Major histocompatibility (MH) polymorphism of common carp

Link with disease resistance

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Dit onderzoek is uitgevoerd binnen de onderzoeksschool Wageningen Institute of Animal Sciences (WIAS).

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. Dr. M. J. Kropff,
in het openbaar te verdedigen
op maandag 24 november 2008
des namiddags te 13.30 uur in de Aula

Major histocompatibility (MH) polymorphism of common carp. Link with disease resistance.
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ISBN: 978-90-8585-244-5

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CHAPTER 1

General introduction

Common carp (*Cyprinus carpio* L.) is one of the most cultured fish species. The annual world production of common carp was estimated to be 3,172,488 tons in 2006 (FAO 2008). The biggest common carp producer in the world is China. Poland, with an average annual production of about 20 thousand tones, is the biggest common carp producer in the European Union. The consumption of carp in Poland has a long standing history and it is closely connected with Christmas, when about 80% of the annual carp production is sold in a few days prior to this holiday (Lirski, 2007).

1. A short history of common carp breeding in Poland

Carp breeding in Poland has a long tradition that was started in the second part of the 12th century. The development of fish farming most likely came from the introduction of carp from Czech and Moravia by the Cistercians, which began to breed fish in ponds (Szumiec, 2005). In the Middle Ages carp production was boosted by a religious rule of fish consumption during frequent fasts, reaching about 50% of the days of the year. One of the first descriptions of carp in the Polish documents came from the writings of Jan Długosz from 1466 (Szczygielski, 1967). The need for the development of technologies for pond construction and for water leveling found response in one of the first technical book in Polish "*O sprawie, sypaniu, wymierzaniu i rybieniu stawów, także o przekopach, o ważeniu i prowadzeniu wody. Książki wszystkim gospodarzom potrzebne*" (English translation: About managing, pond building and stocking, also about digging and water leveling and conducting. Books useful for every farmer). It was written by Olbrycht Strumieński and printed in Cracow in 1573. Huge carp ponds were built for Polish kings and counts (e.g. pond "Zygmunt" in Knyszyn with an area of 460 ha). The majority of the oldest and largest pond farm complexes are located at the central and southern part of the country, like in Zator (near Oświęcim), Gołysz and Landek (near Cieszyn), Łyszkowice (near Łowicz) and Milicz (Fig. 1). Such localization was connected with suitable land configuration and beneficial climate conditions (Nyrek, 1966, Szczygielski, 1967, Łysak, 1996, Szumiec and Augustyn, 2000). Fish ponds were constructed on unproductive land, which could be hardly utilized for typical agricultural purposes. The huge pond complexes also increased the retention of water and prevented floods whereas in periods of drought they were sources of water to irrigate fields (Szumiec, 1996).

Figure 1. A part of the map of the region of upper Vistula elaborated by Jonas Nigrini (1725). Numerous fishponds are present next to the Vistula River. (Copyright by Macierz Ziemi Cieszyńskiej, 2000).

Carp pond culture in Poland has been developing under strong impacts exerted by many external factors, like climate changes, political, social and economical events. The highest development of pond culture occurred in the 14th century and lasted until the end of the 16th century. At the beginning of the 17th century fish farming collapsed due to the Thirty Years War (1618-1648). Many ponds were devastated and dried which resulted in a reduction of the total area of ponds of about 50%. A period of continuous development in carp pond culture began in the middle of the 19th century and is maintained till present (Szczygielski, 1967, Szumiec, 2005). The main stimulus for carp culture development came from Tomas Dubisz (1813-1888) who was working in a fish

farm complex Iłownica-Landek, belonging to the Cieszyn Estate, property of the Habsburgs (today the Fish Culture Experimental Station in Gołysz). He proposed the method of multiple transfers of fish at different ages into ponds of different categories, starting from the transfer of the larvae from spawning ponds to nursery ponds. This new method provided more effective utilization of natural food and shortened the rearing period of the market carp from 5-7 to 3 years (Szumiec, 2005). During this time also the Galician carp breed, which was remarkable for its superior farming traits, was established. These carp were characterized by very good growth rate and moderate dorsal height. In 1880 carps of this breed won prestigious awards at the agricultural exhibitions in Germany (Lirski, 2007). In the 20th century, further steps in the intensification of carp culture, based on introduction of nitrogen-phosphorus fertilization in ponds, increasing stock densities, and feeding fish dry pellets were introduced (Szumiec, 1995). However, in recent years, carp pond farming in Poland is based mostly on semi-intensive methods, which utilize a big share of the natural food in the carp diet (benthos, zooplankton), with additional corn feeding (wheat, barley, rye). The intensification of production is low and does not exceed 700-1000 kg per hectare (Lirski, 2007). Because of this, the carp farming in Poland is considered to be nature-friendly and pro-ecological.

2. Carp live gene bank

The development of the carp live gene bank was started in Gołysz in 1954. At the beginning, stocking material of purely Galician breed, which still existed locally after the Second World War, was used to establish some of the Polish breeding lines. The carp live gene bank has been enlarged by increasing the number of different lines, including common carp from different regions of Poland (Polish lines) as well as different parts of Europe (foreign lines). At present the live gene bank consists of 19 carp lines of various geographical origins (Table 1). Carps from different lines show various morphological characteristics, like different body shape and length, scaliness type and colour (Fig. 2). In Poland, the cultivated mirror forms spread rapidly and became popular because of their good survival and growth rate, but mostly due to market demand.

Table 1. Polish and foreign common carp lines of the carp live gene bank in Gołysz.

Polish lines
Landek line (R2) mirror carp from Southern Poland
Ochaby line (R3) mirror irregular carp from Southern Poland
Gołysz line (R6) mirror carp from Southern Poland
Knyszyn line (K) mirror carp from Northern Poland
Starzawa line (St) full scaled carp from the South-Eastern Poland
Foreign lines
Hungarian line (W) mirror carp imported from fish farm Bikal in Hungary
Hungarian line (R7) Szeged mirror carp from South-Eastern Hungary (HAKI, Szarvas)
Hungarian line (R8) Sumony mirror carp from Southern Hungary (HAKI, Szarvas)
Hungarian line (R0) mirror carp obtained by crossing line R7 with R8 (HAKI Szarvas)
Hungarian line (T) mirror carp from WWFBC, in Szazhalombatta, Hungary
Hungarian line (C) mirror carp of orange or reddish-grey skin coloration
German line (N) mirror carp of greenish-yellow skin coloration
Israeli line (DOR-70) mirror carp imported from Aquaculture Research Station in Dor
French line (F) mirror framed carp of greyish-green and greyish-blue skin coloration
Yugoslavian line (J) mirror framed carp of bright yellowish skin coloration
Ukrainian line (Ur) mirror carp
Ukrainian line (Up) full scaled carp
Lithuanian line (B) mirror irregular carp
Lithuanian line (BVP) mirror carp with irregular scales located around the lateral line

Polish lines R2, R3, and R6 were obtained in Gołysz as a result of breeding selection on local carps, while lines K and St resulted from breeding selection on carp that came from others regions of Poland. In Gołysz, selection was also applied to stocking material brought from various regions of Europe, resulted in establishing e.g. Hungarian line W, German line N, French line F, and Yugoslavian line J. Other carp were imported to Gołysz as existing closed breeding lines (e.g. Hungarian lines R7 and R8, Israeli line DOR-70, Ukrainian line Ur and Up, and Lithuanian line B and BVP). The Hungarian line R0 was the result of hybrid crosses between existing carp lines R7

and R8 (Wohlfarth *et al*., 1980, Irnazarow and Białowąs, 1994 and 1995, Guziur *et al*., 2003).

In each carp line a proper age structure is maintained, which includes mature spawners, in the age of 5 years and older, group of selects in the age of 3-5 years and group of young fingerlings. The number of mature spawners in each breeding line is 150-200 individuals of both sexes, which is in accordance with FAO recommendations of maintaining fish populations (FAO 1981). Each line is regularly reproduced every 6-8 years. Besides the carp live gene bank, a cryopreserved sperm bank of selected important lines has been set up and is maintained in Gołysz since 2002.

The carp live gene bank is an object of extensive experimental work, including analysis of the productive traits of the carp breeding lines, study on the heterosis effect on some quantitative traits of experimental crosses (Białowąs, 1991, Białowąs and Irnazarow, 1993, Białowąs *et al*., 1997), study on the artificial propagation of carp (Brzuska and Białowąs, 2002, Brzuska, 2003, 2004, 2005, and 2006,) and genetic characterization of selected carp lines using different molecular markers (Irnazarow and Białowąs, 1994 and 1995, Irnazarow, 1995). So far, notable success has been achieved as for farming technology and productive traits, nevertheless the impact of diseases caused by a wide range of pathogens (viruses, bacteria and parasites) is still an important problem in the aquaculture of common carp. Studies of the survival rate after the first rearing season, recorded for a period of 15 years revealed that average survival rates of the carp breeding lines ranges from 9 – 68 % survival, approximately (Pilarczyk, 1998). Ichthyopathological studies have also shown notable differences in the level of swimbladder infection and tapeworm infestation between selected carp lines (Pilarczyk, 1998). Wiegertjes and co-authors (1995) demonstrated significant differences in the immune response to the blood parasite *Trypanoplasma borreli* between gynogenetically reproduced homozygous *versus* outbred carps. It has been also described that selected carp lines showed significant differences in susceptibility to *Aeromonas salmonicida,* which cause erythrodermatitis (van Muiswinkel *et al*., 1990; Houghton *et al*., 1991, Wiegertjes *et al*., 1993), and to the ectoparasite *Ichthyophthirius multifilis* (Pilarczyk, 1998). These observations suggest presence of genetic differences in resistance between the carp lines, which recently are studied using DNA marker technologies (Pilarczyk *et al*., 2002).

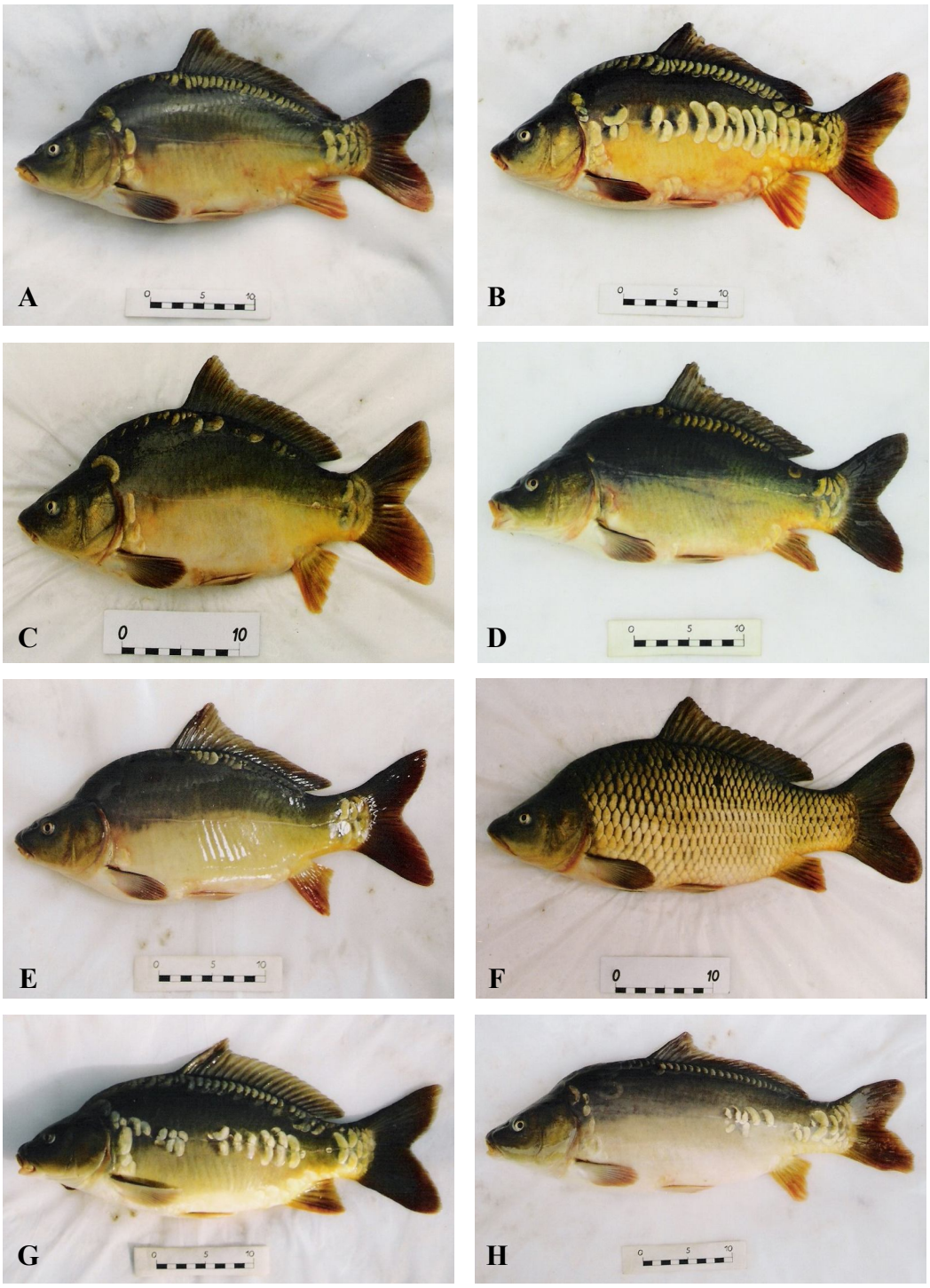

Figure 2. Common carp individuals in the age of 3 years from different carp lines: A- German line 'N', B- Lithuanian line 'B', C- Hungarian line 'R0', D- Ukrainian line 'Ur', E- Polish line 'R6', F- Ukrainian line 'Up', G- Israeli line 'DOR-70', and H- Polish line 'R3'. Different scaliness patterns: mirror (A, C, D, and E), mirror irregular (B, G, and H) and full scaled (F), as well as different skin coloration and body shape is present (courtesy of Dr. H. Białowąs).

3. Control of infectious diseases in carp farming

Antibiotics and other chemotherapeutants are generally cost-effective in controlling some of the diseases that are commonly associated with intensive aquaculture. These practices have consequently become an integral part of modern aquaculture. However, intensive use of these agents is increasing the potential risk of unwanted influences on product quality, environment and human health. In addition, the recognition of farming ponds as valued ecosystems (Szarowski, 1997) argues against pharmacological interventions and strongly in favour of alternative sustainable approaches. An alternative approach is to prevent diseases by improving the immune capacity of fish by vaccination or by genetic selection for more resistant fish. Vaccination is an effective approach only in intensive systems and/or for high market value species, while traditional carp aquaculture is carried out in semi-intensive pond culture conditions. In addition, effective vaccines are available only for a limited range of pathogens.

It is known from studies in mammals that a limited number of major genes, including genes of the major histocompatibility complex (MHC), is involved in the regulation of the immune response and are widely accepted candidate marker genes for studies on associations with disease resistance (Hill, 1998, Parham, 2000). Selective breeding for higher resistance to disease has been applied in aquaculture for only a relatively short period and the data available therefore are limited. Lack of extensive studies on fish diseases, especially infectious diseases, and incomplete elucidation of the immunological reactions in fish have further contributed to the limitation of the data set. When a disease occurs in a fish population, it does not necessarily affect all fish to the same extent; some fish, sometimes even in large numbers, may not be affected. Even the progeny of a single pair of spawners, reared under the same environmental conditions, do not all possess the same level of disease resistance. In these cases, individual variations may serve as starting points for selection programmes.

Family selection of fish for resistance to diseases may prove, and in some cases has already proven, to be an important means of control of fish diseases (Midtlyng *et al*., 2002). Selective breeding of fish for higher resistance to diseases can be dependent on relatively simple mutations at very specific sites, involving one or a few highly polymorphic genes, like the MHC genes. Any gene mutation which could be

accompanied by a change in the synthesis of proteins may cause protein incompatibility of parasite and host. Fish belonging to different strains may differ in their resistance to disease (van Muiswinkel *et al*., 1990, Pilarczyk, 1998, Shapira *et al*., 2005, Jurecka *et al*., 2008). For successful selective breeding it is necessary to obtain fish from genetically distant populations, or fish species from different regions for rearing in experimental fish farms. The availability of a live gene bank, experimental culture ponds and the scientific infrastructure at Gołysz, has permitted the selection studies aimed at obtaining more resistant carp.

4. Structure and functions of MHC molecules

Teleost fish are the first vertebrate group to show an immune system that exerts both innate and adaptive responses. Research has shown that fish and mammalian immune responses and mechanisms share a high level of conservation. Cloning strategies have identified the presence of immune-related genes encoding IgM, TCR, CD3, CD4, CD8, RAG, MHC class I, β_2-microglobulin, MHC class II, MHC class I/II loading pathways, and cytokines or chemokines in fish (Dixon *et al*., 1995, Watts *et al*., 2001, Sun *et al*., 2007, Randelli *et al*., 2008a, and 2008b, Buonocore *et al*., 2008). Unfortunately, the scarcity of fish cell lines and proper antibodies still hampers progress in this field, especially as regards the functioning and interactions between the different cell types of the immune system. Knowledge of the MHC genes is crucial for understanding of antigen presentation and, therefore, of T lymphocyte-dependent adaptive immune responses. As a general rule, in mammals, non-MHC genes appear to regulate the early phase of the immune response to pathogens, whereas the later phase of elimination often depends on the generation of MHC-restricted T cell-mediated immunity. The extensive polymorphism of MHC molecules, observed in many vertebrate species including fish, is most likely favoured by differences between allelic MHC molecules in the efficacy to present antigenic peptides derived from pathogens. Therefore, MHC genes are obvious candidate genes for disease resistance.

