima-DAB*01  ------r-a- -------s*
Icpu-DAB*01  ---------s -------*.. ........
```

Figure 3.—Comparison of deduced amino acid sequences of the platyfish MHC class II β chains with other teleost species. Cyca-DAB4*01 (Van Erp *et al.* 1996), Dare-DAB4*01 (Ono *et al.* 1992), Onmy-DAB*01 (Glamann 1995), Sasa-C-157 (Hordvik *et al.* 1993), Auha-M-231 and Cyfr-T-141 (Ono *et al.* 1993), MosaC-1 (Walker and McConnell 1994), Pore-DB-4-28 (Sato *et al.* 1995), and Icpu-DAB*01 (Godwin *et al.* 1997) were chosen to represent a range of teleost species. Dashes indicate identity with the consensus (simple majority). Dots were inserted for optimal alignment of the sequences. The glycosylation site is underlined, and conserved cysteines involved in disulfide linkages are bold. The numbering system defines the putative mature protein of Xima-DXB*01 as determined by SignalP (Nielsen *et al.* 1997). The proposed domains of the β chain are labeled above the sequence.

TABLE 1

Linkage analysis of *DBX* in *X. clemenciae* × (*clemenciae* × *X. milleri*), (*X. clemenciae* × *X. milleri*) × *X. clemenciae*, and (*X. clemenciae* × *X. helleri*) × *X. helleri* backcrosses

Locus	LG	Parentals	Recombinants	$\chi^2_{1\ \text{d.f.}}$	*r*
ACO1	XIV	24	22	0.09	0.48
ACTB	XVI	9	7	0.25	0.44
ACTBL1	V	33	39	0.50	0.54
ACTBL2	IV	30	29	0.02	0.49
ADA	I	20	22	0.10	0.52
APENDL1	U2	24	18	0.86	0.43
CKM	XI	23	26	0.18	0.53
EGFR	VI	14	20	1.06	0.59
ERBAL2	U4	19	18	0.03	0.49
ERBAL3	XIII	16	23	1.26	0.59
ERCC2	XI	28	28	0.00	0.50
ES1	V	16	20	0.44	0.56
FYNL1	XIII	17	13	0.53	0.43
GALT	VIII	17	17	0.00	0.50
GAPD1	III	40	9	19.61***	0.18
GDA	XII	25	42	4.31*	0.63
GDH1	U2	17	22	0.64	0.56
GLNS	VI	22	27	0.51	0.55
GLYDH	V	18	25	1.14	0.58
GNRH	U22	16	17	0.03	0.52
GOT2	IV	28	21	1.00	0.43
GPI1	IV	41	37	0.21	0.47
GPI2	II	23	26	0.18	0.53
IDH1	IV	11	9	0.20	0.45
IDH2	VII	26	31	0.44	0.54
IIA	III	16	8	2.67	0.33
JUNA2	U4	18	11	1.69	0.38
LIG1	VI	16	19	0.26	0.54
MACR	XXIV	24	25	0.02	0.51
MDH2	V	20	29	1.65	0.59
NP2	VI	47	48	0.01	0.51
PEPA	XIII	20	23	0.21	0.53
PEPS	XII	20	29	1.65	0.59
PGK	XI	20	19	0.03	0.49
PGM	IX	46	30	3.37	0.39
PK1	IV	26	23	0.18	0.47
PK2	II	19	29	2.08	0.60
PVALB2	X	17	38	8.02**	0.69
PVALB3	U4	19	21	0.10	0.53
RBL1	VI	14	16	0.13	0.53
RPS15	VI	11	9	0.20	0.45
TF	VI	16	27	2.81	0.63
TP53	XIV	18	16	0.12	0.47
TPI1	XIII	20	28	1.33	0.58
UMPK	VI	9	10	0.05	0.53
UNG	XII	23	26	0.18	0.53
XRCCL1	XIII	13	6	2.58	0.32

$^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$; *r*, maximum likelihood recombination frequency estimate.

X. clemenciae were made years before our incorporation of many DNA RFLP and AP-PCR polymorphisms as markers; thus, many LG III markers could not be analyzed in the present study. Because *GAPD1* is near the end of LG III in the current Xiphophorus map comprising >20 markers (Morizot *et al.* 1993, 1998), it is likely that *DXB* lies toward the center of the linkage group near *GUK2.* LG III also contains a malic enzyme locus, probably coding for a cytosolic isozyme, which is also the case in human chromosome *6*, carrying both malic enzyme loci and the major histocompatibility complex.

