

## ORIGINAL PAPER

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## Trans-species polymorphism of class II *Mhc* loci in danio fishes

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**Abstract** A characteristic feature of the major histocompatibility complex (*Mhc*) polymorphism in mammals is the existence of allelic lineages shared by related species. This trans-species polymorphism has thus far been documented only in primates, rodents, and artiodactyls. In this communication we provide evidence that it also exists in cyprinid (bony) fishes at the class II *A* and *B* loci coding for the  $\alpha$  and  $\beta$  polypeptide chains of the class II  $\alpha$ : $\beta$  heterodimers. The study has focused on three species of the family Cyprinidae, subfamily Rasborinae: the zebrafish (*Danio rerio*), the giant danio (*D. malabaricus*), and the pearl danio (*D. albolineatus*). The polymerase chain reaction was used to amplify and then sequence intron 1 and exon 2 of the class II *B* loci and exon 2 of the class II *A* loci in these species. Phylogenetic analysis of the sequences revealed the existence of allelic lineages whose divergence predates the divergence of the three species at both the *A* and *B* loci. The lineages at the *B* locus in particular are separated by large genetic distances. The polymorphism is concentrated in the peptide-binding region sites and is apparently maintained by balancing selection. Sharing of this unique *Mhc* feature by both bony fishes and mammals suggests that the main function of the *Mhc* (presentation of peptides to T lymphocytes) has not changed during the last 400 million years of its evolution.

### Introduction

The feature that sets the major histocompatibility complex (*Mhc*) loci apart from all other known loci of the animal

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kingdom is their polymorphism, specifically the large genetic distances between some of the alleles (Klein and Figueroa 1986). The acquisition of over 30 nucleotide differences between alleles could theoretically be explained either by very fast substitution (mutation) rate or long persistence time of allelic lineages (J. Klein et al. 1993). The available evidence fails to support the former but strongly supports the latter explanation. The estimated average nonsynonymous substitution rate for the peptide-binding region (PBR) of the *Mhc* loci is  $5.9 \times 10^{-9}$  substitutions per nonsynonymous site per year (Satta et al. 1991). The average synonymous substitution rate of the *Mhc* class II loci is  $1.18 \times 10^{-9}$  substitutions per synonymous site per year. Both estimates are within the range of those reported for non-*Mhc* loci (J. Klein et al. 1993). The alternative explanation requires allelic lineages to be much older than 2–4 million years (my), which is believed to be the average life span of a species (Stanley 1975); it requires the polymorphism to evolve trans-specifically (Klein 1980, 1987).

The concept of trans-species evolution of the *Mhc* polymorphism was first formulated to explain the occurrence of serologically indistinguishable allelic products (allomorphs) in geographically isolated populations and subspecies of the house mouse (Klein 1980). The first biochemical evidence for the concept was provided by Arden and Klein (1982) who demonstrated that serologically identical H2 molecules from *Mus musculus domesticus*, *M. m. molossinus*, and *M. m. castaneus*, three subspecies believed to have diverged more than 1 my ago, were also indistinguishable by tryptic peptide mapping. Clear evidence for the existence of *Mhc* class II allelic lineages which predated the divergence of mice and rats dated to more than 10 my ago was obtained by Figueroa and Klein (1987) and by Figueroa and co-workers (1988) from a combination of serological and molecular data. Molecular evidence for the allelic lineages predating the divergence of different species of *Mus* was also obtained by Wakeland and co-workers (1987) and McConnell and co-workers (1988). Shortly afterwards, nucleotide sequence analysis demonstrated striking similarities between alleles in differ-

ent species of primates at both class I (Mayer et al. 1988; Lawlor et al. 1988, and others) and class II (Fan et al. 1989; Gyllensten and Erlich 1989; Gyllensten et al. 1991; Kenter et al. 1992; Mayer et al. 1992; Kupfermann et al. 1992; Slierendregt et al. 1992, and others) loci. These studies have led to the conclusion that in primates, allelic lineages at some of the polymorphic loci are probably more than 20 my old (Klein et al. 1990b; Klein and Takahata 1990).

In mammals, trans-species persistence of allelic lineages at the *Mhc* loci is therefore well documented. But for other vertebrate classes the data on trans-species polymorphism are either fragmentary or not available. Only in the cichlid fishes of the Great Lakes in East Africa (D. Klein et al. 1993; Ono et al. 1993a; E. Malaga and J. Klein, unpublished data) and in the Darwin's finches on the Galápagos Islands (V. Vincek, Y. Satta, C. O'hUigin, P. T. Boag, P. R. Grant, B. R. Grant, and J. Klein, unpublished data) is there clear evidence for the interspecific sharing of *Mhc* alleles. But these species are of recent origin and they therefore do not provide any information about long-term persistence of allelic lineages. In an attempt to obtain such information, we decided to compare the polymorphism of the zebrafish (*Danio rerio*) with that of two related fish species, pearl danio (*D. albolineatus*) and giant danio (*D. malabaricus*).

The three species belong to the order Cypriniformes (carp-like fishes), family Cyprinidae and subfamily Rasborinae, which is distributed over most of India, Pakistan, Bangladesh, Sri Lanka, Thailand, the Malay Peninsula, Sumatra, and the Yunnan Province of China (Nelson 1984). The taxonomy of the family has been revised several times. Early on, *D. rerio* was assigned to a separate genus and referred to as *Brachydanio rerio* (Hamilton-Buchanan 1822). Later, the genus *Brachydanio* was fused with the genus *Danio* and the species *D. malabaricus* rendered synonymous with *D. aequipinnatus* (Barman 1991). These fusions are supported by molecular data obtained in a study of mitochondrial (mt) DNA (Meyer et al. 1993, 1995) and of the *Mhc* (this communication). The designation *Danio rerio* for the zebrafish is now used almost uniformly by developmental biologists. Hence to avoid further confusion, here we introduce the designation *Dare* for the *Mhc* of the zebrafish to replace the former designation *Brre* (Ono et al. 1992, 1993a, b; Sülmann et al. 1993, 1994a, b). We retain, however, the name *D. malabaricus* for the giant danio because most recent studies (Meyer et al. 1995) suggest that it might be a distinct species from *D. aequipinnatus* after all.

In our earlier studies (Ono et al. 1992; Sülmann et al. 1993, 1994a, b), we demonstrated the existence of four class II A and six class II B loci in the zebrafish. Of the four class II A loci, *Dare-DAA* is linked to *Dare-DAB*, *Dare-DCA* is linked to *Dare-DCB*, and *Dare-DEA* is linked to *Dare-DEB* (Sülmann et al. 1993, 1994a, b; J. Bingulac-Popovic, F. Figueroa, J. Postlethwait, and J. Klein, unpublished data); the location of the *Dare-DBA* locus is not known. Three of the class II B loci (*Dare-DBB*, *-DCB*, and *-DEB*) seem to be present only in some stocks or subspecies of zebrafish (they were originally detected in a genomic library provided by a commercial supplier and the origin of

the stock used could no longer be established); they are absent from the stocks maintained in our aquarium. Of the three remaining loci, *DAB* and *DBB* are closely linked, whereas *DFB* is located on a chromosome different from that of these two loci (J. Bingulac-Popovic, F. Figueroa, J. Postlethwait, and J. Klein, unpublished data). Only the *DAB* locus has thus far been found to be highly polymorphic and active (represented in a cDNA library). Four allelic lineages have been identified at this locus, which will be referred to here as *Dare-DAB1\*01* through *-DAB1\*04*. In the present study, we have focused on the *DAA*, *DBA*, and *DAB* loci.

## Materials and methods

### Source and isolation of genomic DNA

Specimens of *D. albolineatus* and *D. malabaricus* were obtained from three sources: local dealers in Miami and in Tübingen (Aquarium Rio, Egelsbach, Germany), as well as from the wild. Genomic DNA was isolated as described previously (Ono et al. 1993b with modifications according to Laird et al. 1991).

