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Polymorphism of major histocompatibility complex class II *B* genes in different lines of the common carp (*Cyprinus carpio*)

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Abstract

Regular observation of survival of the carp breeding lines constituting a living gene bank at the Institute of Ichthyobiology and Aquaculture in Golysz (Poland) over a period of at least 15 years showed different survival rates for various lines. In this study, we have examined the polymorphism of the major histocompatibility complex (MHC) gene class II *B* in nine carp lines. The class II *B* gene encodes for the part of the MHC class II molecule which presents peptides from pathogens and protein antigens that are present in the extracellular milieu and have been taken up into the endocytic vesicles of antigen-presenting cells. Polymerase chain reaction was used to amplify *Cyca-DAB* gene fragments comprising part of exon 1, complete intron 1 and almost complete exon 2. Exon 2 encodes for the β_1 domain which is the most polymorphic fragment of MHC class II molecules. Single-strand conformational polymorphism (SSCP) was applied to detect different MHC class II *B* haplotypes. The analysis revealed the presence of seven different haplotypes occurring with various frequencies.

Résumé

Polymorphisme des gènes de classe II B du complexe majeur d'histocompatibilité de différentes lignées de la carpe commune (*Cyprinus carpio*). L'observation régulière, à l'Institut d'Ichtyobiologie et d'Aquaculture à Golysz (Pologne), de la survie des lignées de la carpe, constituant une banque de gènes, sur une période d'au moins 15 ans, a montré différents taux de survie selon les lignées. Dans cetté étude, nous examinons le polymorphisme du gène du complexe majeur d'histocompatibilité (MHC) de classe II B chez 9 lignées de carpes. Ce gène de classe II *B* code pour la partie de la molécule MHC de classe II, qui présente des peptides de pathogènes et les protéines antigéniques qui sont présentes dans le milieu extracellulaire et qui ont été endocytées par des cellules présentatrices des antigènes. Des réactions d'amplification de chaînes polymèrisées ont été utilisées pour amplifier des fragments de gènes *Cyca-DAB* comprenant une partie de l'exon 1, l'intron 1 complet et presque l'exon 2 complet. L'exon 2 code pour la zone β_1 qui est le fragment le plus polymorphe des molécules MHC de classe II. Le polymorphisme de conformation de séquence d'ADN simple brin (SSCP) a été utilisé pour détecter les différents haplotypes MHC de classe II *B* . L'analyse révèle la présence de 7 haplotypes différents observés avec diverses fréquences.

Keywords: Polymorphism; MHC class II *B* genes; *Cyprinus carpio*

1. Introduction

The major histocompatibility complex (MHC) is the most polymorphic region in the vertebrate genome and the gene products are under Darwinian positive selection. The MHC genes encode for cell-surface receptors, capable of binding short peptides for presentation to T-cell receptors. Recognition of the peptide–MHC molecule complex by the T-cell receptor causes T-cell activation and thus initiates a specific immune response resulting in the production of specific antibodies and effector cells. The MHC molecules are heterodimers and belong to two classes that differ in structure, tissue distribution and function. The class I molecules consist of a heavy chain which is composed of three extracellular domains, α_1, α_2, and α_3 and is non-covalently associated with

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a smaller peptide, β_2-microglobulin. The MHC class I molecules are expressed on all nucleated cells and primarily present endogenous peptides to the cytotoxic T cells. The class II molecules are composed of α and β chains, each having two extracellular domains, α_1, α_2 and β_1, β_2, respectively. They are expressed on specific cells of the immune system e.g. B cells, macrophages and other antigen-presenting cells, and primarily present exogenous peptides to the helper T cells.