Assignment of DAB to Xiphophorus newly designated LG U24: DAB was found to be polymorphic in the most extensively mapped Xiphophorus cross type, with >250

TABLE 2

Likage analysis of *DAB* in *X. helleri* × (*X. maculatus* × *X. helleri*)

Locus	LG	Parentals	Recombinants	χ^2	*r*
ACO1	XIV	18	22	0.40	0.55
ACTBL1	V	24	22	0.09	0.48
ACTBL2	IV	20	24	0.36	0.55
CDKN2X	V	22	24	0.09	0.52
ES1	V	19	26	1.09	0.58
ES2	II	21	25	0.35	0.54
GALT1	VIII	17	13	0.53	0.43
GAPD1	III	27	19	1.39	0.41
GDA	XII	22	24	0.09	0.52
GUK2	III	16	15	0.03	0.48
IDH1	IV	17	25	1.52	0.60
IDH2	VII	20	26	0.78	0.57
MACR	XXIV	27	19	1.39	0.41
MPI	II	20	26	0.78	0.57
P	XVII	19	26	1.09	0.58
PGAM1	XI	18	19	0.03	0.51
PGD	I	22	23	0.02	0.51
PGM	IX	25	20	0.56	0.44
PP1B	U22	22	18	0.40	0.45
PVALB2	X	18	25	1.14	0.58
UMPK	VI	23	18	0.61	0.44
XD0098	U20	20	23	0.21	0.54
XD0102	U19	19	24	0.58	0.56
XD0103	U16	19	22	0.22	0.54
XD0104	XIII	19	24	0.58	0.56
XD0111	U18	22	15	1.32	0.41
XD0112	U16	14	18	0.50	0.56
XD0114	IV	15	24	2.08	0.62
XD0147	XV	14	13	0.04	0.48
XD0148	XVI	11	14	0.36	0.56
XD0151	VIII	14	13	0.04	0.48
XD0152	III	13	12	0.04	0.48
XD0154	U24	32	3	24.02***	0.09
XD0155	II	17	21	0.42	0.55
XD0156	XIII	17	20	0.24	0.54
XD0158	IX	17	21	0.42	0.55
XD0162	III	20	17	0.24	0.46
XD0164	U18	21	20	0.02	0.49
XD0168	III	13	11	0.17	0.46
XD0169	III	26	19	1.09	0.42
XD0174	VII	21	23	0.09	0.52
XD0181	V	25	20	0.56	0.45
XD0182	X	23	21	0.09	0.48
XD0188	VII	12	13	0.04	0.52
XD0207	XII	14	15	0.03	0.52
XD0212	VI	23	15	1.68	0.39
XD0213	I	23	15	1.68	0.39
XD0217	XI	16	21	0.68	0.57
XD0219	XVII	14	16	0.13	0.53
XD0220	U20	16	18	0.12	0.53
XD0223	XV	20	19	0.03	0.49
XD0226	U24	36	4	25.60***	0.10
XD0232	XIV	18	26	1.46	0.59
XD0239	XVI	17	14	0.29	0.45
XD0246	U22	17	14	0.29	0.45
XD0253	U19	22	18	0.40	0.45

Pairwise comparisons are with endpoint loci except for LG III, where loci marking the entire length of the linkage group are represented, and for very long linkage groups, where midpoint loci are included.

***$P \leq 0.001$; *r*, maximum likelihood recombination frequency estimate.

informative markers located in 25 multipoint linkage groups, one more than the 24 acrocentric or telocentric chromosome pairs. *DAB* was found to be linked to two AP-PCR markers, *XD0226* and *XD0154*; these three markers here are assigned to the newly designated LG U24. The gene order is uncertain because of small sample sizes, but an order of *XD0154*–12%–*XD0226*–9.8%–*DAB* minimizes multiple crossovers. It is impossible to determine with certainty that *DAB* and *DXB* reside on different chromosomes, particularly since *DAB* yields recombination estimates of <40% with some LG III markers. Additional mapping data will coalesce multipoint linkage groups into 24 chromosomal linkage groups in time, but at present it can be stated with reasonable certainty based upon recombination estimates with all LG III loci (Table 2) that *DXB* and *DAB* are not tightly linked and assuredly are not members of a gene cluster. Bingulac-Popovic *et al.* (1997) performed linkage analysis of MHC class I and class II, and β*2m* genes in zebrafish. These investigators found that MHC class I genes were not linked to class II. They also found *Dare-DAB* and *DDB* (a pseudogene) class II *B* genes to be tightly linked, and *DFB* (a pseudogene) not to be linked to the expressed gene *DAB.* The *Xima-DXB* and *DAB*, however, represent two very different class II *B* genes of different clusters, that are both expressed. The data presented here thus support the possibility that tight clustering of MHC loci in mammals may represent a relatively recent evolutionary arrangement.