### Polymerase chain reaction (PCR)

The class II B intron 1/exon 2 region was amplified using primers based on previously reported *D. rerio* sequences; the primers were provided by F. Figueroa of the Max-Planck-Institut für Biologie, Tübingen. The forward (sense) primer Tu385 (5'-TGCTGTGCA-(A/G)CATTACTGGAAC-3') corresponded to the 3' region of exon 1; the reverse (anti-sense) primer Tu360 (5'-TCGTTTAT-CACG(G/T)ACAGCTGA-3') corresponded to the 3' region of exon 2. PCR was performed in 50 µl of reaction mixture containing 2 mM MgCl<sub>2</sub>, 200 µM dNTP, and 2.5 units *Taq* polymerase (Perkin Elmer, Norwalk, CT). The Tu385/Tu360 primer pair was used initially at an annealing temperature of 55 °C or at 53 °C in some amplifications. Thermocycler conditions were as follows: an initial denaturation for 2 min at 94 °C, then at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min, 40 cycles. The final extension at 72 °C was for 10 min. Exon 2 of class II A genes was amplified using the primer pair A20290 and A20291 based on known *D. rerio* cDNA and genomic sequences (Sülmann et al. 1993). A20291 is a forward (sense) primer (5'-G(C/T)T(A/C)A(A/T)G(C/T)TG-(A/T)(A/G)CA(C/T)(A/G)(A/G)GGAT-3') corresponding to the 5' end of exon 2; A20290 is a reverse (anti-sense) primer (5'-TT(T/G)-CTCT(A/T)(A/C)(A/T)GG(A/T)GA(G/T)TTGTA(G/T)GCCTT-3'). PCR was performed using the Opti-prime kit (Stratagene, La Jolla, CA) to optimize buffer conditions. The reactions were as above with a 10 × buffer consisting of 3.5 mM MgCl<sub>2</sub>, 10 mM Tris-HCl, and 75 mM KCl, pH 8.8. The cycling parameters were 94 °C for 3 min, 50 °C for 2 min, and 72 °C for 1.5 min in the first cycle; then 30 cycles for 1 min at 50 °C and at 72 °C for 1.5 min, followed by a final extension at 72 °C for 10 min.

### Subcloning and sequencing

The PCR products were resolved by electrophoresis on 1% agarose gels and the fragments of interest excised. The agarose blocks were minced and centrifuged through silanized glass wool columns at 6500 rpm for 20–25 min. The isolated DNA was subcloned into a *Sma* I-digested pUC19 vector using the SureClone ligation kit (Pharmacia, Piscataway, NJ). After blunt-end ligation, the reactions were transformed into XL1-Blue MRF' bacteria by standard methods and plated on LB agar containing ampicillin (50 µg/mL), X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and IPTG (isopropyl-β-D-thiogalactopyranoside). Positive (white) colonies were

	1	11	21	31	41	51	61	71	81	91
CONSENSUS ==>	GGCAAGTAA	GCAAAATACT	GAGCAAATAT	ATATAACACC	TATAATTTAT	TAACAATTAT	TTGCTTTATG	TTTTATATGT	ATTCACTGCT	GTTAATCCAA
Dare-DAB1*0203	T	*	G**	**	TAT	TG	A	***	***	***
Dare-DAB1*0205	T	*	**	**	TAT	TG	A	***TC	***	***
Dare-DAB1*0202	T	*	G**	**	TAT	TG	A	***	***	***
Dare-DAB1*0204	T	*	**	**	TAT	TG	A	***	***	***
Daal-DAB1*0201	A	*	**	G*	A	TG	A	***	***	***
Daal-DAB1*0202	A	*	**	G*	A	TG	A	***	***	***
Daal-DAB1*0203	A	*	**	G*	A	TG	A	***	***	***
Daal-DAB1*0204	A	*	**	G*	A	TG	A	***	***	***
Dare-DAB1*0302	A	*	A	A**	C*	TTG	AGC	***	***	***
Dare-DAB1*0303	T	*	A	A**	C*	TTG	AGC	***	***	***
Dare-DAB1*0304	T	*	A	A**	C*	TTG	AGC	***	***	***
Dare-DAB1*0403	*	*	*	*	G	C	TG	A	***	***
Dare-DAB1*0404	*	*	*	*	G	C	TG	A	***	***
Daal-DAB1*0401	*	*	A**	**	G	T	TG	A	***	***
Daal-DAB1*0402	*	*	A**	**	G	T	TG	A	***	***
Daal-DAB1*0403	*	*	A	A**	**	G	T	TG	A	***
Daal-DAB1*0404	*	*	A	A**	**	G	T	TG	A	***
Daal-DAB1*0405	*	*	A	A**	**	G	T	TG	A	***
Daal-DAB1*0406	*	*	A	A**	**	G	T	TG	A	***
Dama-DAB*0101	C	*	A	G	AA	C	AC	C	*	T
Dama-DAB*0102	C	*	A	G	AA	C	AC	C	*	T
Dama-DAB*0103	C	*	A	G	AA	C	AC	C	*	T
Dama-DAB*0104	C	*	A	G	AA	C	AC	C	*	T
Dama-DAB*0105	C	*	A	G	AA	C	AC	C	*	T
Dama-DAB*0106	C	*	A	G	AA	C	AC	C	*	T
Dama-DAB*0107	C	*	A	T	G	AA	C	AC	C	*
Dama-DAB*0108	C	*	A	G	AA	C	AC	C	*	T
Dama-DAB*0109	C	*	A	G	AA	C	AC	C	*	T
Dama-DAB*0110	C	*	A	T	GCA	A	C	***	G	*
Dama-DAB*0111	C	*	A	T	GCA	A	C	***	G	*
Daal-DAB*0201	G	A	**	A	ACC	GT	AT	T**	ACA	*T
Daal-DAB*0202	G	A	**	A	ACC	GT	AT	T**	ACA	*T
Daal-DAB*0203	G	A	**	A	ACC	GT	AT	T**	ACA	*T
Daal-DAB*0204	G	A	**	A	ACC	GT	AT	T**	ACA	*T
Daal-DAB*0205	G	A	**	A	ACC	GT	AT	T**	ACA	*T
Daal-DAB*0206	G	A	**	A	ACC	GT	AT	T**	ACA	*T
Daal-DAB*0207	G	A	**	A	ACC	GT	AT	T**	ACA	*T
Dama-DAB*0301	A	*	A	A	GCA	*	C	*	C	A
Dama-DAB*0302	A	*	A	A	GCA	*	C	*	C	A
Dama-DAB*0303	A	*	A	CT	GCA	*	*	*****	C	G
Dama-DAB*0304	A	*	A	CT	GCA	*	*	*****	C	G
Dama-DAB*0305	A	*	A	T	GCA	*	*	*****	C	G
Dama-DAB*0306	A	*	A	T	GCA	*	*	*****	C	G
Dama-DAB*0307	A	*	A	T	GCA	*	*	*****	C	G
Dama-DAB*0308	A	*	A	T	GCA	*	*	*****	C	G
Dare-DAB1*0101	T	*	T	G	A	*****	G	C	***	TG