The first direct evidence for MHC genes in teleost fishes was published by Hashimoto and co-workers in 1990. They identified MHC genes in common carp with a polymerase chain reaction (PCR) using highly degenerate oligonucleotide primers, based on the comparison of amino acid sequences for class I α_3 and class II β_2 domains from man, mouse and chicken. Today, MHC genes have been isolated and described for all major vertebrate taxa, including cartilaginous fish (Bartl, 1998; Ohta et al., 2000), bony fish (Kruiswijk et al., 2002; Stet et al., 1998), amphibians (Flajnik et al., 1991; Liu et al., 2002; Shum et al., 1993), reptilians (Grossberger and Parham, 1992; Wittzell et al., 1999), birds (Miller et al., 1994) and mammals (Hughes, 2000; Trowsdale, 1995).

The MHC genes of common carp (*Cyprinus carpio*) constitute an extended gene family, with classical and non-classical class I genes, B2m genes, class II *A* and *B* genes. For carp, at least five MHC class II *B* genes have been identified, characterized and named *Cyca-YB*(Hashimoto et al., 1990),*Cyca-DAB1*01*, *Cyca-DAB2*01*(Ono et al., 1993b), and *Cyca-DAB3*01*, *Cyca-DAB4*01*(van Erp et al., 1996). Moreover, it was found that *Cyca-DAB1*01* is linked to *Cyca-DAB2*01* whereas *Cyca-DAB3*01* is linked to *Cyca-DAB4*01* (van Erp et al., 1996). The most polymorphic segment of the coding region of each MHC class II *B* gene is exon 2 which encodes for the β_1 domain (Ono et al., 1993a). The β_1 domain, together with the α_1 domain (encoded by MHC class II *A* gene), creates a peptide-binding groove of MHC class II molecules in which peptides can be anchored (Brown et al., 1993).

The high variability of the MHC genes results in the presence of numerous alleles and, as a consequence, numerous haplotypes within a population. Each allele has the ability to bind and present different groups of peptides in more or less successful ways. Thus, the response of an organism towards certain pathogens can be influenced by the MHC haplotype. In humans, there are 476 alleles for the MHC class I genes (*HLA-A*, *-B*, and *– C*), and 444 for the class II genes (*HLA-DP*, *-DQ*, and *–DR*). The most polymorphic gene of all the *HLA* class II genes is *HLA-DRB1* with 221 defined alleles (Marsh et al., 2000). Polymorphism of MHC class II genes in fishes has been precise established for Atlantic salmon only (Grimholt et al., 1994; Langefors et al., 1998). Stet et al. (2002) described the presence of seven different *Sasa-DAA* and seven *Sasa-DAB* expressed alleles out of 84 individuals sequenced. In addition, they showed that Atlantic salmon express a single MHC class II *B* locus which is closely linked to the class II *A* locus.

This study was undertaken to determine the level of MHC class II *B* (*Cyca-DAB*) genes polymorphism in genetically different lines of the common carp.

2. Materials and methods

2.1. Fish

Common carp (*Cyprinus carpio*) individuals used in this study were obtained from a living gene bank at the Institute of Ichthyobiology and Aquaculture of the Polish Academy of Sciences in Golysz (Poland). The living gene bank includes 19 genetically different carp lines which are regularly reproduced by closed breeding to preserve bio-diversity. Some of these lines were imported to Golysz as existing closed breeding lines; e.g. the Hungarian (R7 and R8) lines were imported from the Fish Culture Research Institute in Szarvas (Hungary), and the Ukrainian (Ur) line was imported with the assistance of Ministry of Agriculture from Ukraine. The Lithuanian (Lit) and Polish (K) lines were imported from fish farms in Bubiai (Lithuania) and Knyszyn (Poland), respectively. Other lines were obtained at Golysz (Poland) as a result of breeding selection on local carps (e.g. the Polish (R6) and (R3) lines) and on imported fishes of unknown origin from various geographic regions (the German (N) line). Further, some carp lines were the result of hybrid crosses between existing lines such as the Hungarian (R0) line-cross between Hungarian (R7) and Hungarian (R8) lines (Bialowas, 1999; Irnazarow and Bialowas, 1994, 1995). All these lines were regularly scored as for survival rate during the breeding history.

In our study, we chose eight carp lines showing clear-cut differences in survival (see also Table 1) to pre-screen the polymorphism of MHC class II *B* genes. Additionally, the Lithuanian (Lit) line, for which no data as for average survival are available, yet, was included into analysis.