The MHC genes encode for two kinds of membrane-bound glycoproteins, class I and class II, responsible for binding and presenting processed antigens to $CD8^+$ and $CD4^+$ T lymphocytes, respectively. The teleost fish MHC is equivalent to its mammalian counterpart (as regards exon–intron organization, polymorphism as well as

protein structure and composition) but the class I and class II genes appear in different linkage groups and are not clustered (Stet *et al*., 2003).

4.1. MHC class I molecules

In human, MHC class I molecules are transmembrane heterodimers of a heavy alpha chain (45 kDa) which is non-covalently associated with a light chain β2-microglobulin (12 kDa). The heavy chain consists of three extracellular domains (alpha-1, alpha-2 and alpha-3), a transmembrane and a cytoplasmic region. The two membrane distal domains, alpha-1 and alpha-2, each approximately 90 residues long, form a peptide binding groove that consists of two anti-parallel alpha-helices that form walls bordering a platform of eight anti-parallel beta-strands. The ends of the class I peptide binding groove are closed which limits the possible length of class I-binding peptides to 8 to 11 amino acids in length. The polymorphic residues of class I molecules are confined to the alpha-1 and alpha-2 domains, where they contribute to variations among different class I alleles. The membrane proximal alpha-3 domain and β2-microglobulin are both homologous with the immunoglobulin C1 domains (Saper *et al*., 1991, Madden *et al*., 1992). MHC class I molecules are expressed on all nucleated cells and present self and non-self endogenous derived peptides to $CD8^+$ T cells. The CD8 receptor interacts with a conserved region of the alpha-3 domain of class I molecule. Recognition of antigen-MHC complex by $CD8^+$ T cell receptor (TCR) is the first step towards developing cytotoxic T cell immune response. In addition, MHC class I molecules also play a role in natural killer (NK) cell mediated responses since specific receptors present on NK cells in humans can detect a down regulation of MHC class I molecules on cell surfaces (Vilches and Parham, 2002).

4.2. MHC class II molecules

MHC class II molecules are transmembrane heterodimers composed of two non-covalently associated polypeptide chains: an alpha chain (32-34 kDa) and a beta chain (29-32 kDa). Each chain consists of two extracellular domains (alpha-1 and alpha-2 or beta-1 and beta-2, respectively), a transmembrane and cytoplasmic region. The membrane distal alpha-1 and beta-1 domains together constitute the platform of eight anti-parallel beta-strands that supports two anti-parallel alpha-helices and form a peptide

binding groove similar to that of MHC class I molecules. However, the ends of the peptide binding groove are open which allows a binding of longer peptides of 12 to 25 or more amino acids in length (Brown *et al*., 1993). In human MHC class II molecules, most of the polymorphism was found in the beta chain, and the fragment encoded for beta-1 domain is the most polymorphic region of class MHC II genes. The membrane proximal alpha-2 and beta-2 domains are both homologous with the immunoglobulin C1 domain and show very low polymorphism. Although the expression of both chains is equivalent, the beta chain has been chosen in most studies, including those in humans, because of its higher degree of polymorphism than the alpha chain. Expression of MHC class II molecules is restricted to macrophages, dendritic cells and B-cells, as well as activated T-cells and epithelial cells and is up regulated e.g. by IFN-gamma. The class II molecules present mostly exogenous derived peptides to $CD4^+$ T cells. The CD4 receptor interacts with conserved region of beta-2 domain of class II molecule.

4.3. MHC or MH

In humans, the MHC (or *HLA*) genes are closely linked in a complex, which is 3.6 Mbp in length and located on chromosome 6. The combination of MHC alleles present on each chromosome is called a MHC haplotype. The MHC is an extensive gene rich region including over 260 genes (MHC Sequencing Consortium, 1999, Horton *et al*., 2004), of which approximately 40% are associated with functions of the immune system. This region is divided into three gene clusters (Margulies, 1999), in order of physical location from centromer to telomer: class II, class III and class I. The class I cluster includes genes encoding the heavy chain of classical (*HLA-A*, *HLA-B*, and *HLA-C*) and non-classical (*HLA-E*, *HLA-F*, and *HLA-G*) class I molecules, several HLA class I pseudogens and various genes apparently not related to the immune system (MHC Sequencing Consortium, 1999, Kelley *et al*., 2005). The class II cluster includes genes encoding for alpha and beta chain (*HLA-DP*, *HLA-DQ*, and *HLA-DR*) of class II molecule, two proteasome subunits genes (*LMP*) and transporter associated with antigen processing (*TAP*) encoding genes (MHC Sequencing Consortium, 1999). The class III cluster, which is the most gene-dense region of the human genome (Xie *et al*., 2003) includes many genes that do not possess an antigen presentation function proper. There are some immune genes like members of complement cascade (*C2*, *C4*, *Bf*) and tumor

necrosis factor family members (*TNF*, *LTA* and *LTB*) (MHC Sequencing Consortium, 1999).

In teleosts, the genomic organization of the MHC genes is different compared to that of humans but also tetrapods and cartilaginous fish (Kulski *et al*., 2002). In teleosts, class I and class II genes are not linked and reside on different chromosomes (Bingulac-Popovitc *et al*., 1997, Sato *et al*., 2000), which is the result of translocation (Kuroda *et al*., 2002). It was also found that *TAP* and *LMP* genes of zebrafish are linked to class I genes (Takami *et al*., 1997), as opposed to humans. Several, but not all, of the zebrafish homologues of the human MHC class III region genes have been identified by Sultmann *et al*. (2000). In zebrafish the MHC class III genes are found distributed throughout their genome although several of these genes were mapped to chromosome 19, which also encompasses the MHC-related genes (Sambrook *et al*., 2005).

The presence of multiple independently segregating histocompatibility loci in fish has already been postulated in the 70's of the last century by Hildemann (1970) and Kalmann (1970) who studied skin transplantation in several fish species. These authors were the first to use the term histocompatibility gene for fish. Together with more recent findings of non-linkage of class I and class II genes Stet *at al*. (2003) proposed, rather than use major histocompatibility complex (MHC), to use the original terminology major histocompatibility (MH) genes for teleost fish species.

4.4 MHC polymorphism and infectious disease association.

MHC genes are the most polymorphic genes present in the genome. The polymorphism at MHC loci is thought to be maintained by pathogen-driven balancing selection (Hughes and Nei, 1989, and 1992, Hedrick, 2002). Several hypotheses have been proposed to explain the possible mechanisms including heterozygote advantage, negative frequency-dependent selection and variable selection in time and/or space (Hedrick *et al*., 1999, Hedrick, 2002). Heterozygote advantage (overdominant selection) indicates that individuals heterozygous at MHC loci have increased immunocompetence through the ability to present wider range of antigenic peptides derived from pathogens or parasites to immune system, than homozygotes. Negative frequency-dependent selection (rare allele advantage) implies that individuals with a rare MHC allele might respond better to pathogens, than individuals with more common MHC alleles, to which

pathogens may have evolved resistance. In such a case, an allele providing a more effective immune response against a pathogen will be selected, increasing its frequency in the population. Consequently, as the selected allele increases in frequency, it becomes a more likely target for parasite adaptation.

The observed patterns of variability on a molecular level provide strong support for a pathogen-driven origin of MHC polymorphism. This is called positive, or Darwinian selection and ensures that random nucleotide substitutions which resulting in amino acids replacements that provide more effective immune defense against the pathogen, will be maintained and even spread in the population. Nucleotide substitutions that do result in amino acid replacements are referred to as non-synonymous. Random mutations will occur throughout all nucleotide sequences and while all amino acids (except methionine and tryptophan) are encoded by multiple codons, some of these mutations (referred to as synonymous) will not result in amino acid replacements and are therefore called silent. The analysis of MHC genes revealed that exons encoding the alpha-1 and alpha-2 domains of the class I molecule and alpha-1 and beta-1 domains of the class II molecule contain a very high degree of non-synonymous nucleotide substitutions especially in codons encoding amino acids directly interacting with the peptide (PBR, peptide binding residues). Thus, polymorphism in MHC molecules primarily affects their peptide binding specificity, which in turn can influence the efficacy of the immune response of an individual organism and thereby the susceptibility or resistance to various pathogens. This concept is substantiated by several examples in the literature.

The first examples of strong association between MHC loci and infectious disease resistance came from studies in birds (Briles *et al*., 1977). The chicken MHC is simple and compact with single dominantly expressed class I and class II genes in common MHC haplotypes (Kaufman *et al*., 1995, and 1999, Kaufman, 2000). It has been shown that the chicken haplotype *B21* is associated with high resistance to Marek's disease virus based upon resistance of MHC to virus mediated downregulation (Kaufman, 2000). An interesting feature of this association is that resistance of haplotype *B21* was confirmed in many studies over many years, in which different experimental and commercial chicken flocks were infected with different field and laboratory MDV strains. Similar well-established associations exist between chicken

MHC haplotypes and resistance to Rous sarcoma virus. These data suggest that the chicken MHC determines life and death in response to exposure to certain infectious pathogens (Kaufman, 2000). For humans, the first relevant association was the discovery of a common protective allele *HLA-B53* against severe malaria in the West African Gambians (Hill *et al*., 1991 and 1992). Also in the Gambian population, the class II allele *HLA-DRB1*1302* was associated with protection from persistent hepatitis B virus (HBV) infection in both adults and children (Thursz *et al*., 1995). Similarly, resistance to persistent hepatitis C virus (HCV) is associated with *HLA-DQB*0301* and *HLA-DRB*11*. Strong associations between MHC and disease progression were also noted in HIV infection. It has been shown that *HLA-B35* and *Cw4* were associated with rapid progression to AIDS (Carrington *et al*., 1999) while *HLA-B57*, *B27* and *C14* may be associated with slower progression (Hendel *et al*., 1999, Migueles *et al*., 2000). Recently, several association studies have been reported for mainly salmonid fish species. Similar to chicken, Atlantic salmon posses "minimal essential" MH loci with a single expressed class I (*Sasa-UBA*) and class II loci (*Sasa-DAA/DAB*) (Grimholt *et al*., 2002, Stet *et al*., 2002). Several challenge experiments using the bacterium *Aeromonas salmonicida*, which causes furunculosis as well as with viral pathogens (e.g. infectious salmon anaemia virus, ISAV) showed significant association of MH class I and II alleles or haplotypes with disease resistance (Langefors *et al*, 2001, Grimholt *et. al*., 2003, Kjøglum *et al*., 2006 and 2008). In addition, the non-linkage of MH class I and class II genes in teleost fish species allows for association studies of only class I or only class II MH genes with disease resistance (Grimholt *et al*., 2003, Stet *et al*., 2003).

4.5 MH class II *B* (*DAB*) genes

MHC class II molecules generally have a broader spectrum of action in the immune system than the MHC class I molecules. MHC class I molecules are specifically involved in the presentation of virus-derived peptides to $CD8^+$ T cells, which in turn are able to eliminate virus-infected cells by cytotoxic mechanisms. MHC class II molecules present pathogen-derived peptides to $CD4^+$ T cells, leading to the activation of phagocytic cells, production of antibodies, and activation of immunological traits that are involved in the elimination of parasites and bacteria, and neutralization of viruses (Klein, 1986).

There are observations that suggest a more intense selection pressure and a more rapid evolution of MH class II than class I alleles in fish. Rainbow trout and brown trout share MH class I allele lineages that predate the separation of the genera *Oncorhynchus* and *Salmo*, while MH class II alleles form closely related, species-specific clusters that probably arose after genera separation (Shum *et al*., 2001). The relative importance of MH class II traits over MH class I traits in fish was supported by a study on the evolution of MH class I and class II genes in a natural animal model, the Lake Tana African large barb species (Kruiswijk *et al*., 2005). This species flock shared MH class I alleles but did not share class II alleles, suggesting MH class II genes are more prone to evolutionary changes than MH class I genes. Therefore, as suggested by Kruiswijk *et al*. (2005), class I molecules might play a less important role in the immune system of fish compared to the class II molecules. Also, in a Baltic sea population of Atlantic salmon, the MH class II alpha locus departed significantly from neutrality, while the MH class I locus did not, suggesting that the MH class II locus was under balancing selection (Vasemägi *et al*., 2005). Most recently, the role of natural selection acting on MH class II- but not on MH class I-linked microsatellite markers in wild Atlantic salmon was also described by de Eyto *et al*., (2007). Thus, the faster evolution of MH class II genes which resulted in the presence of relatively high numbers of new alleles as well as the broader spectrum of pathogens which are presented by MH class II molecules, made us focus rather on MH class II than MH class I genes with regard to association studies with disease resistance in common carp.

5. Pathogens and host responses

Representatives of all major groups of pathogens including viruses, bacteria, fungi and parasites have been found in carp (Jeney and Jeney, 1995, Austin and Austin, 1999, Antychowicz, 2004, Siwicki *et al*., 2005, Kozińska *et al*., 2002, Kozińska, 2007)(see also Table 2). Many of these pathogens are facultative pathogens, which implies that they are almost always present in the water and cause disease mostly when the resistance of fish is decreased. Environmental factors such us quality of water, inadequate oxygenation, accumulation of metabolites (primarily the highly toxic ammonia), and high fish concentration also contribute to the development of epizootics caused by pathogens (Jeney and Jeney, 1995, Kazuń and Siwicki, 2005).

Table 2. Parasitic, fungal, bacterial and viral pathogens of common carp. Pathogens used in laboratory challenge tests described in this thesis are underlined (Jeney and Jeney, 1995; modified).

Parasites	**Fungi**	**Bacteria**	**Viruses**
Protozoa	**Oomycetes**	*Aeromonas salmonicida*	*Rhabdovirus carpio*
Ichthyophthirius multifiliis	*Achlya* spp.	*achromogenes*	Herpesvirus CyHV-1
Ichthyobodo spp.	*Saprolegnia* spp.	Motile Aeromonas	Herpesvirus CyHV-3
Cryptobia spp.	**Branchiomycetes**	*Aeromonas hydrophila*	Irodovirus
Trichodina spp.	*Branchiomyces*	*Aeromonas caviae*	
Trypanoplasma borreli	*sanguinis*	*Aeromonas sobria*	
Trypanosoma carassii		*Edwardsiella tarda*	
Chilodonella spp.		*Pseudomonas*	
Eimeria spp.		*fluorescens*	
Myxozoa		*Pseudomonas putida*	
Myxobolus spp.		*Flavobacterium*	
Myxidium spp.		*columnare*	
Helminths		*Flavobacterium*	
Monogenea		*branchiophilum*	
Dactylogyrus spp.		*Flavobacterium*	
Gyrodactylus spp.		*psychrophilum*	
Trematoda		*Streptococcus* sp.	
Diplostomum spp.		*Mycobacterium* sp.	
Posthodiplostomum spp.			
Sanguinicola spp.			
Clonorchis sinensis			
Opistochis felineus			
Cestoda			
Ligula intestinalis			
Bothriocephalus acheilognathi			
Khawia sinensis			
Caryophillaeus spp.			
Nematoda			
Contracaecum spp.			
Philometra spp.			
Camallanus spp.			
Annelida			
Hirudinae			
Mollusca			
Glochidia			
Arthropoda (Crustacea)			
Argulus spp.			
Ergasilus spp.			
Lernaea spp.			
Tracheliastes spp.			

Differences in survival rates of carp lines grown in ponds can be caused by factors like genotype-environment interactions and/or genetic differences in the capacity to display disease resistance. For successful selection, variation in traits controlled by genetic factors is of prime importance. To identify the effect of genetic factors it is necessary to compare genotypes in uniform environmental conditions. We used various disease models to detect potential differences in resistance to specific pathogens, under laboratory circumstances, thereby minimising environmental variation. This approach

allowed for a precise estimation of the inherited magnitude of differences in resistance to infectious diseases, providing information necessary for selective breeding for enhanced immunity. We have selected four different pathogens which are common for carp and responsible for important diseases with associated high losses in carp aquaculture.

5.1. Infection models

5.1.1. *Aeromonas hydrophila*

Aeromonas hydrophila and related species are Gram-negative bacteria, present in freshwater environments throughout the world. *A. hydrophila* has been associated with tail and fin rot, haemorrhagic septicaemia, motile *Aeromonas* septicaemia (MAS), epizootic ulcerative syndrome (EUS) and red-sore disease (Kozinska A, 1999, Cipriano, 2001). Significant losses from such infections occur annually among cultured and feral fishes, also among cyprinids (Nielsen *et al*., 2001). The spreading of the infection is caused by pond water, diseased fish, and diseased frogs, as well as convalescent frogs and fishes (Cipriano, 2001). *A. hydrophila* can cause disease outbreaks when carp are under stress from crowding, low oxygen, high temperature and (Jeney and Jeney, 1995, Nielsen *et al*., 2001). In most cases *A. hydrophila* infection is secondary to a primary viral or parasitic infection, with sudden change in environmental or nutritional status, or with husbandry changes (Roberts, 1993).