	101	111	121	131	141	151	161	171	181	191
CONSENSUS ==>	AAGCTGGTTT	GCTTTTGACT	AAATATTAAT	ATTCTATGAA	AGTAATATGA	GCAGGTAATCA	GCTAATAGAA	GCACAGTACT	ACAGAGAAGC	TACTACCAA
Dare-DAB1*0203	TAT	TA	*	A	GG	*	T	C	G	T
Dare-DAB1*0205	TAT	TA	*	A	GG	*	T	C	G	T
Dare-DAB1*0202	TAT	TA	*	A	GG	*	T	C	G	T
Dare-DAB1*0204	TAT	TA	*	A	GG	*	T	C	G	T
Daal-DAB1*0201	**	**	**	**	**	**	**	**	**	**
Daal-DAB1*0202	**	**	**	**	**	**	**	**	**	**
Daal-DAB1*0203	**	**	**	**	**	**	**	**	**	**
Daal-DAB1*0204	**	**	**	**	**	**	**	**	**	**
Dare-DAB1*0302	A	*	A	G	G	*	T	G	A	C
Dare-DAB1*0303	A	*	A	G	G	*	T	G	A	C
Dare-DAB1*0304	A	*	A	G	G	*	T	G	A	C
Dare-DAB1*0403	*****	*****	TA	AG	*	T	G	A	C	T
Dare-DAB1*0404	*****	*****	TA	AG	*	T	G	A	C	T
Daal-DAB1*0401	*****	*****	TA	AG	*	T	G	A	C	T
Daal-DAB1*0402	*****	*****	TA	AG	*	T	G	A	C	T
Daal-DAB1*0403	*****	*****	TA	AG	*	T	G	A	C	T
Daal-DAB1*0404	*****	*****	TA	AG	*	T	G	A	C	T
Daal-DAB1*0405	*****	*****	TA	AG	*	T	G	A	C	T
Daal-DAB1*0406	*****	*****	TA	AG	*	T	G	A	C	T
Dama-DAB*0101	*****	*****	*	A	**	A	G	A	*	*
Dama-DAB*0102	*****	*****	*	A	**	A	G	A	*	*
Dama-DAB*0103	*****	*****	*	A	**	A	G	A	*	*
Dama-DAB*0104	*****	*****	*	A	**	A	G	A	*	*
Dama-DAB*0105	*****	*****	*	A	**	A	G	A	*	*
Dama-DAB*0106	*****	*****	*	A	**	A	G	A	*	*
Dama-DAB*0107	*****	*****	*	A	**	A	G	A	*	*
Dama-DAB*0108	*****	*****	*	A	**	A	G	A	*	*
Dama-DAB*0109	*****	*****	*	A	**	A	G	A	*	*
Dama-DAB*0110	C	*****	*****	C	*	G	A	**	A	G
Dama-DAB*0111	C	*****	*****	C	*	G	A	**	A	G
Daal-DAB*0201	***	A	*C	G	TA	TA	G	T	AT	A
Daal-DAB*0202	***	A	*CC	G	TA	TA	G	T	AT	A
Daal-DAB*0203	***	A	*C	G	TA	TA	G	T	AT	A
Daal-DAB*0204	***	A	*C	G	TA	TA	G	T	AT	A
Daal-DAB*0205	***	A	*C	G	TA	TA	G	T	AT	A
Daal-DAB*0206	***	A	*C	G	TA	TA	G	T	AT	A
Daal-DAB*0207	***	A	*C	G	TA	TA	G	T	AT	A
Dama-DAB*0301	***	C	*	C	*	*	*	T	*	T
Dama-DAB*0302	***	C	*	C	*	*	*	T	*	T
Dama-DAB*0303	***	C	*	C	*	*	*	T	*	T
Dama-DAB*0304	***	C	*	C	*	*	*	T	*	T
Dama-DAB*0305	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
Dama-DAB*0306	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
Dama-DAB*0307	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
Dama-DAB*0308	*****	*****	*****	*****	*****	*****	*****	*****	*****	*****
Dare-DAB1*0101	**	---	T	*	A	---	G	A	---	TG

Fig. 1 (For continuation and legend see p. 39)

	201	211	221	231	241	251	261	271	281	291
CONSENSUS ==>	ATAATAGTAG	AAATGAAGAT	GAATACAGAT	ATATTTTTTA	TGACTGATAT	GAAATTATAC	AACAATCTGA	AGCTTCTAAT	GCTATGTTTT	TATGTTTCA
Dare-DAB1*0203	***-----*	-----*	-----*	*-C-----	-----*	T-----	-----*	---G-----	*-C-A-----	C*-T---T-
Dare-DAB1*0205	***-----*	-----*	-----*	*-C-----	-----*	T-----	-----*	---G-----	*-C-A-----	C*-T---T-
Dare-DAB1*0202	***-----*	-----*	-----*	*-C-----	-----*	T-----	-----*	---G-----	*-C-A-----	C*-T---T-
Dare-DAB1*0204	***-----*	-----*	-----*	*-C-----	-----*	T-----	-----*	---G-----	*-C-A-----	C*-T---T-
Daal-DAB1*0201	-----*	-----T--	-----G	-----*	-----A-C	-----TG-	-----*	---G-----	*-C-A-----	C*-T---T-
Daal-DAB1*0202	-----*	-----T--	-----G	-----*	-----A-C	-----TG-	-----*	---G-----	*-C-A-----	C*-T---T-
Daal-DAB1*0203	-----*	G-----T--	-----G	-----*	-----A-C	-----TG-	-----*	---G-----	*-C-A-----	C*-T---T-
Daal-DAB1*0204	-----*	-----T--	-----G	-----*	-----A-C	-----TG-	-----*	---G-----	*-C-A-----	C*-T---T-
Dare-DAB1*0302	---A-----	-----C	---A-----	-----*	-----*	-----T--	-----*	---G-----	*-T-A-----	C*-T---T-
Dare-DAB1*0303	---A-----	---A---C	---A-----	-----*	-----*	-----T--	-----*	---G-----	*-T-A-----	C*-T---T-
Dare-DAB1*0304	---A-----	-----C	---A-----	-----*	-----*	-----T--	-----*	---G-----	*-T-A-----	C*-T---T-
Dare-DAB1*0403	-----G---	-----*	-----*	-----*	-----TC-	---T---TG-	---*---CG	---*---G---	*-C-A-----	C*-T---T-
Dare-DAB1*0404	-----G---	-----*	-----*	-----*	-----TC-	---T---TG-	---*---CG	---*---G---	*-C-A-----	C*-T---T-
Daal-DAB1*0401	-----*	-----*	-----*	T-T-A---	-----C-	---T---TG-	---*---G---	---*---G---	*-C-A-----	C*-T---T-
Daal-DAB1*0402	-----*	-----*	-----*	T-T-A---	-----C-	---T---TG-	---*---G---	---*---G---	*-C-A-----	C*-T---T-
Daal-DAB1*0403	-----*	-----*	-----*	T-T-A---	-----C-	---T---TG-	---*---G---	---*---G---	*-C-A-----	C*-T---T-
Daal-DAB1*0404	-----*	-----*	-----*	T-T-A---	-----C-	---T---TG-	---*---G---	---*---G---	*-C-A-----	C*-T---T-
Daal-DAB1*0405	-----*	-----*	-----*	T-T-A---	-----C-	---T---TG-	---*---G---	---*---G---	*-C-A-----	C*-T---T-
Daal-DAB1*0406	-----*	-----*	-----*	T-T-A---	-----C-	---T---TG-	---*---G---	---*---G---	*-C-A-----	C*-T---T-
Dama-DAB*0101	-----*	*-T-A-T--	-----*	*-C-A---	-----G	---A---	-----*	-----*	---T---	---*
Dama-DAB*0102	-----*	*-T-A-T--	-----*	*-C-A---	-----G	---A---	-----*	-----*	---T---	---*
Dama-DAB*0103	-----*	*-T-A-T--	-----*	*-C-A---	-----G	---A---	-----*	-----*	---T---	---*
Dama-DAB*0104	-----*	*-T-A-T--	-----*	*-C-A---	-----G	---A---	-----*	-----*	---T---	---*
Dama-DAB*0105	-----*	*-T-A-T--	-----*	*-C-A---	---G---G-	---A---	-----*	-----*	---T---	---*
Dama-DAB*0106	-----*	*-T-A-T--	-----*	*-C-A---	---G---G-	---A---	-----*	-----*	---T---	---*
Dama-DAB*0107	-----*	*-T-A-T--	-----*	*-C-A---	---G---G-	---A---	-----*	-----*	---T---	---*
Dama-DAB*0108	-----*	*-T-A-T--	-----*	*-C-A---	---G---G-	---A---	-----*	-----*	---T---	---G-
Dama-DAB*0109	-----*	*-T-A-T--	-----*	*-C-A---	---G---G-	---A---	-----*	-----*	---T---	---*
Dama-DAB*0110	-----*	*-T-A-T--	-----*	*-C-A---	---G---G-	---A---	-----*	-----*	---T---	---*
Dama-DAB*0111	-----*	*-T-A-T--	-----*	*-C-A---	---G---G-	---A---	-----*	-----*	---T---	---*
Daal-DAB*0201	---C---A	*-T---T--	---A---	---C---*	---C---	---G---	---*---	---A*---C---	---C---	**---C---
Daal-DAB*0202	---C---A	*-T---T--	---A---	---C---*	---C---	---G---	---*---	---A*---C---	---C---	**---C---
Daal-DAB*0203	---C---A	*-T---T--	---A---	---C---*	---C---	---G---	---*---	---A*---C---	---C---	**---C---
Daal-DAB*0204	---C---A	*-T---T--	---A---	---C---*	---C---	---G---	---*---	---A*---C---	---C---	**---C---
Daal-DAB*0205	---C---A	*-T---T--	---A---	---C---*	---C---	---G---	---*---	---A*---C---	---C---	**---C---
Daal-DAB*0206	---C---A	*-T---T--	---G---A-	---C---*	---C---	---G---	---*---	---A*---C---	---C---	**---C---
Daal-DAB*0207	---C---A	*-T---T--	---A---	---C---*	---C---	---G---	---*---	---A*---C---	---C---	**---C---
Dama-DAB*0301	T-G-C---A	*-T---CA-	A-----	T-*G---	G-----G-	A---GCA---	-----*	-----*	-----*	---*---A---
Dama-DAB*0302	T-G-C---A	*-T---CA-	A-----	T-*G---	G-----G-	A---CA---	-----*	-----*	-----*	---*---A---
Dama-DAB*0303	---G---C---	*-TA---CA-	-----*	T-*G---	---G---G-	A---CA---	-----*	-----*	---G---	---*---A---
Dama-DAB*0304	---G---C---	*-TA---CA-	-----*	T-*G---	---G---G-	A---CA---	-----*	-----*	---G---	---*---A---
Dama-DAB*0305	-----*	-----*	-----*	T-*C---	-----G-	---G---G-	---A---	---A*---C---	C-----	---*
Dama-DAB*0306	-----*	-----*	-----*	T-*C---	-----G-	---A---G-	---A---	---A*---C---	C-----	---*
Dama-DAB*0307	-----*	-----*	-----*	T-*C---	-----G-	---A---G-	---A---	---A*---C---	C-----	---*
Dama-DAB*0308	-----*	-----*	-----*	T-*CC---	-----G-	---A---G-	---A---	---A*---C---	C-----	---*
Dare-DAB1*0101	*****	**T---T---	*-T---T---	---T---T---	T-AA---C-	---G---C---	---TT-T---	***-----	*T-TC---	---T-T---T-