Table 1
Mean of survival rates of 9/19 carp (*Cyprinus carpio*) breeding lines, at the Institute of Ichthyobiology and Aquaculture in Golysz (Poland). Survival rates are calculated after the first rearing season (Pilarczyk, 1998)

Carp breeding lines (symbol)	Survival (1981–1996)
Polish (K)	16.0 ± 20.9
Polish (R6)	33.3 ± 8.5
Polish (R3)	28.7 ± 18.8
Hungarian (R7)	39.6 ± 11.8
Hungarian (R8)	35.3 ± 11.9
Hungarian (R0)	33.9 ± 12.7
German (N)	67.9 ± 19.8
Ukraine (Ur)	47.9 ± 8.9
Lithuanian (Lit)	No data[a]

[a] The Lithuanian (Lit) line was imported to the Institute of Ichthyobiology and Aquaculture in Golysz (Poland) recently (1995). This line has not been reproduced at the premises and thus no record of survival rate after the first growing season has been made.

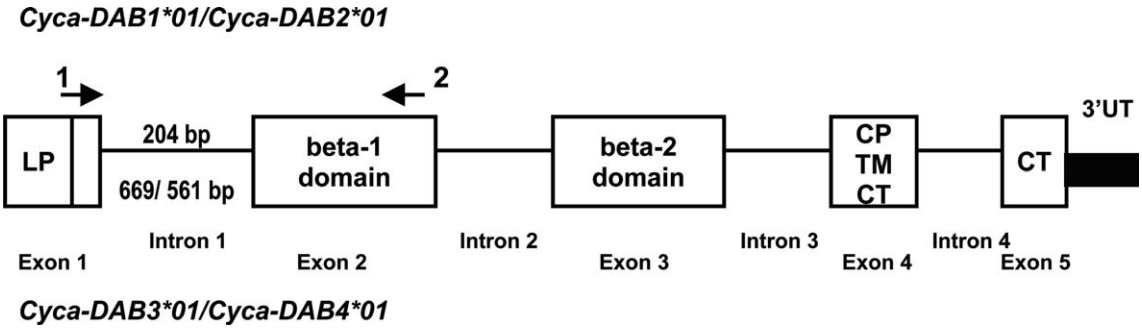

Fig. 1. Genomic organization of *Cyca-DAB* genes based on sequence information of van Erp et al. (1996). Arrows indicate primers used for amplification of part of exon 1, the complete intron 1 and almost complete exon 2 of *Cyca-DAB* genes; 1—forward primer OL93-139, 2—reverse primer OL94-23. LP—leader peptide, CP—connecting peptide, TM— transmembrane region, CT—cytoplasmic domain, 3'UT—3' untranslated region.

2.2. DNA isolation

Randomly picked individuals from each line (n = 8–19) were anaesthetized in tricaine methane sulfonate (TMS, Crescent Research Chemicals, Phoenix AZ, USA) used at 0.25% w/v. Muscle samples were isolated, snap frozen in liquid nitrogen and stored at –80 °C. Total genomic DNA was isolated from muscle samples by proteinase K digestion and purification using phenol:chloroform according to a standard protocol (Sambrook and Russell, 2001).

2.3. Polymerase chain reaction (PCR)

PCR was used to amplify *Cyca-DAB* genes fragments comprising part of exon 1, complete intron 1 and almost complete exon 2 (Fig. 1). Reverse primer OL94-23 was complementary to the end of exon 2 of both *Cyca-DAB3*01* and *Cyca-DAB4*01* genes, whereas the forward primer OL93-139 was complementary to the end of exon 1 of both *Cyca-DAB1*01* and *Cyca-DAB2*01* genes (van Erp et al., 1996) (Table 2). As a template genomic DNA was used. Thirty cycles of PCR (94 °C for, 60 s, 55 °C for 60 s, 72 °C for 120 s) were carried out using 100–500 ng of template DNA, 0.2 mM of each dNTP, 0.2 μM of each primer, 1.5 mM of $MgCl_2$ and 1.5 units of *REDTag* DNA Polymerase (SIGMA-ALDRICH, Germany). PCRs were performed using a Techne machine (Progene, UK).