5.1.2. Cyprinid herpesvirus- 3 (CyHV-3)

The losses of farmed carp and ornamental domesticated varieties (koi) caused by CyHV-3 have a very negative impact on production of carp farms over the whole world. CyHV-3 is a circular double-stranded DNA virus. Viral DNA length is 295 kbp (Aoki *et al*., 2007). Several reports described the properties of virus, including its host range, the effect of water temperature on disease outcome, development of detection models, and novel attempts at control. Recent studies revealed the presence of CyHV-3 virus in wild populations of dace (*Leuciscus leuciscus*) and vimba (*Vimba vimba*) (Kempter and Bergmann, 2007) as well as in several other European cyprinid species (Sadowski- personal communication). In addition, laboratory tests showed that virus can be transferred from vimba (*Vimba vimba*), tench (*Tinca tinca*) and grass carp

(*Ctenopharyngodon idella*) to common carp when they are keep together in one tank (Kempter- personal communication).

5.1.3. *Trypanoplasma borreli*

Trypanoplasma borreli is the extracellular kinetoplastid parasite infecting cyprinid fish (Wiegertjes *et al*., 1995, Saeji *et al*., 2003). *T. borreli* is widespread in farmed populations of common carp: in some European fish farms parasite infestation in juvenile carp may range between 75 and 100%, especially in fish recovering from their first hibernation period (Lom and Dyková, 1992). *T. borreli* caused anaemia which could ended in mortality of infected fish. Under natural conditions *T borreli* is transmitted by leeches, which lead to an initial rise in blood parasitaemia, followed by a decline of parasite numbers.

5.1.4. *Argulus japonicus*

Until recently, crustacean ectoparasites of fish received relatively little attention when compared to the attention that bacterial and viral diseases have received. It is now recognised however that these parasites can also have a significant negative impact on fish farming operations. This impact can either be the direct result of parasitic feeding or can occur in the form of secondary infections transmitted by the parasites. The disease Argulosis is one of the most readily identified diseases of finfish and there is important impact of damage and stress caused by *Argulus* itself. Fish that have been infected with *Argulus spp.* often show several small haemorrhagic regions (Forlenza *et al.*, 2008, Walker, 2008).

6. Marker assisted selection and breeding for improved disease resistance

In natural populations of fish, reproductivity and resistance to infectious diseases are main factors for success. In domesticated populations, productivity is directly related to the maintenance of good health. As a result, management strategies in domesticated fish species have been largely dependent on veterinary interventions such us vaccinations and the use of antibiotics. Artificial selection in domesticated populations has largely ignored health traits in favour of improving (re)productive traits. The existence of genetic variation in disease resistance is a prerequisite for selective

breeding, which can be achieved by producing specific disease resistant strains, or by including disease resistance parameters into a composite breeding index (Midtlyng *et al*., 2002). Several studies have shown the existence of genetically determined variation in resistance of fish to various pathogens (Gjedrem *et al*., 1991, Fjalestad *et al*., 1993, Kirpichnikov *et al*., 1993, Das Mahapatra *et al*., 2008, Sahoo *et al*., 2008). Therefore, an attractive and sustainable approach to increase resistance of carp breeding populations to infectious disease is genetic improvement.

To date, breeding programmes aimed at genetic improvement and selection for disease resistance increasingly make use of genomic information. The most efficient molecular markers are those that directly affect or are closely linked to the desired trait. Such marker genes can then be used in breeding programmes based on marker assisted selection (MAS). For poorly defined traits, such as disease resistance, it is difficult to select candidate genes that may control the trait because a large number of different pathogens and physiological mechanisms contribute to the observed variation. Although there are a number of DNA-based genetic markers available for use in fish breeding programmes (Liu and Cordes, 2004), the most direct approach is to focus on genes that are likely to be functionally relevant to the trait of interest. One group of such candidate gene markers are the major histocompatibility complex (MHC) genes.

When designing a breeding scheme for improved disease resistance the initial step is to define the genetic and the environmental factors influencing the trait. Disease resistance is, among others, to a large extent dependent on the infectious organisms involved, for which reason we selected four different infection models, as described above. Subsequently, suitable (combinations of) genes need to be selected that can represent markers associated with disease resistance. MH genes determine the activity of the immune system and determine the type of immune response crucially involved in protection against infections and other disease-inducing processes. Ideally, first, the heritability and genetic correlations of the immune genes and disease resistance should be determined. Following a promising result, the selection process should be based on these marker genes. During selection, heritability and genetic correlations should be determined regularly as their frequencies could change. Often, the selection program is first based upon the disease trait itself. This procedure has been used so far in our carp live gene bank.

Aim and outline of this thesis

Genes of the major histocompatibility complex (MHC) are candidate genes for disease resistance because they are crucial elements of adaptive immunity, represent the most polymorphic genes in vertebrate genome and have been associated with resistance to various diseases in several vertebrate species, including fish. With the ultimate aim of gaining better control over diseases of common carp we studied polymorphism of MH class II *B* genes and their association with disease resistance, using different carp lines. We hypothesize that the presence of particular MH class II *B* genotypes or alleles can be advantageous in terms of resistance to different pathogens.

In **chapter 1** we described a history of carp breeding in Poland and characterized the carp live gene bank at the Institute of Ichthyobiology & Aquaculture in Gołysz (Poland). We pointed at the survival rate of fish as an important criterion in carp breeding. We described the MH genes as potential markers, which can be used in selection aimed at increasing resistance of common carp. In **chapter 2** we described the polymorphism of two paralogous groups of MH class II *B* genes present in the carp live gene bank and reported that the *Cyca-DAB1*-like but not the *Cyca-DAB3*-like genes are present in all carp lines tested (**chapter 2**). We reported on the development of a simple, but highly sensitive and fast technique based on single strand conformation polymorphism (SSCP) that allows for association studies of polymorphic *Cyca-DAB1*-like genes and disease resistance in large numbers of carp (**chapter 3**). We described a study on the level of antibody production and co-segregation of the two paralogous groups of *Cyca-DAB* genes with the level of antibody response, in backcrosses to high- and low-responsive parental carp lines (**chapter 4**). Both paralogous groups of *Cyca-DAB* genes seem to represent true MH class II *B* genes with respect to sequence conservation, transcription and control of antibody response (**chapter 4**). We then performed a series of laboratory-based challenge tests using bacterium *Aeromonas hydrophila*, the ectoparasite *Argulus japonicus,* the blood parasite *Trypanoplasma borreli* (**chapter 5**) as well as viral pathogen (CyHV-3, also known as KHV) of high importance for modern carp aquculture (**chapter 6**) and studied association of *Cyca-DAB1*-like polymorphism with resistance to these pathogens. In **chapter 7** we discussed the implications of our findings for the future selection of common carp in live gene bank at Poland.

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CHAPTER 2

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

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Aquat. Living Resour. 2003; **16:** 432-437

Abstract

Regular observation of survival of the carp breeding lines constituting a live 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) class II *B* gene 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* genes fragment comprising part of exon 1, complete intron 1 and almost complete exon 2. Exon 2 encodes for the beta-1 domain which is the most polymorphic fragment of MHC class II molecules. Single-strand conformational polymorphism (SSCP) analysis was applied to detect different MHC class II *B* haplotypes. The analysis revealed the presence of seven different haplotypes occurring with various frequencies.

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 effectors 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, α1, α2, and α3 and is non-covalently associated with 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, α1, α2 and β1, β2, 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 polymerase chain reaction (PCR), using highly degenerate oligonucleotide primers. The design of these primers were based on the comparison between amino acid sequences for class I α3 and class II β2 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 nonclassical 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 β1 domain (Ono *et al*., 1993a). The β1 domain, together with the α1 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.

Materials and methods

Fish

Common carp (*Cyprinus carpio* L.) individuals used in this study were obtained from the live gene bank at the Institute of Ichthyobiology and Aquaculture of the Polish Academy of Sciences in Golysz (Poland). The live 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-B) 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 (Białowąs, 1999; Irnazarow and Białowąs, 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-B) line, for which no data as for average survival are available, yet, was included into analysis.

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

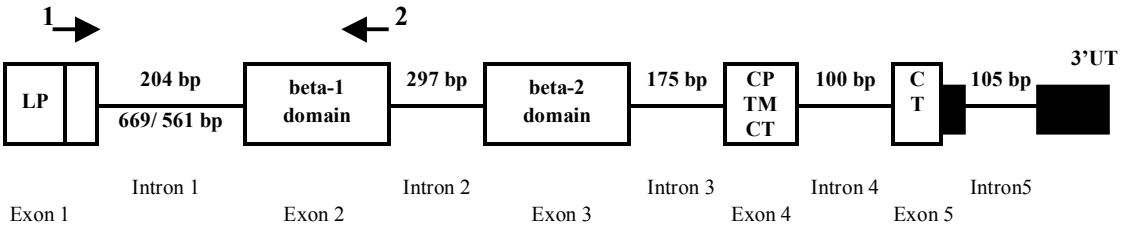
Carp breeding lines (symbol)	Survival (%)(avg ± sd; 1981-1996)
Polish (K)	16.0 ± 20.9
Polish (R6)	33.3 ± 8.5
Polish (R3)	28.7 ± 18.8
Hungarian (R7)	39.6 ± 11.8
Hungarian (R8)	35.3 ± 11.9
Hungarian (R0)	33.9 ± 12.7
German (N)	67.9 ± 19.8
Ukraine (Ur)	47.9 ± 8.9
Lithuanian (Lit-B)	no data*

* The Lithuanian (Lit-B) line was imported to the Institute of Ichthyobiology and Aquaculture in Gołysz (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.

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).

*Cyca-DAB1*01/Cyca-DAB2*01*



*Cyca-DAB3*01/Cyca-DAB4*01*

Figure 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.

Polymerase chain reaction (PCR)

PCR was used to amplify *Cyca-DAB* genes fragment 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).

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

Primer	Sequence (5'- 3')	Position	Comments
OL93-139	CTGATGCTGTCTGCTTTCACTGGAGCA	End of exon 1	Fw primer specific for *Cyca-DAB1*01/DAB2*01*
OL94-23	GATTTGAGCATTATGTTTGCA	End of exon 2	Rev primer specific for *Cyca-DAB3*01/DAB4*01*

Single-strand conformational polymorphism (SSCP)

The SSCP method was used to identify different *Cyca-DAB1* and *Cyca-DAB2* 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 (600V for 3 h at 8 °C) were standardized for optimal resolution of bands.

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 0.01Msilver 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.

Results

Regular score of survival of the 19 carp lines at the Institute of Ichthyobiology and Aquaculture of the Polish Academy of Sciences in Gołysz (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 A., 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 fragment. 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-B), whereas in the remaining carp lines only a single (490 bp) fragment was observed (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).

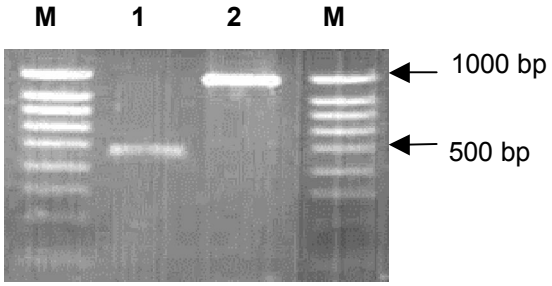


Figure 2. Two PCR products generated by using the forward primer OL93-139 and the reverse primer OL94-23 on genomic DNA of individual carp. 1. 490 bp PCR product representing putative *CycaDAB*01/DAB*02* genes, 2. 900 bp PCR product representing putative *Cyca-DAB*03*/*DAB*04* genes. M. molecular size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

We chose to further analyze *Cyca-DAB1/DAB2* genes. To this end, samples containing the 490 bp PCR products were denatured and separated by the SSCP electrophoresis to identify different *Cyca-DAB1/DAB2* haplotypes. 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/DAB2* genes was the Lithuanian (Lit-B) 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.).

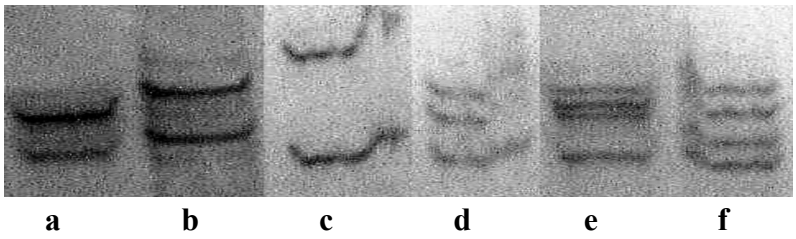


Figure 3. *Cyca-DAB* haplotypes, as detected by single-strand conformational polymorphism (SSCP), present in different breeding lines of common carp (Cyprinus carpio). Characters ('a'-'f') denote haplotypes as unique SSCP patterns. Haplotype 'g' is not depicted.

Table 3. Haplotypes detected by single-strand conformational polymorphism (SSCP), present in different breeding lines of common carp (*Cyprinus carpio*), part of a live 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-B)	3/13			7/13		2/13	1/13
Ukrainian (Ur)	15/15						

Discussion

Freshwater cyprinids are the second major group of finfish cultured in Europe with 301,000 MT 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 the live gene bank at Gołysz (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 major histocompatibility complex 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-*Trypanoplasma borreli*. 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 demonstrate differences in *Cyca-DAB1/Cyca-DAB2* haplotypes between nine breeding lines of common carp that show 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 live gene bank is limited. Irnazarow (1995) examined the genetic variability in seven Polish and Hungarian carp lines, using 13 loci for erythrocyte alloenzymes and transferrin, and found a genetic variation ranging from 0.46 to 0.54.

Major histocompatibility complex 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 J., 1995; Ristow *et al*., 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* genes were described previously (Ono *et al*., 1993b; van Erp *et al*., 1996). There are two unlinked pairs of expressed MHC class II *B* 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 (669 bp 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 fragment comprising part of exon 1, 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.* in preparation).

In order to estimate the level of polymorphism of *Cyca-DAB* genes in our carp lines we applied single-strand conformational polymorphism (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 7 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-B), 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, single-strand conformational polymorphism (SSCP) analysis revealed clear polymorphism for *Cyca-DAB1* and *Cyca-DAB2* genes in different carp lines that represent part of a live 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.

Acknowledgements

We are grateful to Danuta Rajba-Nikiel for technical assistance. Krzysztof Rakus was supported by a Marie Currie Fellowship (QLK5-1999-50416). This work has been supported by the Polish Academy of Sciences.

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CHAPTER

3

Application of PCR-RF-SSCP to study major histocompatibility class II *B* polymorphism in common carp (*Cyprinus carpio* L.)

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Fish Shellfish Immunol. 2008; **24:** 734-744

Abstract

A variety of methods have been applied for the characterization of major histocompatibility (MH) polymorphism in fish. We optimized a technique designated polymerase chain reaction - restriction fragments - single strand conformation polymorphism (PCR-RF-SSCP) for the use of screening large number of individuals for the *Cyca-DAB1* and *Cyca-DAB2* genes polymorphism in common carp. The advantages of this technique are simplicity, high sensitivity and low costs. PCR-RF-SSCP analysis revealed different genotypes consisting of unique combinations of the *Cyca-DAB1* and *Cyca-DAB2* sequences with the number of SSCP bands clearly correlating with the degree of heterozygosity of the *Cyca-DAB1* and *Cyca-DAB2* genes. We found four alleles for *Cyca-DAB1* (**02-*05*) gene but only one allele for *Cyca-DAB2* (**02*) and noted that the *Cyca-DAB2* gene was either homozygous or absent. PCR-RF-SSCP analysis of n=79 carp individuals challenged with *Aeromonas hydrophila* indicated that individuals bearing no *Cyca-DAB2* gene showed higher cumulative mortality and lower bacterial agglutination titers during experiment. We suggest that our PCR-RF-SSCP method can be used to study correlations of different MH class II *B* genotypes/alleles with resistance of common carp to specific pathogens.

Introduction

The Major Histocompatibility Complex (MHC) genes encode for cell-surface glycoproteins, which bind and present endogenous (MHC class I molecules) and exogenous (MHC class II molecules) peptides to T cells, triggering a specific immune response towards the pathogen from which the peptides are derived. One of the most characteristic features of the MHC genes is high polymorphism, which results in the presence of numerous alleles and as a consequence, numerous haplotypes within a population. The highest polymorphism is observed in those exons that encode the peptide binding region of the MHC molecule (Klein, 1986).