picked and grown overnight in LB<sup>-</sup> ampicillin broth and minipreps were performed according to standard protocol (Maniatis et al. 1991). The insert size was then checked by double digestion with *Eco* RI and *Hin* dIII. The inserts were sequenced using the dideoxy nucleotide chain termination method (Sanger et al. 1977) and the Sequenase v2.0 kit (Amersham Buchler, Braunschweig, Germany). Sequencing reactions were electrophoresed on 5% polyacrylamide gels made from 50% Long Ranger gel concentrate (AT Biochem, Malvern, PA) for 4 h and 2 h. Gels were then dried and exposed to X-ray film.

Construction of dendrograms

The nucleotide sequences and inferred protein sequences were aligned using the GCG computer program (Genetic Computer Group, Madison, WI) and the CLUSTAL V program (Higgins et al. 1992). Nucleic acid distances were measured using the two-parameter method (Kimura 1980) for noncoding regions. For coding regions, nonsynonymous substitutional differences were corrected using the Jukes and Cantor method of the MEGA package (Kumar et al. 1993). The evolutionary relationships were then evaluated by the neighbor-joining algorithm (Saitou and Nei 1987). Five-hundred bootstrap replications were performed to determine the reliability of the branching order.

Results and Discussion

Class II B polymorphism

Genomic DNA was isolated from 28 individuals of *D. albolineatus* and *D. malabaricus* (14 from each species), amplified by PCR using the primer pair Tu385/Tu360, and the amplification products were sequenced. Thirty-six un-

Fig. 1 Nucleotide sequences of intron 1 from *Mhc* class II *B* genes of *D. rerio* (*Dare*), *D. malabaricus* (*Dama*), and *D. albolineatus* (*Daal*). The *Dare* sequences are from Ono and co-workers (1992, 1993b). Dashes (-) indicate identity with the simple-majority consensus at the top; dots (.) unavailability of sequence information; and asterisks (\*) indels introduced to achieve optimal sequence alignment

ique sequences were obtained and their identity confirmed by sequencing multiple clones in both directions. The primers were such that they directed the amplification of intron 1 and exon 2 of the  $\beta$  chain-encoding (*B*) class II genes. The length of the amplified products (sequences) ranged from 466 to 520 base pairs (bp), the length variation being effected by the presence of insertions/deletions (indels) in intron 1 – different ones in the various sequence groups. The sequences are designated *Daal* for *Danio albolineatus* and *Dama* for *Danio malabaricus*, in accordance with the proposed nomenclature (Klein et al. 1990a). The *Daal* and *Dama* nucleotide sequences, together with the *Dare* (*Danio rerio*) sequences previously reported (Ono et al. 1992; Sultmann et al. 1994a, b) are given in Figures 1 (intron 1) and 2 (exon 2); the translated amino acid sequences of the exon 2-encoded  $\beta$ 1 domain are given in Figure 3. A phylogenetic tree of intron 1 sequences is shown in Figure 4. A tree of exon sequences from the various *Danio* species, together with homologous sequences of other bony fish, is presented in Figure 5.

The exon codons that specify the residues of the PBR are subject to balancing selection (Hughes and Nei 1988, 1989). These codons are major contributors to the observed