2.4. Single-strand conformational polymorphism (SSCP)

The SSCP method was used to identify different haplotypes. Five microliter of PCR product was mixed with 15 μl of loading buffer (10 mM NaOH, 1 mM EDTA, 95% formamide, 0.1% bromophenol blue, 0.1% xylene cyanole FF). After denaturation at 95 °C for 5 min in a water bath, samples were immediately cooled on ice, and loaded onto 0.4 mm thick, 5% polyacrylamide gels containing 4% glycerol (Zhu and Gasser, 1998). The conditions for electrophoresis (600 V for 3 h at 8 °C) were standardized for optimal resolution of bands.

2.5. Silver staining

Silver staining was used with a slight modification to stain SSCP patterns (Herring et al., 1982). The gels were fixed with 10% ethanol –0.5% acetic acid for 1 h, rinsed twice in distilled water and soaked in 0.01 M silver nitrate for 30 min. Then, gels were rinsed briefly, twice, in distilled water and the reduction carried out with a solution of 0.75 M sodium hydroxide and 0.085 M formaldehyde until the SSCP bands were clearly visible (maximum 10–15 min). The reaction was stopped by transferring the gels to 0.07 M sodium carbonate for 30 min. After digitising (Casio QV 5000 SX), the gels were briefly soaked in a solution of 30% methanol and 5% glycerol and air dried.

3. Results

Regular score of survival of the 19 carp lines at the Institute of Ichthyobiology and Aquaculture of the Polish Academy of Sciences in Golysz (Poland) revealed different survival rates in distinct breeding lines, raised under natural conditions. Survival rates varied from 16.0% in the Polish (K) line to 67.9% in the German (N) line (Pilarczyk, 1998). Based on previously published sequence data by van Erp et al. (1996), we used specific primers in PCRs for the amplification of *Cyca-DAB* genes fragments. PCR amplification was performed on genomic DNA isolated from a total of 119 individuals from nine carp lines (Table 1). Two different PCR products, one of about 490 bp in size and a second product of about 900 bp were detected in two carp lines: Hungarian (R0) and Lithuanian (Lit), whereas in the remaining carp lines only a single (490 bp) fragment was observed

Table 2
Oligonucleotide primers used for amplifying fragment of *Cyca-DAB* genes

Primer	Sequence	Position	Comments
OL93-139	CTGATGCTGTCTGCTTTCACTGGAGCA	End of exon 1	Forward primer specific for *Cyca-DAB1*01/DAB2*01*
OL94-23	GATTTGAGCATTATGTTTGCA	End of exon 2	Reverse primer specific for *Cyca-DAB3*01/DAB4*01*

(Fig. 2). Based on size, the 490 bp PCR product represents putative *Cyca-DAB1*01/DAB2*01* genes, while the 900 bp PCR product represents putative *Cyca-DAB3*01/DAB4*01* genes (van Erp et al., 1996).

We chose to further analyze *Cyca-DAB1*01/DAB2*01* genes. To this end, samples containing the 490 bp PCR products were denatured and separated by the SSCP method to identify different *Cyca-DAB1*01/DAB2*01* alleles. We could detect seven different haplotypes as shown in Fig. 3. Haplotype A was the most frequently occurring haplotype among the carp lines tested. Haplotype B was unique to the Polish line (R3) and was present at a very low frequency (1/119 samples tested). In addition, haplotypes F and G were also very rare (2/119 and 1/119 samples tested, respectively). Three carp lines were monomorphic: the Hungarian (R0) and Hungarian (R7), where a single haplotype (C) was revealed, and the Ukrainian (Ur) where a single haplotype (A) was found. The most polymorphic for *Cyca-DAB1*01/DAB2*01* was the Lithuanian (Lit) line in which four different haplotypes could be detected (A, D, F and G). Moreover, three haplotypes (D, F and G) were unique to this carp line (Table 3).