Allelic MHC molecules bind and present (pathogen-derived) peptides that conform to unique peptide-binding specificities in more or less successful ways. Thus, the response of an organism towards certain pathogens can be influenced by the MHC alleles present in different haplotypes (Jeffery and Bangham, 2000, Nikolich-Žugich *et al*., 2004). The best known associations between MHC genotype and disease resistance have been described for chicken (Briles *et al*., 1977, Kaufman and Wallny, 1996) and humans (Hill *et al*., 1991). More recently, evidence for correlations between major histocompatibility (MH) polymorphism and disease resistance was found for a limited number of mainly salmonid fish species (Langefors *et al*., 2001, Palti *et al*., 2001, Grimholt *et al*., 2003, Miller *et al*., 2004, Wynne *et al*., 2007). In teleost fish species, major histocompatibility (MH) class I and class II genes reside on different chromosomes and segregate independently (Bingulac-Popovic *et al*., 1997, Sato *et al*., 2000, Grimholt *et al*., 2002, Phillips *et al*., 2003), which allows for association studies of only class I or only class II with disease resistance (Grimholt *et al*., 2003, Stet *et al*., 2003).

In common carp (*Cyprinus carpio* L.) two pairs of expressed MH class II *B* loci have been described, which include *Cyca-DAB1*01*/*Cyca-DAB2*01* (Ono *et al*., 1993a) and *Cyca-DAB3*01*/*Cyca-DAB4*01* (van Erp *et al*., 1996) sequences. These two pairs of loci segregate independently from each other. In addition, in some carp individuals transcripts of only *Cyca-DAB1*01*/*Cyca-DAB2*01* or only *Cyca-DAB3*01*/*Cyca-DAB4*01* sequences have been reported whereas in others transcripts of all four sequences have been found (Stet *et al*., 1997). Intron 1 of the *Cyca-DAB3*01* and *Cyca-DAB4*01* sequences has a non-consensus 5' splite-site (GC at the 5' intron border) (van

Erp *et al*., 1996) which could cause lower upregulation of expression of encoded protein (Haviland *et al*., 1991). This could possibly explain why the presence of the *Cyca-DAB3*01* and *Cyca-DAB4*01* seemed to correlate with a lower antibody response whereas the *Cyca-DAB1*01* and *Cyca-DAB2*01* sequences were associated with increased immune responsiveness (Wiegertjes *et al*., 1996). So far, no studies on the level of transcription of *Cyca-DAB1*01*/*Cyca-DAB2*01* genes versus *Cyca-DAB3*01*/*Cyca-DAB4*01* genes have been reported. Recently, we have performed preliminary studies with real-time quantitative PCR using primers specific for *Cyca-DAB1*01/Cyca-DAB2*01* genes or *Cyca-DAB3*01/Cyca-DAB4*01* genes which do not indicate a large difference in basal transcription between these two pairs of genes in immunologically relevant organs (unpublished data). Certainly, these gene-specific primers for RT-qPCR will allow us to study MH class II *B* gene transcription during, for example, infection of common carp.

In the present study we focused on the *Cyca-DAB1* and *Cyca-DAB2* genes polymorphism with the long-term aim of finding correlations with disease resistance. We decided to analyze the polymorphism of exon 2 of the *Cyca-DAB1* and *Cyca-DAB2* genes. Exon 2 encodes for the beta-1 domain of the class II molecule and is the most polymorphic exon of MHC class II *B* genes. A variety of methods have been applied for the characterization of MH polymorphism in fish. Although sequencing may be regarded the most accurate and precise method for investigating nucleotide variations, screening of large numbers of samples is limited by high costs of this type of analysis. Thus, for large-scale analyses alternative mutation detection techniques have been applied which include denaturing gradient gel electrophoresis (DGGE) (Miller *et al*., 1999, Langefors *et al*., 2000), reference strand-mediated conformation analysis (RSCA) (Noakes *et al*., 2003), single-strand conformation polymorphism (SSCP) (Binz *et al*., 2001, Rakus *et al*., 2003), restriction fragment length polymorphism (RFLP) (Langefors *et al*., 1998 and 2000, Quiniou *et al*., 2005) and use of specific minisatellite markers localized in the 3' UTR of class II *A* genes (Stet *et al*., 2002, deEyto *et al*., 2007).

One of the most popular methods for the detection of nucleotide polymorphism is SSCP analysis (Orita *et al*., 1989). The advantages of this method are technical simplicity and relatively high sensitivity. SSCP analysis is based on differences in mobility of single-stranded (ss)-DNA fragments during electrophoresis through a non-

denaturing polyacrylamide gel. The electrophoretic mobility of ss-DNA fragments is determined by their three-dimensional conformation, which depends on the nucleotide sequence composition. Even single base substitutions in ss-DNA fragments can cause a difference in three-dimensional conformation and, as a consequence, difference in electrophoretic mobility.

In our study, the DNA fragment analyzed included exon 2 of the *Cyca-DAB1* and *Cyca-DAB2* genes and was 350 bp in length. One limitation of the SSCP method is that the number of detectable mutations tends to decrease when the length of analyzed fragments increase. The sensitivity of the SSCP method is greater than 90% for DNA fragments of about 200 bp but lower than 80% when DNA fragments of 400 bp or longer are analyzed (Nataraj *et al*., 1999). For our studies we chose to first cleave our 350 bp PCR fragment by enzyme restriction into two shorter fragments, and then perform a SSCP analysis. This combined method is named polymerase chain reaction - restriction fragments- single strand conformation polymorphism analysis (PCR-RF-SSCP) (Tawata *et al*., 1996) and has been used for the analysis of human mitochondrial DNA mutations (Kurihara *et al*., 1999), DNA polymorphism studies on the parasite *Trichinella* (Wu *et al*., 2000) and for studies on mutations detection in rice waxy mutants (Sato and Nishio, 2003).

The aim of the present study was to establish a fast, cost-effective and sensitive method to detect MH class II *B* polymorphism in carp, focusing on exon 2 of the *Cyca-DAB1* and *Cyca-DAB2* genes. We also performed initial studies on putative correlations between the *Cyca-DAB1* and *Cyca-DAB2* polymorphism and disease resistance to examine whether the newly developed method could be applied for future association studies of carp MH polymorphism and disease resistance.

Materials and methods

Fish

European common carp (*Cyprinus carpio carpio* L.) individuals used in this study were obtained from the live gene bank of the Institute of Ichthyobiology and Aquaculture in Gołysz (Poland). In this study we refer to European common carp as common carp. The live gene bank includes 19 different carp lines of various geographical origins. For our study we have chosen 159 individuals (n=79 experimental group, n=80 control group)

from eight carp lines (Polish lines 'K' and 'R2', Hungarian lines 'R0', 'R7', and 'R8', Israeli line 'DOR-70', Ukraine line 'Ur' and German line 'N') that have shown clear differences in survival under pond conditions (Pilarczyk *et al*., 1998). Fish were grown in recirculation systems and fed at a rate of 1% body weight/day. Fish were twelve months of age (mean weight 307 g) at the time of infection.

Challenge experiment

Three weeks prior to challenge fish were moved to a quarantine unit and randomly distributed over two identical recirculating systems of 10 aquaria at 21±2 °C. Virulent *Aeromonas hydrophila,* strain BSK-10 were used (Nielsen *et al*., 2001). Bacteria were grown on tryptose-soy agar (TSA) at 28 °C for 24h. The colonies were used for inoculation of 250 ml of Luria Broth (LB) medium at 28 °C with shaking at 200 rpm to obtain high numbers for the challenge experiment. Concentration of bacteria was estimated by optimal density measurement at 540 nm using the McFarland scale. Exact number of viable bacteria was determined by plate count analysis.

Fish (n=79) were intraperitoneally injected with $3x10^7$ colony forming units of *A. hydrophila* in 100 µl of phosphate buffered saline (PBS; BIOMED, Lublin, Poland) per fish. Control fish (n=80) were injected with 100 µl of sterile PBS per fish. Mortality was monitored daily during the experiment.

Bacterial agglutination test

Serum was collected by caudal vena puncture at day 0 and at 5, 11, 18, 25 and 50 days post-injection with *A. hydrophila*. The bacterial agglutination test was carried out in 96-well round-bottomed microtitre plates (MEDLAB, Raszyn, Poland). 50 µl of phosphate buffered saline (PBS; BIOMED, Lublin, Poland) was added to the columns 2-12 and 50 µl of serum was added to the columns 1 and 2. Using a multi-channel pipetter the contents of the column 2 wells were serially diluted (1:2) through column 11. This resulted in dilutions ranging from 1 to 1/1024 and 50 µl in every well. The 50 µl of PBS only in column 12 served as a negative control. A volume of 50 µl of bacterial suspension (10^9 formalin-killed *A. hydrophila*/ml PBS) was added to each well and plates were incubated at 25 °C for 12 hours. A clear button of antigen at the bottom

indicated a negative reaction. Titers were recorded as the negative $\log_2$ of the last well in which visible agglutination occurs and scored as 1 to 11.

Polymerase chain reaction

PCR was used to amplify the complete exon 2 of the *Cyca-DAB1* and *Cyca-DAB2* genes. Forward and reverse primers were chosen as to amplify both *Cyca-DAB1* and *Cyca-DAB2* genes. Forward primer DAB12-F (5'-TCTGACATAACTGTAATGCTGC-3') was complementary to the end of intron 1 of both *Cyca-DAB1* and *Cyca-DAB2* genes and reverse primer DAB12-REV (5'-CAGGAGAGATCAGAGTCTTG-3') was complementary to the beginning of intron 2 of both *Cyca-DAB1* and *Cyca-DAB2* genes. Total genomic DNA was extracted from fin clips by proteinase K digestion and purification using phenol:chlorophorm:isoamyl alcohol (Sambrook and Russell, 2001). PCR was carried out in a total volume of 25 µl containing 200-500 ng of template DNA, 200 nM of each primer, 200 µM of each dNTP, 1.5 mM $MgCl_2$ and 1 unit of *REDTaq* polymerase (Sigma, Germany). The PCR profile included an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, and a final extension step at 72 °C for 7 min. PCRs were performed using a Techne machine (Progene, UK).

PCR-RF-SSCP analysis

Before SSCP analysis, 6 µl of PCR product was mixed with 0.5 µl (5 units) of restriction enzyme *Pst* I and 1 µl of buffer SH (10x) and incubated at 37 °C for 2 h. Next, 14 µl of denaturing buffer (95% formamide, 0.1% bromophenol blue, 0.1% xylene cyanole FF, 1 mM EDTA, 10 mM NaOH) was added to the samples. Samples were denatured at 95 °C for 5 min and after that immediately cooled on ice and loaded on 0.7 mm thick, 9% polyacrylamide gel (9%T, 2%C) containing 5% of glycerol. Electrophoresis was performed in 1 x TBE buffer under optimized conditions (600V for 2h 15 min at 6 °C). Prior to silver staining (Herring *et al.*, 1982) gels were fixed with 10% ethanol-0.5% acetic acid for 1 h, rinsed twice in deionized water and soaked in 0.01 M silver nitrate for 30 min. Next, gels were rinsed twice in deionized 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 soaking in solution of 30% methanol and 5% glycerol, gels were scanned. Identification of the *Cyca-DAB1/Cyca-DAB2* genotypes was performed based on SSCP bands patterns of the longer fragment (239 bp) of digested PCR product.

Cloning and sequencing

For each *Cyca-DAB1/Cyca-DAB2* genotype PCR products from 2 independent PCR reactions were ligated into pGEM-T Easy vector (Promega, Madison, USA) and cloned into *JM109* competent cells (Promega, Madison, USA) according to the standard protocol. Plasmid DNA was isolated from single positive colonies by alkali lyses and ethanol precipitation. A number of at least eight clones for each PCR product were selected for sequencing using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, USA) and the T7 primer in an ABI 377 automated sequencer (Applied Biosystem, USA).

All sequences were examined and corrected using BioEdit 7.0.5.3 software (Hall, 1999) available at http://www.mbio.ncsu.edu/BioEdit/bioedit.html. Nucleotide sequences and predicted amino-acids residues were aligned using ClustalX (Thompson *et al*., 1997) and the phylogenetic trees were constructed using MEGA 2.1 software (Kumar *et al*., 2001). Phylogenetic relationships using p-distances for amino acids sequences with confidence in individual nodes assessed by 1,000 bootstrap replications were constructed by the neighbor-joining algorithm (Saitou and Nei, 1987). The analysis of molecular variance AMOVA (Weir and Cockerham, 1984) and the exact test of sample differentiation based on genotype frequencies (Raymond and Rousset, 1995) were performed using the Arlequin version 3.1 software available at http://cmpg.unibe.ch/software/arlequin3/.

Statistical analysis

All statistical analysis were performed by using STATISTICA version 6.0 and MedCalc version 9.3.0.0 software. Calculations were based on a significance level of $\alpha = 0.05$. Relationships between cumulative mortality, individual genotype and heterozygosity status, was accessed using Kruskal-Wallis test. Relationship between bacterial agglutination titers with carp origin and MH loci configuration was tested by two and

one-way ANOVA using an area under the curve (AUC) values. Bonferroni corrected significance levels and the Tukey post-hoc tests were used for multiple comparisons and calculations of pairwise differences.

Results

PCR-RF-SSCP analysis

Specific primers were designed to amplify the *Cyca-DAB1* and *Cyca-DAB2* gene fragments. The amplified PCR products were 350 bp in size and included a short sequence of intron 1, the complete exon 2 and a short sequence of intron 2. Different PCR products from different carp lines, representing unique *Cyca-DAB1* and *Cyca-DAB2* sequences, were used for optimization of the PCR-RF-SSCP technique. These PCR products differed between 1 and 29 nucleotide substitutions. The enzyme *Pst* I, which restriction site (5'-CTGCA/G-3') is located in the conserved region of exon 2 of the *Cyca-DAB1* and *Cyca-DAB2* genes, was chosen to digest the PCR products into two fragments of 239 bp and 111 bp in size. After several optimization steps, 9% polyacrylamide gel (9%T, 2%C) containing 5% of glycerol (electrophoresis at 600V, 2h 15min, 6 °C) was found to give the best resolution of the different SSCP band patterns. Identification of the *Cyca-DAB1/Cyca-DAB2* genotypes was based on the SSCP band patterns of the longer fragment (239 bp) of digested PCR product.

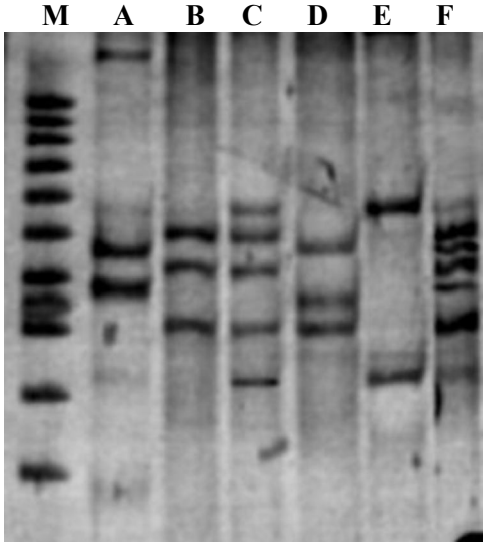

Figure 1. Major histocompatibility *Cyca-DAB1/Cyca-DAB2* genotypes, as detected by PCR-RF-SSCP. Different genotypes are characterized by unique SSCP patterns and named A-F. M=marker (TrackIt 100bp DNA Ladder, Invitrogen).

By the use of optimized PCR-RF-SSCP analysis, six different *Cyca-DAB1/Cyca-DAB2* genotypes (A-F, see Fig. 1) were identified in n=79 carp individuals. The most common genotype was genotype C (overall frequency 0.33), present in all carp lines except lines German 'N' and Israeli 'DOR-70' (Table 1). The overall frequency of other genotypes varied between 0.01 and 0.28. The highest number of different *Cyca-DAB1/Cyca-DAB2* genotypes within a single carp line (n=10 individuals tested per carp line) was 4 genotypes observed in the Hungarian carp lines 'R7' and 'R8' (Table 1).

Table 1. Distribution and frequency of major histocompatibility *Cyca-DAB1/Cyca-DAB2* genotypes as determined by PCR-RF-SSCP analysis in eight common carp (*Cyprinus carpio* L.) lines (n=10 individuals/carp line). Genotypes are A-F as denoted in Fig 1.

Carp lines (symbol)	*Cyca-DAB1/Cyca-DAB2* genotypes					
	A	B	C	D	E	F
Polish (K)		0.4	0.3		0.3	
Polish (R2)[a]			0.55	0.45		
Hungarian (R0)		0.1	0.9			
Hungarian (R7)	0.1	0.2	0.3		0.4	
Hungarian (R8)	0.1		0.4		0.4	0.1
Israeli (DOR-70)		0.7			0.3	
Ukrainian (Ur)		0.8	0.2			
German (N)	0.4			0.6		

[a] n=9 individuals

Cyca-DAB1/Cyca-DAB2 sequence analysis

Unique *Cyca-DAB1/Cyca-DAB2* genotypes identified by PCR-RF-SSCP were cloned and sequenced. Sequence analysis of exon 2 revealed the presence of four *Cyca-DAB1* (**02-*05*) (GenBank accession numbers EU203666- EU203669) and one *Cyca-DAB2* (**02*) (GenBank accession number EU203670) sequences, which were aligned with the previously known *Cyca-DAB1*01* and *Cyca-DAB2*01* sequences (Fig. 2). Among the 276 nucleotides, 36 (13%) nucleotide positions were variable (Fig 2). Nucleotide sequences were translated into amino acids (Fig 3). The highest variability (64%) was observed in the positions known to code for peptide binding residues (PBR)

in humans, as indicated in Fig. 3, spanning a region of 24 aa of which 14 (58%) aa were variable. Variability at each position was limited to a maximum of three different amino acids. Almost all (35 out of 36) nucleotide substitutions resulted in changes at amino acids level and included both PBR (n=23) and non-PBRs (n=12). The neighbour-joining tree (Fig. 4) indicates that the two *Cyca-DAB2* alleles are divergent from the *Cyca-DAB1* alleles and are found in a separate cluster supported by a high bootstrap value.