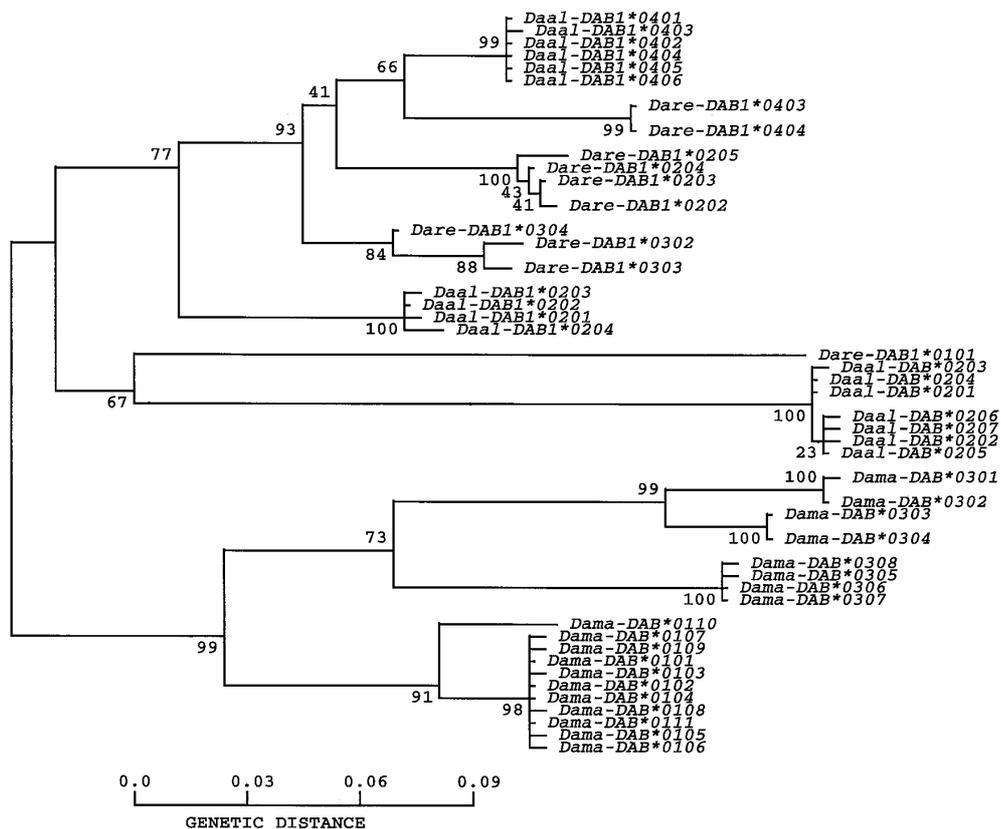


	3	13	23	33	43	53	63	73	83
CONSENSUS ==>	GADGYNYTK	AECVYSTSDY	SDMVFLDSYS	FNKVVDIQFN	STVGKFGVGYT	EQGVKFAENF	NKDPALQQE	KAQVDTFCRH	NAEISD
Dare-DAB1*0101-	-----Q--M	L--I-----	---L-E-G-	-----V-Y-	-----Y-	-----I--R--	--NQ-Y--R	---ES---	--Q---
Daal-DAB1*0201	-----D-VM	T--I-----	---E-Q-	-----V-Y-	-----S-	-----Q-I---	---Y---	---A-L-	
Dare-DAB1*0201	-----D---	Y--I-----	---Q---	---I--VKC-	-QEV-C---	-----Q-I---	--L--A---	---F-	
Dare-DAB1*0202	-----D---	F--I-----	F---Y-V-L-	---F--KC-	-EV-C---	-----Q-I---	--DL--S---	---F-	
Dare-DAB1*0203-	-----L-	G--I-----	---YIV-L-	---F--VKC-	-EV-C--F-	-----C---	--Q-V--NL	---S--L--N	
Dare-DAB1*0204	-----D---	V--I-----	---E-F-	---F--KC-	-QEV-C--L-	-----TD-L	--K-F--NL	---S--*	
Dare-DAB1*0205	-----H-M-	Y--I-----	---Y-K-F-	---F--VKC-	-EV-C--F-	K-----	--NQ-L--DL	---S---	
Dare-DAB1*0301	-----S---	---S---	---L-I---	-----T---	---S---	-----S---	--NQ-Y--L	---G---	--Q---
Dare-DAB1*0302	-----M-	Q--F--S---	---L-F---	---D--T---	---S---	-----D---	--NQ-Y--L	---G---	--Q-L-
Dare-DAB1*0303	-----CDMN	---F-----	---L-A---	---AL-T---	---S--A---	-----Q-Y--W	---A---	---Q-W-	
Dare-DAB1*0304	-----GN	Q--F--S---	---L---	---A---	---S---	-----NQ-Y--L	---GQ*--TN	---Q---	
Daal-DAB1*0401	-----D-M-	I-----	---Y-L---	-----S-E---	-----R-YV--Q	---S--I*---	---G---	---G---	
Daal-DAB1*0402	-----D-M-	I-----	---Y-L---	C-----	---S-E---	-----R-YV--Q	---S--I*---	---G---	
Daal-DAB1*0404	-----D-M-	I-----	N---Y-L---	-----S-E---	-----R-YV--Q	---S--I*---	---G---	---G---	
Daal-DAB1*0405	-----D-M-	I-----	---Y-L---	-----S-E---	-----R-YV--Q	---S--I*---	---G---	---G---	
Daal-DAB1*0406	---E--D-M-	I-----	---Y-L---	-----S-E---	-----R-YV--Q	---S--I*---	---G---	---G---	
Dare-DAB1*0403	-----D-I-	QQ-F-----	---Y-A---	-----T---	---S---	---LI---	--Q-Y-H-L	---Q---	---Q-W-
Dare-DAB1*0404	-----D---	N--F-----	---Y-L---	-----T---	---S---	---L---	--NQ-V--V	---N--R---	---Q---
Dama-DAB*0102	-----H-NIL	-A-I-NK---	---Y-V-L-	---LI---	-----N	-----I	--NK-FV--Q	---SL--H--A	---CRS-N
Dama-DAB*0104	-----H-NIL	-A-I-NK---	---Y-V-LF	---LI---	-----N	-----I	--NK-FV--Q	---SL--H--A	---CRS-
Dama-DAB*0107	-----H-NIL	-A-I-NK---	---Y-V-L-	---LI---	-----N	-----I	--NK-FV-RQ	---SL--H--A	---CRS-
Dama-DAB*0109	-----H-NIL	-A-I-NK---	---Y-V-L-	---LI---	---E--N	-----I	--NK-FV--Q	---SL--H--A	---CRS-
Dama-DAB*0110	.V--H-NIL	-A-I-N-	---Y-V-L-	---LI--**\	-----N	-----I	--NK-FV--Q	---SL--H--A	---CRS-
Dama-DAB*0111	-----H-NIL	-A-I-NK-\	---Y-V-L-	---LI---	-----N	-----I	--NK-FV--Q	---SL--H--A	---CRS-
Daal-DAB*0201	-----F--M	F-----G-	---A-F-	---QI-EL---	-----A-Q-H-	---Q-LI--T	---S-----	---IN--K---	
Daal-DAB*0202	-----F--M	F-----G-	---A-F-	---QI-EL---	-----A-Q-H-	---Q-LI--T	---S-----	---IN--K---	
Daal-DAB*0203	-----F--M	F-----G-	---A-F-	---QI-EL---	-----A-Q-H-	---Q-LI--T	---S-----	---IN--K---	
Daal-DAB*0204	-----F--M	F-----G-	---A-F-	---QI-EL---	-----C--A-Q-H-	---Q-LI--T	---S-----	---IN--K---	
Dama-DAB*0301	-----YHV	N--F-----	---Y-I-S-	---L-EY-Y-	---C--A-	---Q-IM--W	---A-----	---T-	
Dama-DAB*0302	-----YHV	N--F-----	---Y-I-S-	---L-EY-Y-	---NC--A-	---Q-IM--W	---A-----	---T-	
Dama-DAB*0303	-----Y-L	YD-F-----	---I-S-	---L-EY-Y-	---NC--A-	---Q-IM--W	---C-----	---W-	
Dama-DAB*0304	-----Y-L	YD-F-----	---I-S-	---L-EY-Y-	---NC--A-	---Q-IM--W	---Y-----	---W-	
Dama-DAB*0305	-----EE-M	S-----	---Y-I-L-	---HL---	---D--E-	K---Y--L	---QTL-E-L	---D--R---	---Q-L-