It is uncertain whether more than one copy of *DXB* and *DAB* exists in the Xiphophorus genome. *DXB* exhibits only one strongly hybridizing fragment on Southern blots with the 190-bp cDNA probe used (Figure 4A), and likely is present as a single copy. *DAB*, on the other hand, exhibits several fragments in addition to three obviously polymorphic fragments (Figure 4B). Whether the additional fragments represent pieces of *DAB*, which is quite possible as a full-length cDNA was used for probe, or a gene duplicate cannot be determined at present. Cloning of the genomic DAB sequence and location of restriction sites will help to resolve this issue. Sato *et al.* (1995) present evidence suggesting that the MHC class II *B* sequences found in guppy are encoded at a single locus, the *DAB*-like locus. Guppy may also possess the *DXB* locus in addition to the *DAB* locus characterized by Sato *et al.* (1995). Exon 3 in *Xima DXB*01*, encoding the more conserved β2 domain, is 61% similar to exon 3 in the guppy (*Pore*-DB-4-28), a surprisingly low degree of identity between two very similar species until considering that *DXB* and the guppy *DAB*-like gene are two very different loci. The detection of one predominant set of bands in our Southern blots supports the detection of a single *DXB* locus without cross-hybridization to *DAB.*

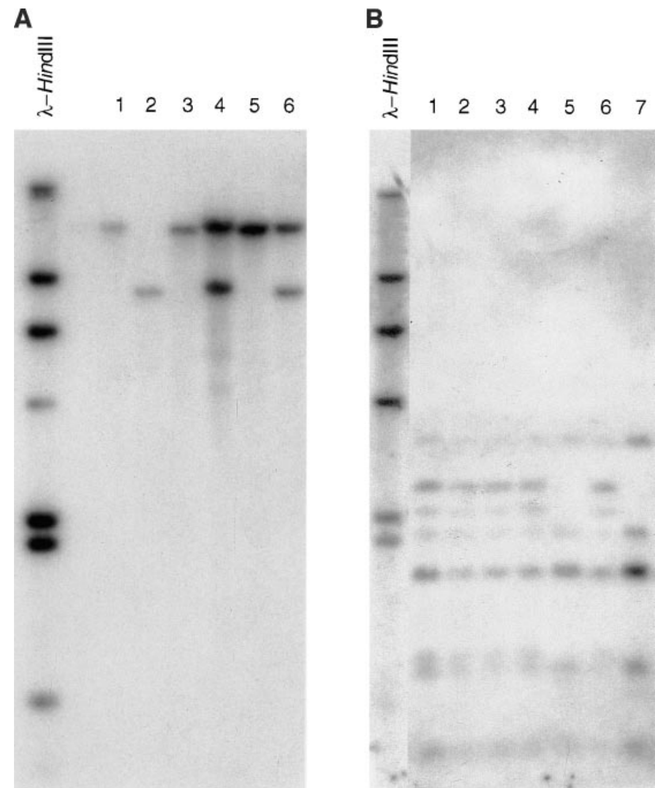

Figure 4.—(A) Representative Southern blot of the *DXB* cross-hybridizing fragments using the 190-bp probe described in the text. Lane 1, *X. clemenciae*; lane 2, *X. milleri*; lanes 3–6, backcross hybrids from *X. clemenciae* × (*X. milleri* × *X. clemenciae*) crosses. Lanes 3 and 5 are homozygotes for *X. clemenciae* alleles, while lanes 4 and 6 are heterozygotes. (B) Representative Southern blot of the *DAB* cross-hybridizing fragments using a full-length cDNA probe. Lanes 1–7, backcross hybrids from *X. helleri* × (*X. maculatus* × *X. helleri*) crosses. Lanes 1–4 and 6 are heterozygotes, while lanes 5 and 7 are homozygotes for *X. helleri* alleles.