Table 2. Frequency of major histocompatibility *Cyca-DAB1/Cyca-DAB2* genotypes as determined by PCR-RF-SSCP analysis in n=79 common carp (*Cyprinus carpio* L.) individuals. Genotypes are A-F as denoted in Fig.1 were determined by sequencing and named in Fig. 2.

Symbol	Genotypes		Number of fish	Frequency
	Cyca-DAB1	*Cyca-DAB2*		
A	*DAB1*04/DAB1*04*	not present	6	0.075
B	*DAB1*02/DAB1*02*	*DAB2*02/DAB2*02*	22	0.278
C	*DAB1*02/DAB1*05*	*DAB2*02/DAB2*02*	26	0.329
D	*DAB1*03/DAB1*03*	*DAB2*02/DAB2*02*	10	0.126
E	*DAB1*05/DAB1*05*	not present	14	0.177
F	*DAB1*02/DAB1*04*	*DAB2*02/DAB2*02*	1	0.012

Each *Cyca-DAB1/Cyca-DAB2* genotype as determined by PCR-RF-SSCP consisted of unique combinations of the *Cyca-DAB1* and *Cyca-DAB2* sequences (Table 2). Genotypes A and E were shown homozygous for the *Cyca-DAB1* gene and no *Cyca-DAB2* gene was observed (14 clones sequenced for each genotype). Genotypes B and D consisted of homozygous *Cyca-DAB1* and homozygous *Cyca-DAB2* genes, whereas genotypes C and F consisted of heterozygous *Cyca-DAB1* and homozygous *Cyca-DAB2* genes. We found only one allele for *Cyca-DAB2* gene (*Cyca-DAB2*02*) and noted that the *Cyca-DAB2* gene was either homozygous or absent. We could not amplify the *Cyca-DAB2* sequences from 20 out of 79 individuals.

Clearly, the degree of heterozygosity of the *Cyca-DAB1* and *Cyca-DAB2* genes correlated with the number of SSCP bands (see also Fig. 1): (i) two SSCP bands represented homozygous *Cyca-DAB1* and absence of *Cyca-DAB2*, (ii) three SSCP bands represented homozygous *Cyca-DAB1* and homozygous *Cyca-DAB2*, and (iii) five SSCP bands represented heterozygous *Cyca-DAB1* and homozygous *Cyca-DAB2*. We

observed only one SSCP band for the *Cyca-DAB2*02* allele, which could be due to a very similar conformation of two DNA strands of this sequence. However there is no theoretical basis for predicting the three-dimensional structure of single stranded DNA under the conditions of SSCP electrophoresis.

```
Cyca-DAB1*01 CTAATGGTTACTACCATTCTTGGTGGACTAAATGCATCCACAGCTCCCGTGATTTCAGTG
Cyca-DAB1*02 ..............................................................
Cyca-DAB1*03 ..................C...........................................
Cyca-DAB1*04 .......A.......G....C......A...................................
Cyca-DAB1*05 .......A......TGG...C.....G........T.....G.....................
Cyca-DAB2*01 .......A.......G....C......A...................................
Cyca-DAB2*02 .......A.......G....C......A...................................

                              30                              40
Cyca-DAB1*01 ACATGGTGTACATTGATAACTATATCTTCAATAAAGATGTGTACATACAGTTCAACAGCA
Cyca-DAB1*02 ..............................................................
Cyca-DAB1*03 ..............................................................
Cyca-DAB1*04 ..............................................................
Cyca-DAB1*05 .........T.G.....................................................
Cyca-DAB2*01 .........T.G.............................C.....................
Cyca-DAB2*02 .........T.G.............................C.....................

                            50                                60
Cyca-DAB1*01 CTGTGGGGGAGTATGTGGGGTACACTGCACTTGGAGTATATAACGCAGAGAGATTTAACA
Cyca-DAB1*02 ..............................................................
Cyca-DAB1*03 ..................................................TT..CG....
Cyca-DAB1*04 ............T..................A...................TT..GG....
Cyca-DAB1*05 ...............................A.......C............T.........
Cyca-DAB2*01 ............T.........................C............TT..GG....
Cyca-DAB2*02 ............T.........................C............TT..GG....

                           ▼ ▼ ▼ ▼ ▼                              80
Cyca-DAB1*01 AAGATCCCAACATTCTGCAGCAAGAGAGAGCTCAGGTGGAGAGATACTGCAAACATAATG
Cyca-DAB1*02 ..............................................................
Cyca-DAB1*03 ..............................................................
Cyca-DAB1*04 ..................................G.............................
Cyca-DAB1*05 ............C.....................G.............................
Cyca-DAB2*01 .....A...C.GG..........................CTC..T..................
Cyca-DAB2*02 .....A...C.GG..........................CTC..T..................

                            90
Cyca-DAB1*01 CTGAACTCTATCAGTCAGCGATCCGTGATAAAACAG
Cyca-DAB1*02 ........................GC...........
Cyca-DAB1*03 ..C..A..AGA............GC...........
Cyca-DAB1*04 ........................GC...........
Cyca-DAB1*05 ..C..AA.AGA............GC...........
Cyca-DAB2*01 ..C..A..AGA.............................
Cyca-DAB2*02 ..C..A..AGA............GC...........
```

Figure 2. Alignment of nucleotide sequences of exon 2 of common carp (*Cyprinus carpio* L.) major histocompatibility *Cyca-DAB1* and *Cyca-DAB2* genes. Novel sequences were aligned with known sequences *Cyca-DAB1*01* and *Cyca-DAB2*01* (Ono *et al*., 1993a, underlined). Dots indicate identity to *Cyca-DAB1*01*. The restriction site for *Pst* I (5'-CTGCA/G-3') is indicated by small arrows. Numbering denotes codon numbers within putative mature protein.

```
                10        20        30        40        50        60
                * * *           * * *     **        *         *  **
Cyca-DAB1*01 NGYYHSWWTKCIHSSRDFSDMVYIDNYIFNKDVYIQFNSTVGEYVGYTALGVYNAERFNK
Cyca-DAB1*02 ............................................................
Cyca-DAB1*03 ......R.....................................................LS..
Cyca-DAB1*04 ....R.R.N.................................F.....H......LW..
Cyca-DAB1*05 ....W.R.A..F.G........FV.......................H..H...I...
Cyca-DAB2*01 ....R.R.N.............FV.........H.........F........H...LW..
Cyca-DAB2*02 ....R.R.N.............FV.........H.........F........H...LW..

                70        80        90
              * *  **  *   *  **  ** **
Cyca-DAB1*01 DPNILQQERAQVERYCKHNAELYQSAIRDKT
Cyca-DAB1*02 ........................A...
Cyca-DAB1*03 ....................QIR....A...
Cyca-DAB1*04 ..........E................A...
Cyca-DAB1*05 ...L......E.........QNR....A...
Cyca-DAB2*01 .TTG........DSF.....QIR........
Cyca-DAB2*02 .TTG........DSF.....QIR....A...
```

Figure 3. Alignment of deduced amino acid sequences of exon 2 of common carp (*Cyprinus carpio* L.) major histocompatibility *Cyca-DAB1* and *Cyca-DAB2* genes. Novel sequences were aligned with known sequences *Cyca-DAB1*01* and *Cyca-DAB2*01* (Ono *et al*., 1993a, underlined). Dots indicate identity to *Cyca-DAB1*01*. Asterisks indicate amino acids known to interact with peptide in the human MHC class II beta chain (Brown *et al.,* 1993). Numbering is as in Fig. 2.

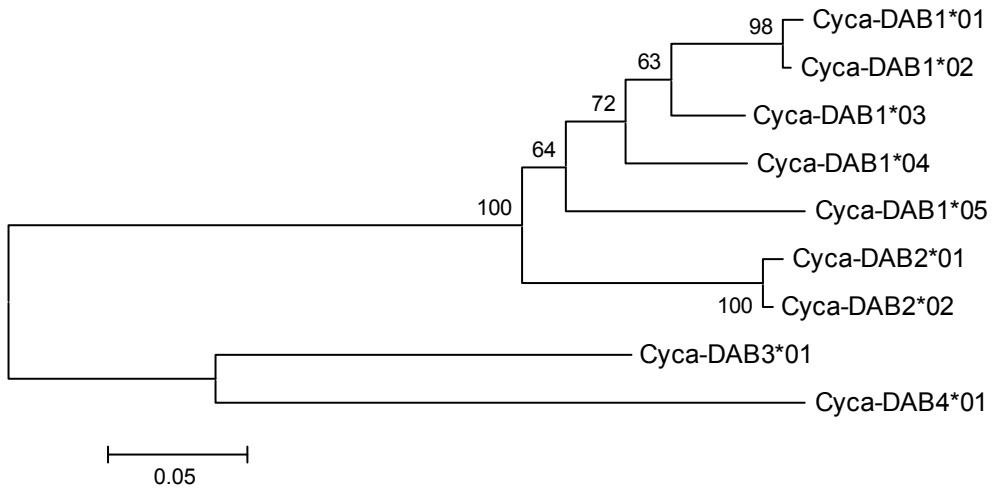

Figure 4. Neighbor-joining tree based on *Cyca-DAB* amino acid sequences. The topology of the tree is supported by bootstrap *P* values (1000 iterations). *Cyca-DAB1*01* and *Cyca-DAB2*01* (Ono *et al.,* 1993a) and *Cyca-DAB3*01* and *Cyca-DAB4*01* (vanErp *et al.*, 1996) alleles are included.

To estimate population differences between the carp lines we performed an Analysis of Molecular Variance (AMOVA). AMOVA analysis was performed only for the *Cyca-DAB1* locus and showed that most (80.2% of the total) of the variation was apparent within individuals. The proportion of variation attributable to differences between carp lines (18.9%) was much less pronounced. The test of sample differentiation based on allele frequencies and molecular differences showed that the most distinct lines were German carp line 'N', Polish carp line 'R2' and Hungarian carp line 'R0'.

Verification of PCR-RF-SSCP method for studying associations with disease resistance

To verify the suitability of the newly developed PCR-RF-SSCP genotyping method for correlation studies on the *Cyca-DAB1* and *Cyca-DAB2* polymorphism with differences in disease resistance, all MH-typed fish (n=79 individuals) were individually injected with *A. hydrophila*, ensuring infection of each fish, and their individual survival/mortality were scored during challenge. During the challenge, fish showed frequent clinical signs of disease, such as darkened skin and ulcerative skin lesions. Mortality was most frequent up to 8-9 days post-infection. Differences between carp lines in cumulative mortality were not statistically significant. Average cumulative mortality over all carp lines was 38±13%.

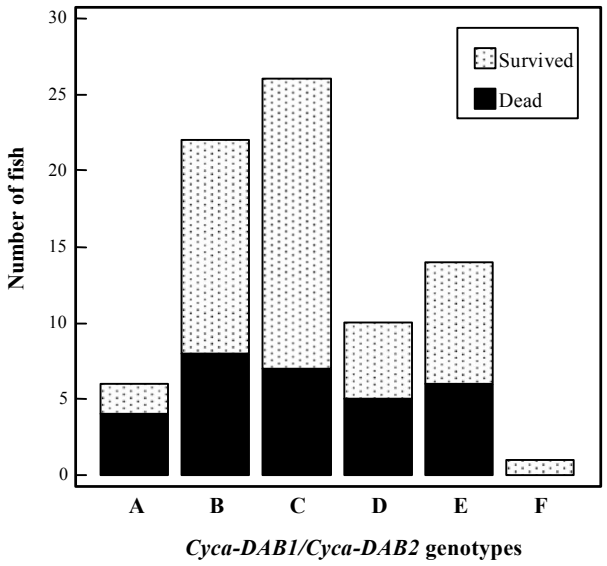

Figure 5. Distribution of six different *Cyca-DAB1/Cyca-DAB2* genotypes revealed by PCR-RF-SSCP analysis in dead and survived common carp individuals challenged with *Aeromonas hydrophila.*

Notably, genotypes C was present in an almost three times higher frequency in surviving fish (n=19) than in mortalities (n=7) (Fig. 5). In addition, analysis of the correlations between cumulative mortality and status of the *Cyca-DAB1* gene (heterozygous/homozygous) and the *Cyca-DAB2* gene (presence/absence) showed, although not significantly, that the lowest cumulative mortality (26%) was observed in the group of fish (n=27) carrying both a heterozygous *Cyca-DAB1* gene and a homozygous *Cyca-DAB2* gene. The cumulative mortality observed in the group of fish (n=32) with a homozygous *Cyca-DAB1* and a homozygous *Cyca-DAB2* gene as well as the cumulative mortality in the group of fish (n=20) with a homozygous *Cyca-DAB1* gene only and no *Cyca-DAB2* gene was higher (40% and 50%, respectively). Interestingly, the bacterial agglutination titer was signifantly lower in fish having no *Cyca-DAB2* gene compared to the fish carrying *Cyca-DAB2* gene (Fig. 6). This suggested that the presence of the *Cyca-DAB2* gene could be advantageous in developing immune-based resistance after bacterial challenge.

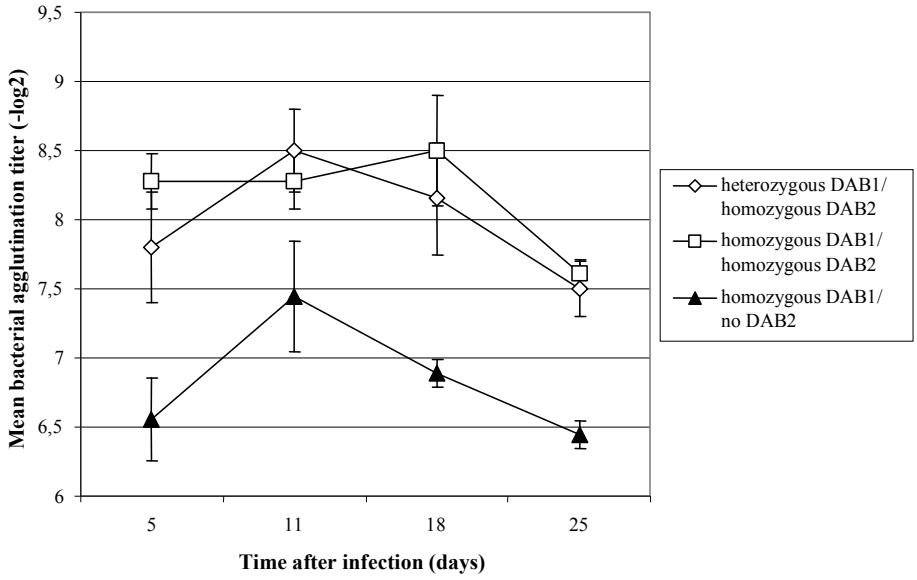

Figure 6. Mean bacterial agglutination titre of three groups of common carp (*Cyprinus carpio* L.) characterized by different status of *Cyca-DAB1* (heterozygosity/homozygosity) and *Cyca-DAB2* (presence/absence) genes, infected with *Aeromonas hydrophila.* Blood samples were taken from fish (n= 49) available for the full period of the experiment (day 0-50).

Discussion

Several reports show that disease resistance could be genetically determined in fish (Standal and Gjerde, 1987, Gjedrem *et al*., 1991, Kirpichnikov *et al*., 1993) and that the major histocompatibility (MH) genes may play a major role in this process (Grimholt *et al*., 2003, Wiegertjes *et al*., 1996). We established a fast, cost-effective and sensitive method to detect MH class II *B* polymorphism in common carp. We have shown that the combined method designated polymerase chain reaction - restriction fragments - single strand conformation polymorphism (PCR-RF-SSCP) can reliably be applied for the investigation of the *Cyca-DAB1* and *Cyca-DAB2* polymorphism. Although the highest per cent of detected mutations by SSCP analysis is indicated for short DNA fragments (about 200 bp) (Nataraj *et al*., 1999), we confirm that the analysis of longer DNA fragments without loss of sensitivity is possible by digestion of the analyzed DNA fragments with restriction enzyme before the SSCP analysis. The current study demonstrated that appropriate restriction of 350 bp in length DNA fragments allowed us to distinguish between all known *Cyca-DAB1* and *Cyca-DAB2* sequences by a different SSCP banding pattern. The optimized PCR-RF-SSCP technique provided reliable and repeatable results and can be used for screening of large number of samples for new alleles prior to sequencing. We suggest that this combined method could be applied for future association studies of carp MH polymorphism and disease resistance.