Dama-DAB*0307	-----EE-M	S-----	---Y-I-L-	---HL---	---D--E-	K---Y--L	---QTL-E-L	---D--R---	---Q-L-
Dama-DAB*0308	-----EE-M	S-----	---Y-I-L-	---HL---	---E--K---	---Y--L	---QTL-E-L	---D--R---	---Q-L-
Dare-DFB	-----SS-W	SQ-I--YP-F	R--E-IVG-Y	---WM---	-----Q-L--M	---S--SI---	---YE		
Cyca-DEB*01	A--N--HSW	TK-IH-SR-F	---Y-I-N-I	---D-Y---	---EY---	AL--YN--R	---NI---	R-Q-ERY-K	---LYQ
Cyca-DEB*02	A--N--RSR	NK-IH-SR-F	---V-N-I	---D-H---	---E--AL--HN--LW	---TTG---	R-Q--S--K	---Q-RQ	
Dare-DEB	-----HSRL	TK-IFQLQ-L	--IGVH-N-I	---D-Y-R-	--L-Y---	H--YN-QLW	KQRVQL-E-	R-HE-R--KY	---DY
Sasa-c144	-T---FYHVM	TQ-R--SK-L	QGIELT--V	---QAEN-R-	-----H--N--AW	---G*--AG	LGVLERY-KF	---P-DY	
Sasa-c157	-T---FEQVV	RQ-R--SK-L	QGIE-I--V	---AEY-R-	-----L--N--AW	---S-A-V-AV	RG-LERY-K	---DLHY	
Sasa-c22	-T---FEQVV	RQ-R--SK-L	QGIE-I--V	---AEYVR-	---Y--L--N--AW	---G*--AV	LG-LER-K	---DLHY	
Sasa-DB15	-----L	QGIE-I--V	---AEY-R-	---Y--L--N--AW	---G*--AG	LG-LERV-K	---P-DY		
Sasa-DB05	-----L	QGIE-I--V	---AEYVR-	---Y--L--N--AL	---G*--AV	LG-LERY-KL	TLLSTT		
Sasa-DB03	-----L	QGIE-IH--V	---QAEN-R-	---Y--L--N--AW	---G*--AG	LG-LER-K	---DLHY		
Sasa-DB06	-----L	QGIE-IH--V	---QAEN-R-	---Y--L--N--AW	---G*--AG	LG-LER-K	---DLHY		
Sasa-DB11	-----L	QGIE-I--V	---AEY-R-	---Y--L--N--AW	---G*--AV	LG-LER-K	---A-YY		
Sasa-DB10	-----L	QGIE-I--V	---AEYVR-	---Y--L--N--AW	---G*--AV	LG-LER-K	---A-YY		
Sasa-DB09	-----L	QGIE-IH--V	---QAEN-R-	---Y--L--N--AW	---G*--AV	LG-LER-K	---A-YY		
Sasa-DB08	-----L	QGIE-IH--V	---QAEN-R-	---Y--L--N--AW	---G*--AV	LG-LERY-K	---D-DY		
Sasa-DB12	-----L	QGIE-I--V	---AEY-R-	---Y--L--N--AW	---G*--ARA	LG-LERV-K	---P-YY		
Sasa-DB02	-----L	QGIE-I--V	---QAEN-R-	---Y--L--N--AL	---G*--AV	LG-LERY-KL	.....		
Sasa-DB14	-----L	QGIE-I--V	---AEYVR-	---Y--L--N--AW	---G*--AG	LG-LERV-K	---P-DY		
Sasa-DB13	-----L	QGIE-I--V	---AEYVR-	---Y--L--N--AW	---G*--ARA	LG-LERV-K	---P-YY		
Sasa-DB07	-----L	QGIE-IH--V	---QAEN-R-	---Y--L--N--AW	---G*--AV	LG-LER-K	---D-DY		
Mosa-C-1	.G--FL--AV	NR--FNST-P	KNIEYIY-HY	Y--LEIAR-S	---S-EY---	F--Q-KYW	---S--ARR	S-QKE-V-Q	---IN-DY
Mosa-C-2	---FL--AV	GR--FNST-P	KNIEYIY-EY	Y--LEIAR-S	---S-E---	Y-L-Q-KYW	---S--A-M	R-AKERYSQ	---IN-DY
Mosa-C-22	---FL--AV	GR--FNST-P	KNIEYIY-EY	Y--LEIAR-S	---S-E---	Y-L-Q-KYW	---S--A-M	R-AKERYSQ	---IN-DY
Mosa-R-41	---FRYFET	DR--FNST-L	K-IKYIR-EY	Y--LEIAR-S	---S-E---	L-LRW-KYW	---NN-SY-A-M	RG-KERY-Q	---IGNWY
Mosa-S-1	---FRYFET	DR--FNST-P	KNIEYIY-EY	Y--LEIAR-S	---SE-E---	L--N--R-	---SY-A-R	R--KERY-LT	---IN-DY
Mosa-S-2	---FLS-DI	NR--FNST-P	KNIEYIY-EY	Y--LEIAR-S	---SE-E---	L--N--R-	---TY-A-R	R--KERY-LT	---IN-DY
Pore-4-28	.FREFAVFAV	DR--F-SPEL	K-IQ-IR-C	Y--LEF-R-D	---NL--Y---	L--N--RW	---TSLIAM	---QRE-Y-LN	---VGNDY
Pore-18-1	.FRE-EV-EV	DR--F-SPEL	K-IEYIR-NY	Y--LEIAR-D	---SL-R---	F--Q-NYW	---TSFIAAL	N-QREAV-LT	TVG-DY
Pore-W3-2R	-----DR--FNSTEL	K-IQYIY-AF	Y--LEYAR-D	---NL--Y---	---F--Q-NYW	---SN-S-IARR	---QREGY-LN	HVTA..	
Pore-W1-1	-----DR--FNSTEL	K-IQ-IR-IY	Y--LEI-R-T	---SL-R---	---Y--MN--RW	---TSIIGAM	A-QREGY-MN	---IG--	
Pore-W2-2	-----TR--FNS-EL	K-IQ-IR-C	Y--LEF-R-D	---N---	---L--N--IW	---SN-SQIAS	R-LRE-Y-L	---VG--	
Pore-W4-1	-----SR--FNSTEL	K-IE-IR-NY	Y--LEIAR-D	---NL--Y---	---F--Q-NYW	---TSYSYAL	---QKEAV-LT	---VG--	
Dare-DBB	---H-H-GFVQ	FT-H*MLGSL	QNVEVYIY	-DTTELLR-	---EN-A-A-	---YAM-W-NDL	---Q-KW-HEQ	VEKNIAD-KL	FG-TYF
Dare-DCB	.VC-N-G-LQ	SQ-R*VL-ST	KKVELIF-FI	---IEY-RY-	---DQ-I---	---F-E--V--Y	*--NNTFVLVL	AEFGIYN-KK	I-KALI