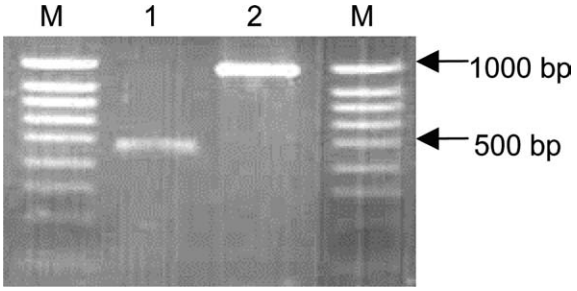

Fig. 2. Two PCR products generated by using the forward primer OL93-139 and the reverse primer OL94-23 on genomic DNA of individual carp. (1) 490 bp PCR product representing putative *CycaDAB*01/DAB*02* genes, (2) 900 bp PCR product representing putative *Cyca-DAB*03/DAB*04* genes. M. molecular size marker: Smartladder SF.

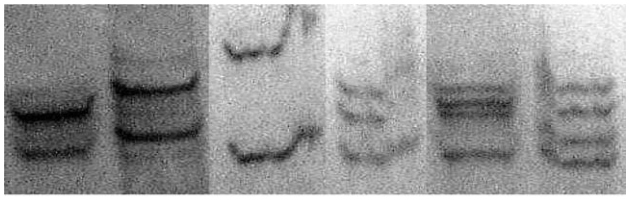

Fig. 3. *Cyca-DAB* haplotypes, as detected by SSCP, present in different breeding lines of common carp (*Cyprinus carpio*). Characters (A–F) denote haplotypes as unique SSCP patterns. Haplotype G is not depicted.

4. Discussion

Freshwater cyprinids are the second major group of finfish cultured in Europe with 301 000 t of production in 2000 (FAO) and the common carp is the major cultured species in this group. Regular score of survival of the carp breeding lines constituting a living gene bank at Golysz (Poland) over a period of at least 15 years showed that particular carp lines have a consistently high survival while others have a consistently low survival. There is some evidence for association between MHC alleles and disease resistance in fishes. Wiegertjes et al. (1996), suggested that high antibody responsiveness could be associated with *Cyca-DAB1*01/Cyca-DAB2*01* genes whereas *Cyca-DAB3*01/Cyca-DAB4*01* genes seemed to correlate with a low antibody response to DNP-KLH and to a blood parasite. Langefors et al. (2001a) and Lohm et al. (2002) revealed that certain MHC class II *B* alleles are associate with resistance/susceptibility to *Aeromonas salmonicida*, which causes the disease furunculosis, in salmonids.

In this study, we demonstrated differences in MHC class II *B* haplotypes between nine breeding lines of common carp that showed differences in survival. However, survival can be under the influence of many genetic and environmental parameters, and it will become very important to unravel genetic and environmental effects. So far, our knowledge of genetic differences between the various lines of common carp constituting our living gene bank is limited. Irnazarow (1995) examined the genetic variability in seven Polish and Hungarian carp lines, using 13 loci for erythrocyte transferrin and alloenzymes, and found a genetic variation ranging from 0.46 to 0.54.

MHC class II *B* genes have now been described for many teleost species including Atlantic salmon (*Salmo salar*) (Hordvik et al., 1993; Langefors et al., 2001b), rainbow trout (*Oncorhynchus mykiss*) (Glamann, 1995; Ristow et al.,

Table 3
Haplotypes detected by SSCP, present in different breeding lines of common carp (*Cyprinus carpio*), part of a living gene bank. Numbers denote the numbers of individuals with a particular SSCP profile out of the numbers of individuals tested

Carp breeding lines (symbol)	SSCP haplotypes						
	A	B	C	D	E	F	G
Polish (K)	9/15		6/15				
Polish (R6)	16/18		2/18				
Polish (R3)	18/19	1/19					
Hungarian (R7)			11/11				
Hungarian (R8)	2/8		5/8		1/8		
Hungarian (R0)			8/8				
German (N)	4/12				8/12		
Lithuanian (Lit)	3/13			7/13		2/13	1/13
Ukrainian (Ur)	15/15						