PCR-RF-SSCP analysis of n=79 carp individuals showed the presence of six different *Cyca-DAB1/Cyca-DAB2* genotypes, with frequencies ranging between 0.01 and 0.33. Our previous preliminary examination of putative polymorphism of MH class II *B* genes among different carp lines, based on SSCP analysis of 490 bp PCR fragment, covering the whole intron 1 and only part of exon 2, also showed differences in frequencies among revealed genotypes. The most frequently occurring genotype was present with a frequency of 0.56 and was found in seven out of nine carp lines examined whereas two genotypes were found only in a very low number of individuals of specific carp lines (one and two individuals respectively) (Rakus *et al*., 2003). Differences in frequencies of MH haplotypes have also been described in other fish species. Stet *et al*. (2002) described seven unique MH class II *Sasa-DAA/Sasa-DAB* haplotypes in 84 Atlantic salmon individuals, with frequencies ranging from 0.01 to 0.49.

Sequence analysis showed that each *Cyca-DAB1/Cyca-DAB2* genotype consisted of unique combinations of the *Cyca-DAB1* and *Cyca-DAB2* sequences. Four novel *Cyca-DAB1* (**02-*05*) and one novel *Cyca-DAB2* (**02*) sequences were found. To our knowledge, this is the first paper describing MH class II *B* allelic variation in different carp lines. Studies on different teleost fish populations revealed that MH genes generally show relatively high levels of genetic differentiation (Ono *et al*., 1993b, Dixon *et al*., 1996, Hedrick and Parker, 1998, Kim *et al*., 1999, Dorschner *et al*., 2000). We detected a total number of 4 *Cyca-DAB1* and 1 *Cyca-DAB2* alleles in 8 different carp lines. Miller *et al.* (2001) found a total number of 11 different class II *B* sequences (exon 2) in 31 populations of Sockeye salmon. This suggests that screening a higher number of carp lines could lead to the detection of further allelic MH class II *B* diversity in carp.

Noteworthy, the *Cyca-DAB2* gene was either homozygous or absent. In theory, the absence of a PCR product could be due to variation in the sequence spanning the primer regions (false negative). Southern blot analysis would provide more information on the genomic presence of *Cyca-DAB2* gene only when *Cyca-DAB2*-specific probe could be used, which is not possible due to the relatively high sequence similarity between *Cyca-DAB1* and *Cyca-DAB2* genes. Southern blot analysis would not overrule a false negative result obtained by PCR because additional bands in an expected restriction pattern could not be positively assigned to *Cyca-DAB2* gene due to for example, possible occurrence of *Cyca-DAB1* allelic variants within the same individual. Therefore, PCR analysis of genomic DNA still remains the method of choice to confirm the absence of *Cyca-DAB2* gene. In fact, when we designed new primers on conserved regions of *Cyca-DAB1* and *Cyca-DAB2* genes to amplify a product overlapping the polymorphic exon 2 region examined in the present study, again we were unable to find a *Cyca-DAB2* sequence, even after sequencing more than 40 clones per individual (unpublished data). In human it is well established that not all people contain the same number of the *HLA-DRB* genes. Only the *DRB1* gene is present in all people but in some human individuals the *DRB3*, *DRB4* or *DRB5* can be detected in addition to the *DRB1*. Thus, there can be between one and four different *HLA-DR* molecules expressed in humans (Parham, 2000). For fish species other than the common carp variation in the number of MH loci has been described as well (Sato *et al*., 1998, Málaga-Trillo *et al*.,

1998, Figueroa *et al*., 2001). Although it will remain very difficult to positively confirm the individual absence of the *Cyca-DAB2* gene, we believe that variation in the number of MH class II *B* loci is not uncommon in carp.

The highest variability in the *Cyca-DAB* nucleotide sequences was observed in the positions known to code for peptide binding residues (PBR) in humans. Because the PBR of MH class II molecules have not been defined in common carp, we used the model described for the human *HLA-DRB* (Brown *et al*., 1993) and its application for cyprinid fish (Dixon *et al*., 1996) to show the putative PBRs in the beta-1 domain of common carp. Most of the positions defined as PBRs in humans corresponded to the most variable sites in the *Cyca-DAB1* and *Cyca-DAB2* sequences. Nevertheless, the very high level of non-synonymous substitutions (35 out of 36) in both PBR (n=23) and in non-PBRs (n=12) suggest that the definition of MH class II PBR for common carp is still incomplete and that additional residues may be involved in the binding of peptides by the teleost MH class II molecule.

We performed initial studies on putative correlations between the *Cyca-DAB1* and *Cyca-DAB2* polymorphism as detected by the PCR-RF-SSCP technique and resistance to *A. hydrophila* to examine whether the newly developed method could be applied for future association studies of carp MH polymorphism and disease resistance. The influence of MH diversity on resistance to pathogens (parasites) has been clearly demonstrated in three-spined stickleback, which possess up to six class II *B* loci (Sato *et al*., 1998, Binz *et al*., 2001). Kurtz *et al*. (2004) found that fish with low MH class II *B* diversity suffered more from parasite infection and suggested that optimal rather than maximal MH diversity confers the highest resistance to parasites. We found no clear and significant correlations between the *Cyca-DAB1/Cyca-DAB2* genotypes and resistance of carp to *A. hydrophila*, although subsequent studies using higher number of animals do suggest a genetic correlation (unpublished data). We did note that genotype C was almost three times more prevalent among survived fish than in the mortalities. Genotype C consisted of a heterozygous *Cyca-DAB1* gene and a homozygous *Cyca-DAB2* gene. We noted a trend that fish with a heterozygous *Cyca-DAB1* gene and a homozygous *Cyca-DAB2* gene showed a lower cumulative mortality compared to the other genotypes (homozygous *Cyca-DAB1*/homozygous *Cyca-DAB2* and homozygous *Cyca-DAB1*/no *Cyca-DAB2*). The highest cumulative mortality (50%) was noted within

the group of fish with a homozygous *Cyca-DAB1* gene and bearing no *Cyca-DAB2* gene. This percentage, however, was not significantly different from the rest and future studies require higher numbers of fish to verify this finding. Nevertheless, we did find a statistically significant influence of the *Cyca-DAB2* gene on the immune response as measured by bacterial agglutination titers. Fish with bearing no *Cyca-DAB2* gene had a significantly lower bacterial agglutination titer than fish that did have the *Cyca-DAB2* gene present. We suggest therefore, that MH diversity as exemplified by the presence rather than absence of the *Cyca-DAB2* gene could be advantageous in developing immune-based resistance of carp against *A. hydrophila*.

In conclusion, the optimized PCR-RF-SSCP is a reliable technique that can be used for screening large number of individuals for investigating the *Cyca-DAB1* and *Cyca-DAB2* genes polymorphism in common carp. The advantages of this technique are simplicity, high sensitivity and low costs. The PCR-RF-SSCP analysis revealed *Cyca-DAB1/Cyca-DAB2* genotypes consisting of unique combinations of the *Cyca-DAB1* and *Cyca-DAB2* sequences and the degree of heterozygosity of the *Cyca-DAB1* and *Cyca-DAB2* genes clearly correlated with the number of SSCP bands.

We suggest, on the condition that higher numbers of fish are used to enhance the power of the statistical analysis, our PCR-RF-SSCP method can be used to study correlations of different MH class II *B* genotypes/alleles with resistance of common carp to specific pathogens.

Acknowledgements

We thank the staff at the hatchery of the Institute of Ichthyobiology & Aquaculture in Golysz (Poland) and Ms. Danuta Rajba-Nikiel (II&A, Poland) for technical assistance. We are grateful to Dr. M. Nielsen (Royal Veterinary and Agricultural University, Denmark) for providing the virulent strain of *Aeromonas hydrophila*. Dr Vitaliy Bekh was supported by NATO Science Fellowship Programme. This study was supported by the State Committee for Scientific Research in Poland (KBN; grant number 3 P06K 005 25).

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CHAPTER 4

Classical crosses of common carp (*Cyprinus carpio* L.) show co-segregation of antibody response with major histocompatibility class II *B* genes

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Fish Shellfish Immunol.
doi:10.1016/j.fsi.2008.08.011

Abstract

In cyprinids, two paralogous groups of major histocompatibility (MH) class II *B* genes, *DAB1* and *DAB3*, have been reported but have not been studied in detail. In our study on MH association with immune responsiveness in common carp (*Cyprinus carpio* L.) we have taken a long-term approach using divergent selection for antibody production. We report the co-segregation of *Cyca-DAB1*-like and *Cyca-DAB3*-like genes with antibody response, in backcrosses to high- and low-responsive parental carp lines. We show that the presence of *Cyca-DAB1*-like, but not *Cyca-DAB3*-like genes, preferentially leads to a high DNP-specific antibody response in carp. Background genes other than *Cyca-DAB* genes also influenced the level of antibody response. Our data support the hypothesis of a genetic control by *Cyca-DAB* genes on the antibody response measured. We could not detect an association of the *Cyca-DAB* genes with disease resistance to the parasite *Trypanoplasma borreli*. Sequence information, constitutive transcription levels and our co-segregation data indicate that both paralogous *Cyca-DAB1*-like and *Cyca-DAB3*-like groups represent functional MH class II *B* genes. Previously defined differences in allelic diversity between *Cyca-DAB1*-like genes, especially, identify *Cyca-DAB1* as the most interesting *DAB* gene for further study in common carp.

1. Introduction

Genes of the major histocompatibility complex (MHC) are considered crucial to acquired immunity to pathogens and are frequently studied for their linkage to disease resistance. High polymorphism is one of the characteristic features of classical MHC genes, and specific MHC alleles can be linked with disease resistance. In most vertebrate species, MHC diversity resides in the presence of multiple loci and a considerable number of alleles at each locus. This diversity is especially observed in the gene fragments encoding the peptide binding grooves of the MHC molecules in which self or foreign peptides are bound and presented to T cell receptors (Klein *et al*., 2007). Studies on anti-peptide immune responses in mice have been linked with MHC diversity, showing a preferential binding of specific hapten-carrier antigens by particular MHC molecules (McDevitt and Sela, 1965). Association studies, initially in humans (Hill *et al*., 1991) and chicken (Briles *et al*., 1977, Kaufman and Wallny, 1996), but more recently also in (mainly salmonid) fish (Langefors *et al*., 2001, Grimholt *et al*., 2003, Wynne *et al*., 2007, Kjøglum *et al*., 2006 and 2008), have pointed at the influence of particular major histocompatibility (MH) alleles or haplotypes on disease resistance.

In cyprinid fish species at least two paralogous groups of MH class II *B* genes (*DAB1* and *DAB3*) are present (Dixon *et al*., 1996). Genes of both *DAB1* and *DAB3* groups show high polymorphism between different cyprinid fish species and are transcribed (Ottová *et al*., 2005, Šimková *et al*., 2006). Divergence estimation using exon 3 sequences of *DAB* genes from 11 cyprinid species in Central Europe indicated that separation of *DAB1* and *DAB3* groups occurred around 50 MYA, which is before the differentiation of cyprinids (Ottová *et al*., 2005). In common carp (*Cyprinus carpio* L.), four different *Cyca-DAB* sequences; *Cyca-DAB1*01* and *Cyca-DAB2*01* (here denoted as *Cyca-DAB1*-like genes) (Ono *et al*., 1993) as well as *Cyca-DAB3*01* and *Cyca-DAB4*01* (here denoted as *Cyca-DAB3*-like genes) (van Erp *et al*., 1996) have been described. These sequences most likely are the result of a much more recent duplication event (1-2 and 10-12 MYA, respectively) (van Erp *et al*., 1996). In a preliminary study, we examined the allelic polymorphism for the *Cyca-DAB1*-like and *Cyca-DAB3*-like genes and found a ubiquitous presence and high polymorphism of *Cyca-DAB1*-like but not *Cyca-DAB3*-like genes in different European common carp lines (Rakus *et al*., 2003). So far, all the evidence for a functional role for both *Cyca-*

DAB1-like and *Cyca-DAB3*-like genes has been based principally on sequence information.

Previously, we performed a long-term divergent selection for antibody production in fish. We immunized a base population of common carp with the hapten-carrier complex dinitrophenyl-keyhole limpet haemocyanin (DNP-KLH) and identified particular individuals as early/high or late/low responders. Hapten-carrier complexes typically are informative in studies on B-T cell cooperation, where the B cell receptor binds the hapten and where peptides derived from the protein carrier are presented by MHC molecules to the T cell receptor. As a result, the activated T cells will stimulate the production of hapten-specific antibodies. We have reported a genetically determined difference in the ability to produce a hapten-specific humoral response to the selection antigen DNP-KLH, but also to pathogens such as the blood parasite *Trypanoplasma borreli*, in homozygous high- and low-responder inbred carp lines (Wiegertjes *et al*., 1996a). Subsequent PCR-based typing for MH class II *B* (*Cyca-DAB*) genes suggested that high immune responsiveness and resistance to infection were associated with the presence or absence of particular *Cyca-DAB* genes (Wiegertjes *et al*., 1996a).

Our long-term divergent selection for antibody production successfully resulted in carp lines with a different immune response, but the association between *Cyca-DAB* diversity and immune responsiveness could have been circumstantial. True correlation can be studied with classical crosses using F_2 hybrids, or backcrosses to parental lines, collecting evidence for a single mode of Mendelian inheritance. In the present study, we examined co-segregation of *Cyca-DAB* genes with antibody responsiveness and/or disease resistance. To this end we crossed carp inbred lines that have either *Cyca-DAB1*-like or *Cyca-DAB3*-like genes to produce a heterozygous F_1 generation, which was used to produce backcross generations to each of the parental inbred lines. Fish from all five groups (two parental lines, F_1 and two backcrosses) were immunized with DNP-KLH and their DNP-specific antibody response measured. The same fish were infected with the parasite *Trypanoplasma borreli* to also study co-segregation of *Cyca-DAB* genes with specific disease resistance. Our data support the hypothesis of a genetic control by *Cyca-DAB* genes of the antibody response to DNP-KLH, but not disease resistance to *T. borreli*. We examined *Cyca-DAB1*-like and *Cyca-DAB3*-like genes transcription by real-time quantitative PCR and found that constitutive gene

transcription levels were high. This supported the hypothesis that both *Cyca-DAB1*-like and *Cyca-DAB3*-like genes have retained their function as true MH class II *B* genes in common carp. We discuss the observation that *Cyca-DAB1*-like genes, more so than *Cyca-DAB3*-like genes are associated with increased immune responsiveness in carp.

Materials and Methods

Fish

Five groups of European common carp (*Cyprinus carpio carpio* L.) were reproduced on the same day and reared in the central fish facility of Wageningen University at 25 ± 2 ^{o}C in a recirculation system with UV-treated water and fed pelleted dry food (Trouvit, Nutreco) daily. The groups included: (*i*) gynogenetic homozygous progenies of the E4 carp line (Komen *et al*., 1991) previously typed as low responders to DNP-KLH (Wiegertjes *et al*., 1995), (*ii*) gynogenetic homozygous progenies of the 69-45 carp line, previously typed as high responders to DNP-KLH (Wiegertjes *et al*., 1996a), (*iii*) F_1 heterozygous cross between a female from the E4 line with a sex-reversed male from the 69-45 line, (*iv*) backcross to low responders (F_1 x E4), and (*v*) backcross to high responders (F_1 x 69-45). Backcrosses were obtained by using sex-reversed males from the gynogenetic carp lines (E4 and 69-45).

All fish were grown in a common environment until 16 months of age and weighed 200-500 grams at the time of immunization. Groups were mixed over n = 5 aquaria prior to immunization or infection, to ensure equal representation while avoiding confounding tank effects. Fish were anaesthesized in 0.3 g l^{-1} tricaine methane sulfonate (TMS, Crescent Research Chemicals, Phoenix, USA) buffered with 0.6 g l^{-1} sodium bicarbonate (Sigma, Germany) prior to tattooing for individual identification, immunization treatment, or bleeding for MH genotyping, antibody production or parasitaemia. Prior to infection, carp were acclimatized for 2 weeks to 20 ± 2 °C in a quarantine unit.

Determination of antibody response

Fish were injected intramuscularly with 10 µg dinitrophenyl-keyhole limpet haemocyanin (DNP_{494}KLH, Calbiochem, La Jolla, CA, USA) in a 1:1 mixture with Freund's incomplete adjuvant as previously described (Wiegertjes *et al*., 1994). Blood

samples were collected by vena puncture of the caudal vessel at 0 and at 12, 21 and 28 days post-immunization (d.p.i.) and serum stored at -20 ºC for antibody measurement. DNP-specific antibody production was measured by enzyme-linked immunosorbent assay (ELISA) in serum samples as described previously (Wiegertjes *et al*., 1994). Briefly, to detect DNP-specific antibodies in serum samples, 96-well ELISA plates were coated overnight at 37 °C with 0.10 μg ml^{-1} $DNP_{44}BSA$ (DNP-Bovine Serum Albumin, Calbiochem, Germany). After a blocking step, plates were incubated with carp serum (two-fold serial dilutions starting at 1:50), next with mouse monoclonal antibody against carp immunoglobulin (WCI-12, 1:500) (Secombes *et al*., 1983, Koumans-van Diepen *et al*., 1994), and subsequently with goat anti-mouse horseradish peroxidase (GAM-HRP, Bio-Rad, Richmond, CA, USA). Orthophenylenediamine substrate incubation was stopped with sulphuric acid, and colour development (optical density OD) was detected at 492 nm with a spectrophotometer (Anthos Reader 2001 Anthos Labtech Institute, Straussberg, Austria). Titres (OD values) were determined by regression at a serum dilution factor of 1:800.