Fig. 2 Nucleotide sequences of exon 2 from *Mhc* class II *B* genes of *D. rerio* (*Dare*), *D. malabaricus* (*Dama*), and *D. albolineatus* (*Daal*). The numbering follows Ono and co-workers (1992). For symbol explanations, see Figure 1

Fig. 3 Amino acid sequences translated from nucleotide sequences in Figure 2. A *backslash* (\) indicates a frameshift mutation. Symbols are explained in Figure 1. The amino acid residues are given in the international *single-letter code*. *Cyca* is the carp (Ono et al. 1993 c), *Mosa* the striped bass (Walker and McConnell 1994), *Sasa* the Atlantic salmon (Grimholt et al. 1994), and *Pore* the guppy (Sato et al. 1996)

**Fig. 4** Phylogenetic tree based on genetic distances between intron 1 nucleotide sequences in Figure 2. Genetic distances were determined from the number of substitutions between sequences (Kimura 1980) and the dendrogram was constructed using the neighbor-joining method of the MEGA package (Kumar et al. 1993). Numbers on branches are bootstrap values



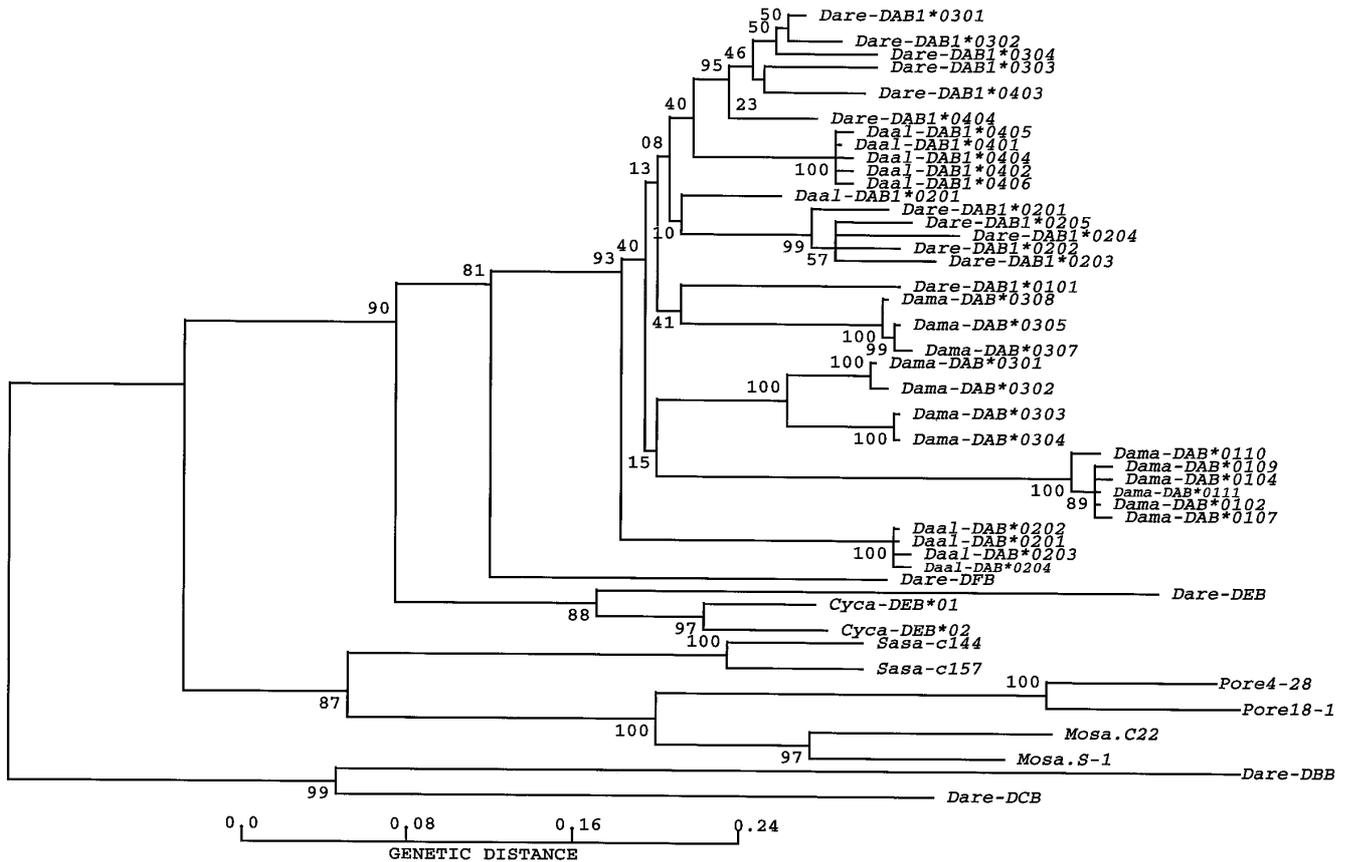
convergences between *Mhc* alleles in primates (O'hUigin 1995; Klein and O'hUigin 1995) and their influence tends to reduce apparent evolutionary distances between distant alleles which happen to share PBR motifs, an influence that is most obvious when short exons are used to measure distances. Although the PBR region is not yet defined in fish, the most polymorphic codons are implicated in its construction. We constructed trees in which all codons are included (not shown) and others in which the ten most polymorphic codons were removed. The trees resulting from the latter approach were more conservative with respect to the extent of transspecies polymorphisms and these were therefore used in this manuscript. The most polymorphic codons assessed by the method of Wu and Kabat (1970) for the  $\alpha$  chains are those at positions 12, 14, 25, 32, 53, 55, 58, 61, 62, and 68. For the  $\beta$  chains, codons 9, 11, 13, 29, 31, 39, 68, 72, 75, and 85 are excluded.

In an earlier study (Ono et al. 1992), the *Dare-DAB* sequences could be divided into clusters, originally designated *DAB1* through *DAB4*. At that stage it could not be decided whether the four clusters constituted allelic lineages at a single locus or alleles at separate loci. More recent results from linkage and segregation analysis in haploids (J. Binguac-Popovic, F. Figueroa, and J. Klein, unpublished data) indicate that the sequences represent alleles at a single locus which we designate *Dare-DAB1*, the lineages being *DAB1\*01* through *DAB1\*04*.

In Figures 4 and 5, the cluster of alleles in the *Dare-DAB1* lineages is interspersed with sequences from *Daal*

(and to a lesser degree with those of *Dama*). We conclude that these are allelic products of the *Daal-DAB1* locus (and possibly *Dama-DAB1*). A cluster of six *Daal-DAB1* alleles shows a higher degree of similarity to *Dare-DAB1\*04* than to other lineages. We designated these alleles *Daal-DAB1\*0401* through *Daal-DAB1\*0406*. A second group of *Daal* alleles clusters close to the *Dare-DAB1\*02* lineage and these are named *Daal-DAB1\*0201* through *Daal-DAB\*0204*. The *Dare-DAB1\*0101* allele is the most distinctive of the *Dare-DAB1* alleles, clustering at the base of the *DAB1* tree. Although several *Daal* (Fig. 4) and *Dama* (Fig. 5) alleles cluster with the *Dare-DAB\*0101* sequence, the clades are not robust and we prefer leaving the locus designation indeterminate in these cases. Among the remaining alleles we distinguish three groups corresponding to the *Daal-DAB\*02*, *Dama-DAB\*03*, and *Dama-DAB\*01* lineages/loci. The closer similarity of *Dare-* and *Daal-DAB1* alleles to each other rather than to *Dama* alleles supports the sister group relationship of *Dare* and *Daal* proposed by Meyer and co-workers (1993, 1995).

No *Dama* or *Daal* genes corresponding to the *Dare-DBB*, *-DCB*, *-DDB*, *-DEB*, and *-DFB* genes were found, possibly because the primers were originally chosen to amplify preferentially the *DAB* genes. While no exon 2 sequences were obtained for *DDB*, as seen in Figure 5, the other four genes are quite distinct from the *DAB* genes. On the other hand, *DAB* is apparently the main, if not the only, functional class II *B* family of *Danio rerio*. The facts that lead us to this conclusion are first, that *DAB* genes are



found in all individuals tested, while some of the other loci may be absent in some stocks or species (Sültmann et al. 1994a, b). Second, *DABI* is thus far the only *Dare* class II *B* locus for which transcripts (cDNA) could be found (Ono et al. 1992, Sültmann et al. 1994a, b). Third, the *Dare-DABI* gene does not contain any apparent defect which could interfere with its functionality (Ono et al. 1992). And fourth, the *DAB* locus is polymorphic in all three species tested, while the other loci are either mono- or oligomorphic (Ono et al. 1992; Sültmann et al. 1994a, b; and the present study).

The intermingling of *DABI* allelic lineages from different species in Figures 4 and 5 constitutes evidence of trans-species evolution of class II *B* polymorphism in cyprinid

**Table 1** The number of synonymous ( $K_s$ ) and nonsynonymous ( $K_a$ ) substitutions per site for PBR and non-PBR codons of all pairwise comparisons of 18 *Dare* and *Daal* *DABI* alleles\*