Phylogenetic analysis: To examine the relationship of *Xima* class II *B* sequences with those of other fishes, a dendrogram was constructed employing the neighbor-joining method (Saitou and Nei 1987) on distances based on the p-distance algorithm as calculated by MEGA (Kumar *et al.* 1993). The phylogenetic tree shown in Figure 5, based on exon 3 sequences, demonstrates the wide disparity between the Xiphophorus *DXB* sequences and the *DAB* sequences. An analysis using the Jukes-Cantor (Jukes and Cantor 1969) and Tajima-Nei (Tajima and Nei 1984) methods of correction for multiple substitutions led to a dendrogram with identical topology as shown in Figure 5. Sequences of a class II *B* locus in carp, and of closely related loci in zebrafish, cluster together, while Xiphophorus *DXB* genes and the closely related guppy *Poecilia reticulata DB-4-28* (Sato *et al.* 1995) (both species are in the Family Poeciliidae, Subfamily Poeciliinae, Tribe Poeciliini; Nelson 1994) are shown as unrelated in Figure 5. Xiphophorus-*DAB* and the guppy sequence *DB-4-28* are, however, shown as closely related. The dendrogram also places the Xiphophorus-*DXB* sequences as ancestral to those of the class II sequences of representative members of the orders Salmoniformes and Perciformes, with the very low

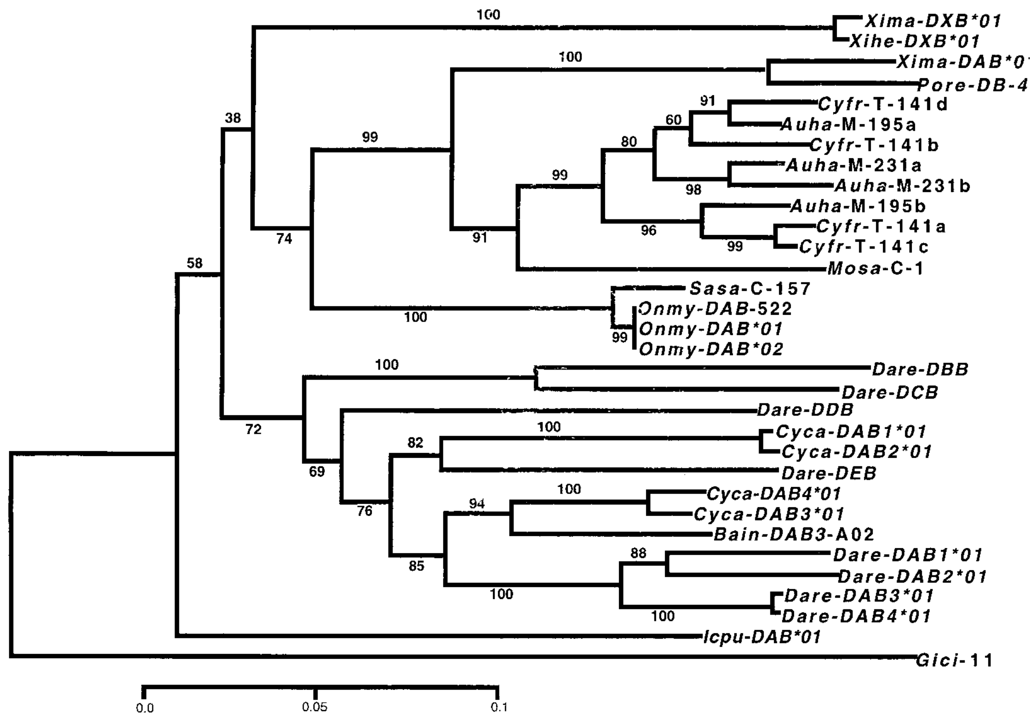

Figure 5.—A phylogenetic tree based on the genetic distances between exon 3 nucleotide sequences from different teleosts. The tree was constructed as described under materials and methods. Numbers on nodes indicate frequency with which this node was recovered per 100 bootstrap replications in a total of 500 replications. References correspond with those given in Figure 2, with additional references: *Auha*-M-195a, *Auha*-M-195b, *Auha*-M-231a, *Auha*-M-231b, *Cyfr*-T-141a, *Cyfr*-T-141b, *Cyfr*-T-141c, *Cyfr*-T-141d (Ono *et al.* 1993); *Onmy-DAB*02*, *Onmy-DAB*-522 (Glamann 1995); *Dare-DAB1*01*, *Dare-DAB2*01*, *Dare-DAB3*01* (Ono *et al.* 1992); *Dare-DBB*, *Dare-DCB*, *Dare-DDB*, *Dare-DEB* (Sültmann *et al.* 1994); *Cyca-DAB1*01*, *Cyca-DAB2*01*, *Cyca-DAB3*01* (Van Erp *et al.* 1996); *Bain-DAB3*-A02 (Dixon *et al.* 1996); and *Gici*-11 (Bartl and Weissman 1994).

bootstrap value of 38. In fact, this placement of the Xiphophorus *DXB* genes is the only grossly inconsistent feature of this dendrogram, which otherwise fits with presumed phylogenetic relationships based on a cladistic analysis of morphological characters (Nelson 1994). The basal placement of *DXB* on the teleost dendrogram implies a very early duplication event with much subsequent independent evolution of the *DXB* and *DAB* genes. This timing is consistent with intron-exon structure that we discussed earlier. Our findings raise an important caution for investigators using MHC genes to make phylogenetic inferences: proper assignment of alleles to the correct homologous gene will be critical when tracing MHC evolution. Future experiments will test for the presence *DXB* (and *DAB*, if necessary) in other species of fish to determine the evolutionary history of these two loci relative to one another and relative to MHC class II *B* loci in tetrapods. The "*X*" family designation of the *DXB* locus will need to be reassigned as more is learned about the evolutionary relationship of this gene to other class II *B* genes in other species. Also, the possibility of different functions for these two loci will be investigated. The levels of polymorphism of *DXB* versus *DAB*, tissue expression patterns, and cellular trafficking patterns will lead to a more detailed understanding of the function of the products of the *DXB* locus.

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