Infection of carp with the blood parasite Trypanoplasma borreli.

Infection experiments were performed at 20 ± 2 ^{o}C in a quarantine unit. *T. borreli* (Steinhagen *et al*., 1989) was maintained by syringe passage through naïve carp. Parasites were counted in blood using a Bürker counting chamber. Fish were intra-peritoneally (i.p.) injected with a dose of 2 x 10^3 parasites per fish. Infected fish were bled for parasitaemia at 14, 21 and 28 days post-infection (d.p.i.); the experiment was terminated at 35 d.p.i. Fish were checked daily and removed from the experiment when lethargic, to prevent unnecessary suffering. Previous experiments have shown lethargic fish typically have developed lethal parasitaemia (> 10^8 ml^{-1}). Number of fish removed was recorded and remaining fish were counted as survivors and given a survival time of 35 d.p.i.

Major Histocompatibility (MH) class II B (Cyca-DAB) genotyping

Genomic DNA was extracted using the Wizard® genomic DNA purification system (Promega, Madison,USA). Briefly, heparinized whole blood samples were spun down to collect blood cells. Volumes of 20 μl of cells were lysed in 300 μl ice-cold Nuclei

Lysis Buffer, homogenised and incubated for 30 min at 65 °C. RNase Solution was added and the cells incubated for another 30 min at 37 °C and cooled to room temperature. Proteins were precipitated by adding Protein Precipitation Solution and samples spun down for 4 min at 14,000 rpm. Supernatant was mixed with isopropanol and DNA spun down for 1 min at 14,000 rpm. The pellet was washed twice with 70% ethanol, dissolved in milliQ and stored at 4 °C.

Primers specific for amplification of *Cyca-DAB1*-like and *Cyca-DAB3*-like genes were designed based on four previously described *Cyca-DAB* sequences; *Cyca-DAB1*01* (GenBank accession number CAA88847), *Cyca-DAB2*01* (CAA88848), *Cyca-DAB3*01* (X95431) and *Cyca-DAB4*01* (X95434) (see also Table 1). Because of high sequence similarity, primers were not designed to distinguish between *Cyca-DAB1* and *Cyca-DAB2* on one hand and *Cyca-DAB3* and *Cyca-DAB4* on the other hand. Thus, we aimed to study the segregation of two groups: *Cyca-DAB1*-like and *Cyca-DAB3*-like genes, rather than all four individual *Cyca-DAB* sequences. PCR amplification was performed in 100 µl of solution containing 100-200 ng of gDNA, 1 x reaction buffer, 200 nM of each primer, 200 µM of each dNTP, 1.5 mM $MgCl_2$, and 1 U Taq polymerase (Eurogentec, Seraing, Belgium). Amplification was carried out using a thermal cycle profile (1 min 95 °C, 1 min 55 °C, 2 min 72 °C) for 30 cycles, with an additional extension step of 10 min at 72 °C.

Table 1. Primers for PCR-based Major Histocompatibility typing and primers for real-time quantitative PCR (RT-qPCR) analysis of constitutive transcription of *Cyca-DAB1*-like and *Cyca-DAB3*-like genes (Fw- forward, Rev- reverse).

Genes	Sequence 5'→3'	Function
Cyca-DAB1-like	CTGATGCTGTCTGCTTTCACTGGAGCA	PCR: *DAB1*01*/*DAB2*01* (Fw)
	CTGTTTTATCACGGATCGCTGACTG	PCR: *DAB1*01*/*DAB2*01* (Rev)
	GACA+TGG+TGTACGTTGA+TAACTATAT	RT-qPCR: *DAB1*01*/*DAB2*01* (Fw)
	ATCTCTCTGCGTTATGTACTCCAAGTG	RT-qPCR: *DAB1*01*/*DAB2*01* (Rev)
Cyca-DAB3-like	ATGCTGTCTGCATTTACTGGAACAG	PCR: *DAB3*01*/*DAB4*01* (Fw)
	CTCTGCTGCAGTTCTGCC	PCR: *DAB3*01* (Rev)
	TGTCCACTGAAGTTTTCAGA	PCR: *DAB4*01* (Rev)
	GATAT+GGT+GTATCTTGT+GTCACTTTCC	RT-qPCR: *DAB3*01*/*DAB4*01* (Fw)
	GTTCTCTGCATCTTTCACTCCTTCCTC	RT-qPCR: *DAB3*01*/-*DAB4*01*(Rev)
40S	CCGTGGGTGACATCGTTACA	RT-qPCR (Fw)
	CAGGACATTGAACCTCACTGTCT	RT-qPCR (Rev)

'+': indicates the base with LNA modification (underlined)

RNA isolation, cDNA synthesis and RT-qPCR analysis

To study constitutive transcription of *Cyca-DAB1*-like and *Cyca-DAB3*-like genes in immune organs, RNA was isolated from carp (n = 5) known to express both paralogous groups (cross R3xR8, unpublished data). Constitutive gene transcription was studied by Real Time-quantitative PCR (RT-qPCR) as described previously (Forlenza *et al*., 2008). In short, total RNA was isolated from different immune organs using the RNeasy Mini Kit (Qiagen, Leusden, The Netherlands) including on-column DN*ase*. Prior to RNA isolation from liver a Proteinase K treatment was also included. RNA concentration and purity was determined at OD_{260nm}/OD_{280nm}. RNA integrity was determined by electrophoresis on 1% agarose gel. Prior to cDNA synthesis, a second DN*ase* treatment was performed using DN*ase* I, Amplification Grade (Invitrogen, Breda, The Netherlands). Synthesis of cDNA was performed with Invitrogen's Superscript™ III First Strand Synthesis Systems for RT-PCR including a non-reverse transcriptase control for each sample. cDNA samples were further diluted 50 times (25 times for PBL samples) in nuclease-free water before use as template in RT-qPCR.

RT-qPCR was performed using a Rotor-Gene™ 2000 (Corbett Research, Mortlake, Sydney, Australia) and Brilliant® SYBR® Green QPCR (Stratagene, La Jolla, CA, USA) as detection chemistry according to the previously described protocol (Forlenza *et al*., 2008). The primers used for RT-qPCR are presented in Table 1. Master-mix for each PCR run was prepared as follows: 1 µl of each primer (4.2 µM), 7 µl Master SYBR Green I mix and 5 µl of diluted cDNA. The following amplification program was used: after 15 min of denaturation at 95 ºC, 40 cycles of RT-qPCR with three-step amplification were performed: 15 s at 95 ºC for denaturation, 30 s at 60 ºC for annealing and 30 s at 72 ºC for elongation followed by a final holding step of 1 min at 60 ºC. A melting step was then performed and in all cases, the amplifications were specific and no amplification was observed in negative controls (non template control and non-reverse transcriptase control). Fluorescence data from RT-qPCR experiments were analyzed using Rotor-Gene version 6.0.21 software. The cycle threshold C_t for each sample and the reaction efficiencies (E) for each primer set were obtained upon Comparative Quantitation Analysis from the Rotor-Gene version 6.0.21 software (40S: E = 1.72; *Cyca-DAB1*-like: E = 1.71; *Cyca-DAB3*-like: E = 1.72). Basal transcription of immune-related genes was calculated as a ratio of reference gene versus target gene in

different organs (Pfaffl, 2001, Tichopad *et al*., 2003). The 40S ribosomal protein S11 was used as an internal reference gene.

Statistics

Differences in basal transcription levels between *Cyca-DAB1*-like and *Cyca-DAB3*-like genes in different organs were tested for significance by a *t* test for independent samples. Comparison of differences in mean antibody production between genetic groups was made using repeated measures ANOVA analysis. When differences between means were significant, a post-hoc comparison was applied to assess which of the means contributed to the effect (i.e. which groups were particularly different from each other). The optical density values for the antibody production were log-transformed. The effect of MH genotype on antibody production in backcrosses and parental lines was evaluated using repeated measures ANOVA analysis. All analyses were performed using Statistica 6.0 software (StatSoft). Differences were considered significant at $P < 0.05$.

Results

Cyca-DAB1-like and Cyca-DAB3-like genes show Mendelian patterns of inheritance

The high- and low-responder parental inbred lines were proven by PCR-based typing to have either *Cyca-DAB1*-like or *Cyca-DAB3*-like genes, respectively, and all individuals in the F_1 cross have both *Cyca-DAB1*-like and *Cyca-DAB3*-like genes (data not shown). More informative, 50% of the fish in each backcross carried both *Cyca-DAB1*-like and *Cyca-DAB3*-like genes, while the remaining 50% were typed positive for either *Cyca-DAB1*-like (backcross to high responders; $n = 16/34$) or *Cyca-DAB3*-like genes (backcross to low responders; $n = 17/32$). Thus, our PCR-based typing of the *Cyca-DAB* genotypes confirmed Mendelian inheritance noticed previously (Wiegertjes *et al*., 1996a) and showed an independent segregation of these *Cyca-DAB* genes.

Constitutive transcription of Cyca-DAB1-like and Cyca-DAB3-like genes is comparable

Clearly, mRNA transcription of both *Cyca-DAB1*-like and *Cyca-DAB3*-like genes was high in the selected immune organs (Fig. 1). The internal reference gene (40S) was detectable already after about 18 PCR cycles (not shown) and the ratio of 40S

versus *Cyca-DAB* genes expression was even ≥1 in most of the investigated organs. This indicates that *Cyca-DAB* genes are transcribed at high constitutive levels (17-18 Ct values), comparable to the reference gene. In general, *Cyca-DAB1*-like genes consistently showed slightly higher mRNA transcription than *Cyca-DAB3*-like genes, with significant ($P < 0.05$) differences in head kidney, thymus and spleen.

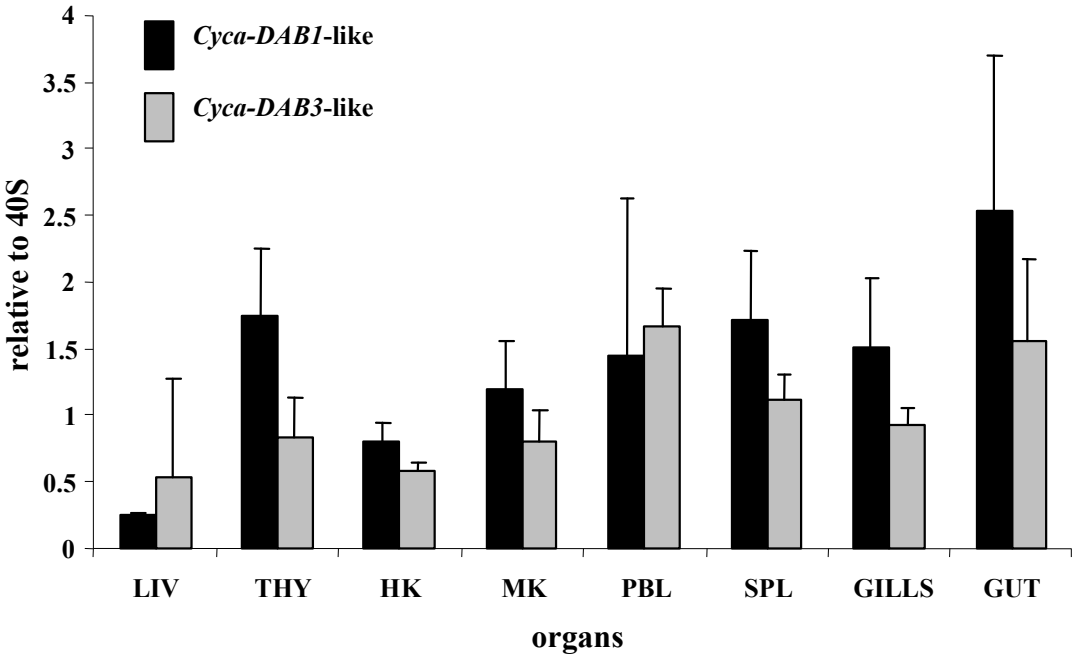

Figure 1. Real-Time quantitative PCR analysis of constitutive gene transcription of *Cyca-DAB1*-like and *Cyca-DAB3*-like genes in liver (LIV), thymus (THY), head kidney (HK), mid kidney (MK), Peripheral Blood Leukocytes (PBL), spleen (SPL), gills and gut from $n = 5$ healthy common carp individuals, heterozygous for *Cyca-DAB1*-like and *Cyca-DAB3*-like genes. mRNA levels are expressed relative to the house keeping gene 40S. Asterisks indicate statistically significant ($P < 0.05$) differences between *Cyca-DAB1*-like and *Cyca-DAB3*-like genes in the same organ.

Carp with Cyca-DAB1-like genes have a higher DNP-specific antibody response than carp with only Cyca-DAB3-like genes.

Fish of all five crosses (*i.e.* two parental lines, F_1 and two backcrosses) were immunized with DNP-KLH and the anti-DNP antibody response measured by ELISA. We observed significant differences ($P < 0.01$) between all genetic groups in mean level of antibody production. Presumed high-responders (parental 69-45 line; *Cyca-DAB1*-like genes only), showed a higher level of DNP-specific antibodies than presumed low-responders (parental E4 line; *Cyca-DAB3*-like genes only) (Fig. 2). As could be

expected in a heterozygous F_1 group (fish with both *Cyca-DAB1*-like and *Cyca-DAB3*-like genes), the mean level of antibody production was intermediate between the parental high- and low-responders, although significantly closer to the high responders ($P < 0.01$). In the backcross to high-responders, the mean level of antibody production was high, significantly higher ($P < 0.001$) than the mean level of antibody production in the parental high-responders (69-45 line).

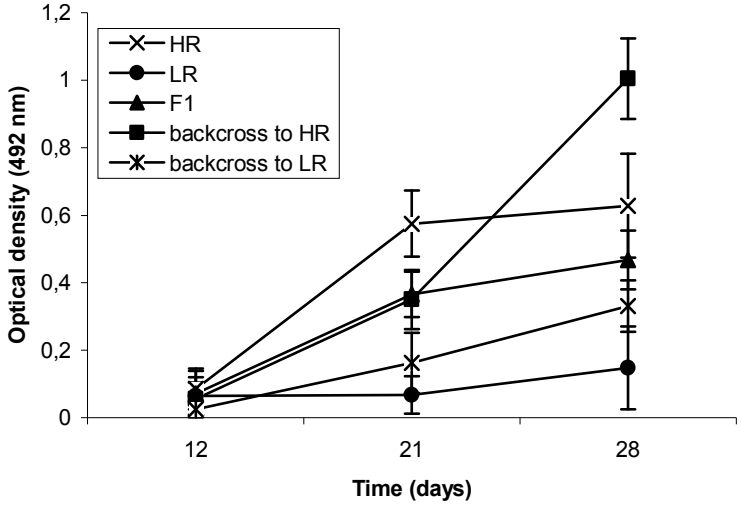

Figure 2. Determination of DNP-specific antibody response in *Cyca-DAB*-typed classical crosses of common carp injected with $DNP_{494}KLH$. DNP-specific antibody response was measured by ELISA in fish from five crosses (*i.e.* two parental lines, F_1 and two backcrosses). Presumed low-responder (LR) carp were crossed with presumed high responders (HR) to obtain an F_1 generation. Fish from the F_1 generation were backcrossed to low- and high-responder fish. Serum samples were collected at day 0 and at 12, 21 and 28 days. OD values (0.065 ± 0.031) at time 0 are not shown.

Co-segregation of the DNP-specific antibody response type with *Cyca-DAB* genes was studied in the two backcross generations. Differences in mean level of antibody production between fish with both *Cyca-DAB1*-like and *Cyca-DAB3*-like genes and fish with *Cyca-DAB3*-like genes only were not significant in the backcross to low responders (F1 x E4). However, in the backcross to high responders, a significant difference ($P < 0.05$) was observed between fish with both *Cyca-DAB1*-like and *Cyca-DAB3*-like genes and fish with only *Cyca-DAB1*-like genes (Fig. 3). Both genotypes in this backcross showed a higher level of antibody production than the parental high-responders (69-45 line, Fig. 3). This suggests a positive correlation exists between *Cyca-DAB* genes and the level of antibody production against DNP-KLH.