	PBR	Non-PBR
No. of codons	16	69
$K_a$	0.397	0.078
$K_s$	0.556	0.169

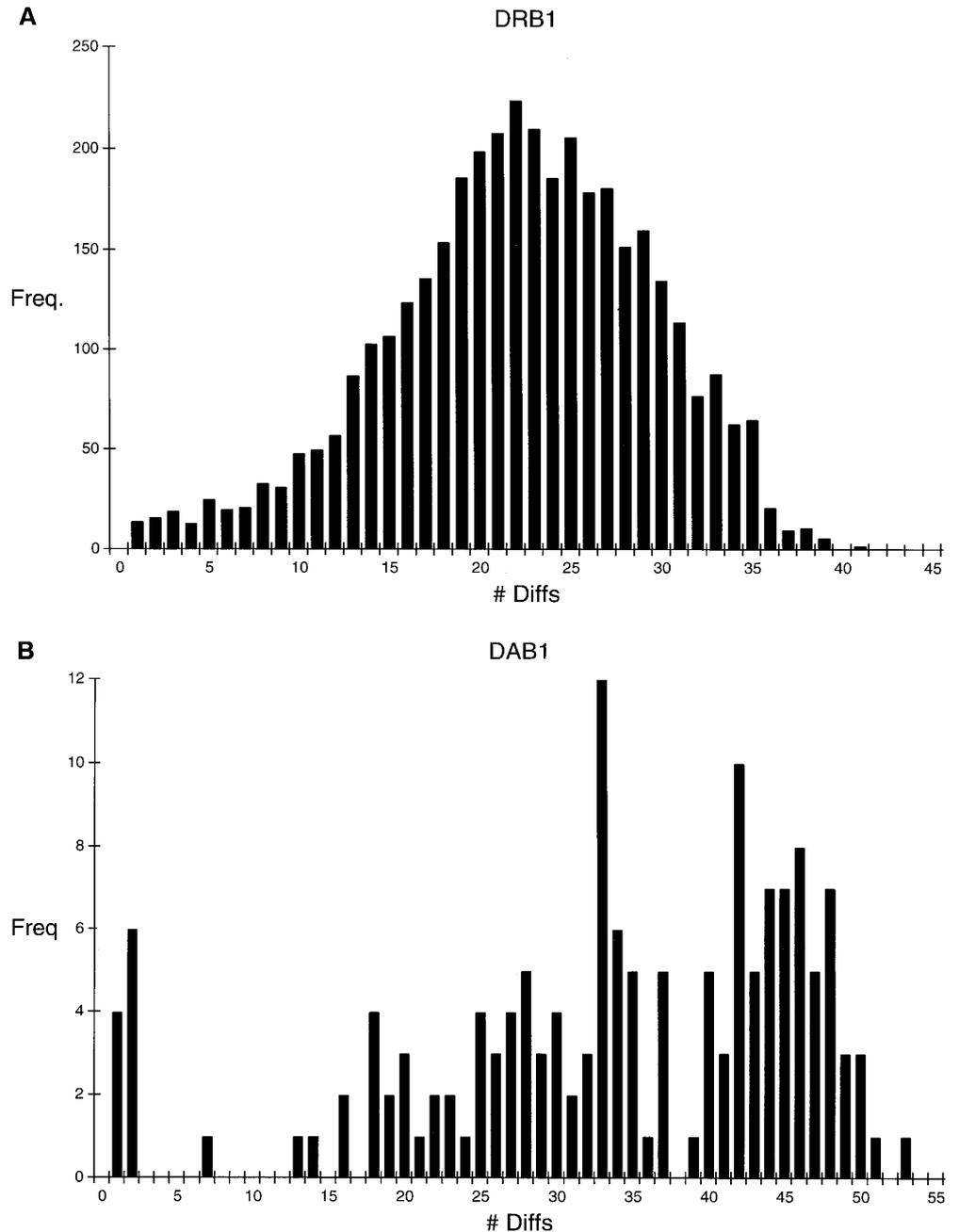
Average  $g = 1.64$

\* The location of the PBR sites in fish *Mhc* genes is uncertain. The Brown (1988) model of the PBR in mammals is preferred to the Brown (1993) model because it more fully predicts the most polymorphic codons in fish.  $\gamma$  is the ratio of PBR nonsynonymous substitution frequency (per site) to the overall synonymous substitution frequency.  $\gamma$  values greater than 1.0 are indicative of positive selection

**Fig. 5** Phylogenetic tree based on nonsynonymous genetic distances between sequences in Figure 3, as well as other bony fish exon 2 sequences. Genetic distances were determined from the number of nonsynonymous substitutions between sequences following exclusion of the ten most polymorphic residues. The dendrogram was constructed using the neighbor-joining method of the MEGA package (Kumar et al. 1993). Numbers on branches are bootstrap values. Species designations are given in Figures 2 and 3. *Mosa*, *Morone saxatilis* (striped bass; Hardee et al. 1995); *Sasa*, *salmo salar* (Atlantic Salmon; Grimholt et al. 1994)

fishes. Had the polymorphism not evolved trans-specifically, one would expect the *Dare*, *Daal*, and *Dama* sequences to form three separate clades. An alternative explanation – that the relatedness of allelic lineages in the different species is the result of convergent evolution (similar mutations being selected for independently in the three species) – is not supported by the data. This type of relationship could not have occurred at intron 1 sequences of the *DABI* locus, yet the sequences (Fig. 1) and the phylogenetic tree based on them (Fig. 4) reveal both similar clustering and similar affinities among the clusters at the exon 2 ( $\beta 1$  domain) sequence. The same is also true for the exon 2 synonymous sites (data not shown). Nonsynonymous PBR sites, the primary target of convergent evolution in the *Mhc*, are largely omitted in exon 2 trees by the removal of the ten most polymorphic sites. We conclude therefore that the trans-species mode of evolution of *Mhc* polymorphism is not limited to mammals, but occurs also in bony fishes.

**Fig. 6A, B** Pairwise comparison of differences between **A** the *Dare (Daal)*-*DAB1* exon 2 sequences in Figure 2 or **B** 87 primate *DRB1* exon 2 sequences (consisting of Old World monkey, ape, and human sequences). The number of combinations differing at a given number of nucleotides are plotted on the y and x axes, respectively



In mammals, *Mhc* polymorphism is maintained over long periods of time by positive (balancing) selection at the nonsynonymous sites specifying the PBR of the *Mhc* molecule. Evidence for the effect of balancing selection is provided by the observation that at the PBR sites (but not in the remainder of the gene), the rate of nonsynonymous substitutions ( $K_a$ ) significantly exceeds that of synonymous substitutions ( $K_s$ ; Hughes and Nei 1988, 1989). Table 1 indicates that at the *DAB1* locus, too, the  $K_a/K_s$  ratio is greater than 1, as would be expected if the locus were evolving under balancing selection.

The allelic lineages at the primate *DRB1* and certain other class II loci are characterized by the presence of

sequence motifs at the sites specifying the PBR (Klein and O'hUigin 1995; O'hUigin 1995). Sequence motifs are also present in the *Dare/Daal/Dama* lineages (Fig. 3) at positions presumably constituting the PBR. None of the fish motifs, however, resembles any of the primate class II motifs, suggesting that the fish and primate PBRs deal with different sets of peptides. (In primates, similar motifs apparently arise independently by convergent evolution in distantly related genes; see O'hUigin 1995 and Klein and O'hUigin 1995).

In comparison with primate class II *B* allelic lineages, the *Dare/Daal/Dama* lineages appear to encompass large sequence differences. In pairwise comparisons of all the





uous evidence of trans-species polymorphism. The *DAA* locus sequences fall into three lineages, here designated *DAA\*01* through *\*03*. Trans-species polymorphism is indicated by the intermingling of the lineages from the three species (i.e., the lack of segregation into three clusters according to the species) and the presence of sequences from different species in the same allelic lineage. Thus the *DAA\*02* lineage contains sequences from *D. malabaricus*, *D. albolineatus*, and *D. rerio*. The *DBA\*03* lineage contains sequences from both *D. malabaricus* and *D. albolineatus*. The interspecies genetic distances of the alleles in each of these two lineages are very small. In general, the branch lengths of the class II *A* sequences are shorter and the segregation into lineages less distinct than those of the class II *B* sequences (compare Figures 5 and 9). This observation may be an indication of a more conservative evolution of the class II *A* loci in comparison with the class II *B* loci. Such a tendency has also been noted for mammalian class II loci, its extreme example being provided by the *DR* gene family in which the *DRB* loci are highly polymorphic, while the *DRA* locus is virtually monomorphic (reviewed in Klein 1986). Either the *DAA*, the *DBA* or both (assuming they represent one locus) appear to be the main functional class II *A* loci of the *Danio* fish group (transcripts have thus far been found only for these genes; Sülthmann et al. 1993, 1994a, b). It may, therefore, be presumed that one or both of their products associate with those of the *DAB* locus to form functional  $\alpha:\beta$  heterodimers. Both the *DAA* and *DBA* loci, like *DAB*, are under positive selection as indicated by the relative prevalence of nonsynonymous over synonymous substitutions at the PBR sites, and the polymorphism is concentrated in the putative PBR (data not shown).

In conclusion, we find strong evidence for trans-species evolution of class II gene polymorphism at both the *A* and *B* loci in cyprinid fishes. The polymorphism is restricted to apparently functional loci; it is focused on the PBR sites and is apparently maintained by balancing selection. These observations suggest that the *Mhc* carries out the same function in bony fishes as it does in mammals and that this function relies on the complexing of peptides with the *Mhc* molecules.

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