1999), large barbus (*Barbus intermedius*) (Dixon et al., 1996), channel catfish (*Ictalurus punctatus*) (Godwin et al., 1997), striped bass (*Morone saxatilis*) (Walker et al., 1994), cichlids (Cichlidae) (Figueroa et al., 2000; Málaga-Trillo et al., 1998), zebrafish (*Danio rerio*) (Bingulac-Popovic et al., 1997; Ono et al., 1992), and common carp (*Cyprinus carpio*) (Rodrigues et al., 1995; Stet et al., 1997). However, clear polymorphism of the class II *B* genes has been described only for Atlantic salmon, and seven different expressed alleles for *Sasa-DAB* were found (Stet et al., 2002). In carp, sequence data for MHC class II *B* were described previously (Ono et al., 1993b; van Erp et al., 1996). There are two different pairs of MHC class II *B* expressed loci. One represents *Cyca-DAB1*01* and *Cyca-DAB2*01* genes, whereas, at the second *Cyca-DAB3*01* and *Cyca-DAB4*01* genes are found. The most significant difference to distinguish between the linked *Cyca-DAB1*01*/*Cyca-DAB2*01* and *Cyca-DAB3*01*/*Cyca-DAB4*01* genes is the length of intron 1. Both *Cyca-DAB1*01* and *Cyca-DAB2*01* genes show a 204 bp intron 1, in contrast to the *Cyca-DAB3*01* and *Cyca-DAB4*01* genes that have a much longer intron 1 of 669 and 561 bp, respectively (van Erp et al., 1996). Based on this sequence information we designed primers for use in PCR reactions, amplifying *Cyca-DAB* genes fragments comprising part of exon 1 and the complete intron 1 and almost complete exon 2. As template we used genomic DNA from 119 individuals collected from nine genetically different carp breeding lines. Two different PCR products, one of about 490 bp in size and the second of about 900 bp, were observed. Sequencing of the PCR products confirmed that, the amplified 490 bp PCR products correspond to *Cyca-DAB1*01*/*Cyca-DAB2*01* genes and the 900 bp PCR products correspond to *Cyca-DAB3*01*/*Cyca-DAB4*01* genes. These genes are co-dominantly expressed (Rakus et al. unpublished communications).

In order to estimate the level of polymorphism of *Cyca-DAB* genes in our carp lines we applied SSCP analysis. This analysis allows the detection of single base polymorphisms in short DNA stretches due to mobility differences of single-stranded DNA fragments during electrophoresis in polyacrylamide gels (Orita et al., 1989). The sensitivity (detection rate) of SSCP analysis tends to decrease with increasing length of DNA fragments and is 80% approximately for fragments of 400–500 bp in size. Therefore, we only used the 490 bp and not the 900 bp samples for our SSCP analysis. We could describe a number of seven different haplotypes which showed clearly different frequencies in the investigated carp lines. The most frequently occurring haplotype (A; frequency 0.56) was found in all lines except the two Hungarian lines (R7 and R0). In contrast, other haplotypes (B and G) were found only in single individuals of specific carp lines Polish (R3) and Lithuanian (Lit), respectively. In order to exclude the PCR errors, existence of haplotypes B, F and G were confirmed by making the three independent PCR reactions and running the three SSCP gels.

In conclusion, SSCP analysis revealed clear polymorphism for *Cyca-DAB1*01* and *Cyca-DAB2*01* genes in different carp lines that represent part of a living gene bank at the Institute of Ichthyobiology and Aquaculture in Golysz (Poland). At least seven haplotypes were detected. The exact degree and nature of this *Cyca-DAB* polymorphism was confirmed by sequencing (Rakus et al., in preparation). Future research will focus on examining putative correlations between specific MHC class II *B* haplotypes and resistance against specific pathogens in carp lines with a history of high or low survival.

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