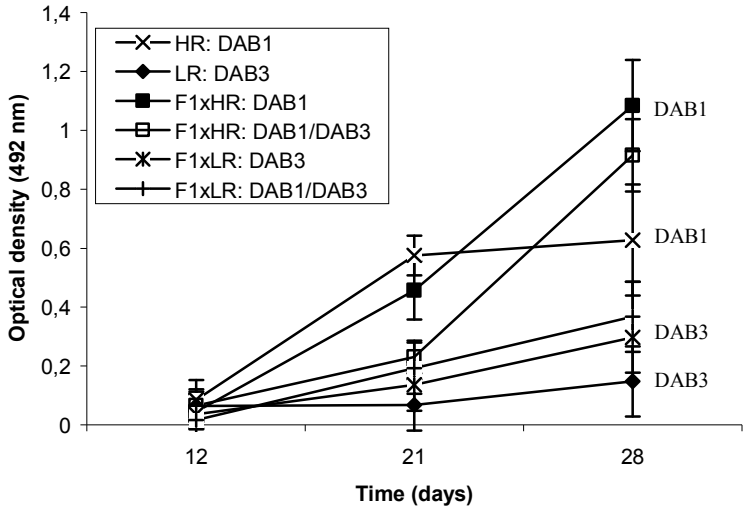

Figure 3. Determination of DNP-specific antibody response in *Cyca-DAB*-typed classical crosses of common carp injected with $DNP_{494}KLH$. DNP-specific antibody response in fish from the two parental lines and two backcrosses in which MH genes segregate into two groups: fish with both *Cyca-DAB1*-like and *Cyca-DAB3*-like genes and fish with only *Cyca-DAB1*-like genes or only *Cyca-DAB3*-like genes. Serum samples were collected at day 0 and at 12, 21 and 28 days post-immunization (d.p.i.). OD values (0.052 ± 0.01) at time 0 are not shown.

Background genes influence resistance to Trypanoplasma borreli

Fish of all five crosses (*i.e.* two parental lines, F_1 and two backcrosses) were infected with *T. borreli* and resistance evaluated by calculating parasitaemia at 21 d.p.i., mean survival time (MST) and mortality at 35 days post-infection. Differences in resistance to *T. borreli* could be observed, but not between presumed high- and low-responder inbred lines (Table 2). Within the backcrosses, fish with *Cyca-DAB1*-like genes only (50% of the backcross to high-responders) and fish with *Cyca-DAB3*-like genes only (50% of the backcross to low-responders) showed a similar pattern of resistance. Thus, resistance to *T. borreli* did not co-segregate with *Cyca-DAB* genes. Interestingly, within the fish that carried both *Cyca-DAB1*-like and *Cyca-DAB3*-like genes (all F_1 and 50% of each backcross generation), F_1 fish showed the highest resistance. In both backcrosses, resistance was higher than in the corresponding homozygous parental lines. This clearly suggested that heterozygous background genes other than the *Cyca-DAB* genes only, could influence carp resistance to *T. borreli*.

Table 2. Infection of *Cyca-DAB*-typed common carp with *Trypanoplasma borreli*. Carp were presumed high antibody responder (HR), presumed low antibody responder (LR), heterozygous F_1 or backcross generations to each of the parental inbred lines. Fish were injected with $2x10^3$ parasites and bled for parasitaemia at 14, 21 and 28 days post-infection (d.p.i.). Only parasitaemia at 21 d.p.i. is shown. The experiment was terminated at 35 d.p.i. Fish were checked daily and removed from the experiment when lethargic to prevent unnecessary suffering. Number of fish removed was recorded and remaining fish were counted as survivors with a survival time of 35 d.p.i.

Cross	n	Parasitaemia 21 d.p.i. (x 10^5)	Mean Survival Time (days)	Mortality 35 d.p.i. (%)
***Cyca-DAB1*-like only**				
high resp	20	94	28	89
backcross to HR	18	32	31	65
***Cyca-DAB 3*-like only**				
low resp	23	17	29	95
backcross to LR	17	51	32	50
***Cyca-DAB1-like and Cyca- DAB3*-like**				
F1	33	20	33	38
backcross to HR	16	50	31	79
backcross to LR	15	13	32	100

Discussion

In cyprinid fish species at least two paralogous groups of major histocompatibility (MH) class II *B* genes (*DAB1* and *DAB3*) are present (Dixon *et al*., 1996, Ottová *et al*., 2005). Given the time of divergence (> 50 MYA) between the *DAB1* and *DAB3* groups it would have been likely for one of the two paralogous gene groups to lose function due to accumulation of deleterious mutations or, acquire new function via directional selection (Bailey *et al*., 1978, Lynch and Conery, 2000). Analysis of the polymorphism of *DAB* genes in 11 cyprinid species from Central Europe revealed the presence of a total number of 1-3 distinct *DAB* transcripts per fish. When three different transcripts were detected in one individual, either two *DAB1* and one *DAB3* or one *DAB1* and two *DAB3* sequences were present (Ottová *et al*., 2005). Sequence analysis revealed that for sequences from both *DAB1* and *DAB3* groups, the exon 2 region was positively selected (dN/dS > 1), while the exon 3 region was undergoing purifying selection (dN/dS < 1) (Ottová *et al*., 2005). Since in functional

MHC genes the exon 2 region, but not the exon 3 region, encodes the peptide binding residues, positive selection on this region would imply both *DAB1* and *DAB3* groups could function as true MH class II *B* genes.

For the European common carp, the major cyprinid fish species cultivated in Europe, four *Cyca-DAB* sequences; *Cyca-DAB1*01* and *Cyca-DAB2*01* (here denoted as *Cyca-DAB1*-like genes) (Ono *et al*., 1993) as well as *Cyca-DAB3*01* and *Cyca-DAB4*01* (here denoted as *Cyca-DAB3*-like genes) (van Erp *et al*., 1996) have been described. *Cyca-DAB3*-like sequences have in common a non-consensus splice site sequence for intron 1 (5' GCAAGT) (van Erp *et al*., 1996). The same 5' splice site has been found for intron 1 in orthologous sequences of the Lake Tana barbel (*Bain-DAB3*) and Portuguese barbel (*Babo-DAB3*) (Dixon *et al*., 1996). In theory, this could lead to incomplete splice variants for *DAB3*-like genes or could have an impact on transcription levels of these genes. In practice, the presence of correctly spliced cDNA transcripts of *Cyca-DAB3*-like genes (van Erp *et al*., 1996) as well as *Bain-DAB3* and *Babo-DAB3* (Dixon *et al*., 1996) argues in favour of normal transcription. To gain further insight in the functioning of the *Cyca-DAB1*-like and *Cyca-DAB3*-like genes, we designed primers for real-time quantitative PCR transcription studies. For these primers to be specific for each group we made use of locked nucleic acid (LNA) modifications, which enhance the discriminatory power between similar transcripts. There were no differences in tissue distribution between *Cyca-DAB1*-like and *Cyca-DAB3*-like genes. In general, constitutive transcription for both *Cyca-DAB1*-like and *Cyca-DAB3*-like genes was high. We could detect a higher transcription level of *Cyca-DAB1*-like than *Cyca-DAB3*-like genes in head kidney, thymus and spleen. The differences, however, seem minor with respect to the generally high transcription levels. Yet, the potential biological consequences of the small but significant differences in transcription levels in these immunologically important tissues require further investigation. Our transcription studies support the hypothesis that both *Cyca-DAB1*-like and *Cyca-DAB3*-like genes have retained their function as true MH class II *B* genes in the cyprinid common carp.

Sequence information and gene transcription studies can provide indicative but not conclusive evidence on the functioning of MH class II *B* genes. Thus, we used our long-term divergent selection for antibody production in carp to further study the function of *Cyca-DAB1*-like and *DAB3*-like genes. Many studies have demonstrated

correlations between MHC and antibody production against a variety of antigens including hapten-carriers antigens or sheep red blood cells in mammals (Klein, 1986), mice (Puel *et al*., 1996 and 1998) and chicken (Loudovaris *et al*., 1990, Zhou and Lamont, 2003). The use of clonal inbred carp lines allowed for a 'single major gene' analysis by examining the pattern of immune responsiveness (antibody response to DNP-KLH) and analysis of Mendelian inheritance in classical backcrosses to both parental lines. Indeed, we could confirm an independent segregation of the two paralogous *Cyca-DAB* groups and we could show a Mendelian pattern of inheritance both in the base population (Wiegertjes *et al*., 1996a) and backcross generations (this study), supporting a single major gene control. Individuals from the backcross to high-responders, typed as only *Cyca-DAB1*-like (50% of the offspring), showed significantly higher DNP-specific antibody production than individuals from the backcross to the low-responders, typed as only *Cyca-DAB3*-like (50% of this offspring). This indicated that *Cyca-DAB1*-like and *Cyca-DAB3*-like genes co-segregated with the DNP-specific antibody response and supports the hypothesis of a major gene control by *Cyca-DAB* genes. It does not mean, however, that *Cyca-DAB1*-like genes always correlate with high- and *Cyca-DAB3*-like genes always correlate with low antibody responses in carp. More likely, the divergent selection favoured an MH genotype with a groove shaped to fit the KLH protein best. Of course, further studies into the function of *Cyca-DAB* genes are required, for example using the phage display technique to assist in the definition of peptide-ligand binding motifs (Zhao *et al*., 2008). Fish in the backcross to high-responders, that all may be considered heterozygous for background genes, often showed a significantly higher antibody production than their homozygous counterparts. Therefore, not surprisingly, background genes also contributed to the antibody response. In conclusion, our co-segregation studies support the hypothesis that the *Cyca-DAB* genes are true MH class II *B* genes in the cyprinid common carp.

We also studied *Cyca-DAB* co-segregation with resistance to the parasite *T. borreli* but did not find the same pattern of segregation as seen with the DNP-specific antibody response. Of course, in wild type carp, MH class II could play a role in the resistance against this parasite via alleles not studied here. Our observations show that the use of MH-typed inbred fish lines is more informative in the analysis of the antibody response to a simple hapten than in the analysis of complex traits such as disease

resistance. Indeed, the divergent selection was based on the principle that a genetic control of the antibody response would be more easily detected using the hapten-carrier DNP-KLH than with a complex antigen such as the parasite *T. borreli*. It is known that antibody production to *T. borreli* does play an important role in the protective immune response against this parasite (Joerink *et al*., 2007). Most likely, both *Cyca-DAB1*-like and *Cyca-DAB3*-like genes are required for a proper presentation of all parasite-derived antigens. There are several approaches that can be taken to study the genetic influence on disease resistance in fish, including selective breeding, family selection and association studies (Wiegertjes *et al*., 1996b). During recent years, several association studies, especially in salmonid fish, have detected an effect of particular MH haplotypes on resistance to various pathogens (Palti *et al*., 2001, Grimholt *et al*., 2003, Miller *et al*., 2004, Wynne *et al*., 2007, Kjøglum *et al*., 2006 and 2008, Johnson *et al*., 2008). Association studies often makes use of MH typing techniques other than sequencing, such as analysis of specific minisatellite markers localized in the 3' UTR of the *Sasa-UBA* and *Sasa-DAA* loci (Grimholt *et al*., 2002, Stet *et al*., 2002, Grimholt *et al*., 2003). In a preliminary study, we examined the allelic polymorphism for the *Cyca-DAB1*-like and *Cyca-DAB3*-like genes and found a ubiquitous presence and high polymorphism of *Cyca-DAB1*-like but not *Cyca-DAB3*-like genes in different European common carp lines (Rakus *et al*., 2003). Although *Cyca-DAB3*-like proteins may have a groove more suited to antigens other than the ones studied here, the differences in allelic diversity, especially, identify *Cyca-DAB1* as the most interesting *DAB* genes for further study in carp. We recently developed a single strand conformation polymorphism (SSCP)-based typing method to detect allelic polymorphism for *Cyca-DAB1*-like genes in large numbers of fish (Rakus *et al*., 2008). The suggestion that the *Cyca-DAB* genes are true MH class II *B* genes and the previously observed allelic polymorphism for *Cyca-DAB1*-like genes, especially, will drive further studies into the association of *Cyca-DAB1*-like allelic polymorphism with disease resistance of common carp.

Acknowledgements

We thank the central fish facilities of Wageningen University "De Haar-Vissen" for excellent care of the carp. The authors would like to thank Marieke Mossink, Mascha Jansen and Trudi Hermsen for technical assistance. Brian Dixon, University of

Waterloo (Canada) is gratefully acknowledged for carefully reading the manuscript. This work was supported by the European Commission within the project IMAQUANIM (EC contract number FOOD-CT-2005-007103) and by the State Committee for Scientific Research in Poland (KBN; grant number 3 P06K 005 25).

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CHAPTER 5

Major histocompatibility (MH) class II *B* gene polymorphism influences disease resistance of common carp (*Cyprinus carpio* L.)

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Submitted

Abstract

Genes of the major histocompatibility complex (MHC) are crucial elements of adaptive immunity. High polymorphism renders the MHC genes highly suitable for studies on association with disease resistance. In common carp (*Cyprinus carpio* L.), there are two paralogous groups of MH class II *B* genes, *Cyca-DAB1*-like and *Cyca-DAB3*-like genes. The *Cyca-DAB1*-like genes especially, could be linked to high polymorphism and increased antibody responsiveness. The aim of the present study was to analyze association between *Cyca-DAB1*-like genotypes, as revealed by polymerase chain reaction – restriction fragments – single strand conformation polymorphism (PCR-RF-SSCP), and disease resistance. We used a large number of individuals of different genetic background and performed standardized disease challenges with the bacterium *Aeromonas hydrophila*, the ectoparasite *Argulus japonicus,* and the blood parasite *Trypanoplasma borreli*. Genotyping revealed the presence of $n = 9$ unique *Cyca-DAB1*-like genotypes, of which three genotypes (B, D, and E) were most common. We could detect significant associations between genotype E and abundance of *A. japonicus* and between genotype D and higher level of parasitaemia after *T. borreli* infection. The *Cyca-DAB1*-like group of genes comprises (at least) two gene copies, *Cyca-DAB1* and *Cyca-DAB2*. We observed a significant association between *Cyca-DAB1* heterozygosity and lower level of parasitaemia after *T. borreli* infection. In general, *Cyca-DAB2* was often homozygous or absent. Our data suggest that *Cyca-DAB1* allelic polymorphism can be used as a potential genetic markers in future breeding programs of common carp.

Introduction

The impact of fish diseases is an important issue in developing a sustainable aquaculture industry of economic importance. Aquaculture of common carp (*Cyprinus carpio* L.) is carried out mostly in open environment (earthen ponds), strongly limiting the possibilities for disease control measures. As a consequence, losses can be as high as 90% of the initial stock during the first rearing season, and can reach a further 30% mortality of the remaining population during the second rearing season (Pilarczyk, 1998). The main limitations for an effective prevention of diseases in carp aquaculture are: *i*) lack of possibilities to isolate infected from healthy fish to protect breeding stock from infections, *ii*) difficulties in rapid diagnosis and monitoring of diseases and *iii*) practical limitations in the use of chemotherapeutants and/or antibiotics. An attractive alternative route that could lead to improved disease resistance of carp is genetic selection, which can be considered a sustainable approach to disease control in semi-intensive carp pond farming.

Genetic resistance of fish to various pathogens has been described frequently (Fjalestad *et al*., 1993, Das Mahapatra *et al*., 2008, Sahoo *et al*., 2008) and indeed, genetic selection for disease resistance has been included in breeding programs for Atlantic salmon, rainbow trout (Midtlyng *et al*., 2002) and common carp (Kirpichnikov *et al*., 1993). There are, however, a number of difficulties associated with direct breeding for genetic disease resistance of common carp. The most important are the long generation intervals (slow genetic progress) and the need to apply combined, or family, selection (labour intensive) with infection challenge tests in each generation (high costs) (Vandeputte, 2003). Therefore, selection based on marker genes is an attractive alternative to direct breeding aimed at providing stronger and more robust carp (Midtlyng *et al*., 2002).

Genes of the major histocompatibility complex (MHC) are candidate marker genes for studies on association with disease resistance. The MHC contains some of the most polymorphic genes known to date and are considered crucial to adaptive immunity. MHC molecules bind both self and foreign peptides and present them to T lymphocytes (T cells). MHC class I molecules present endogenously derived peptides to $CD8^+$ T cells, while MHC class II molecules present exogenously derived peptides to $CD4^+$ T cells. Each MHC molecule has the ability to bind and present different groups

of peptides in more or less successful ways. This can influence the immune response of an organism since the peptides derived from a certain pathogen may either not be presented by specific MHC molecules, which can result in higher susceptibility or, may be bound with a high affinity by specific MHC molecules which could lead to increased resistance to the pathogenic organism (Klein *et al*., 2007). The best-known associations between resistance to infectious disease and MHC genes have been described for malaria infection of humans (Hill *et al*., 1991) and for viral infection of chicken (Kaufman and Wallny, 1996, Briles *et al*., 1997). In teleost fish species, the genomic organization of the MH genes is different. Here, MH class I and class II genes reside on different chromosomes and segregate independently (Bingulac-Popovitc *et al*., 1997, Sato *et al*., 2000), which allows for association studies of only class I or only class II MH genes with disease resistance (Grimholt *et al*., 2003, Stet *et al*., 2003). This has lead to evidence for correlations between MH polymorphism and disease resistance in several, but mostly salmonid, fish species (Langefors *et al*., 2001, Grimholt *et al*., 2003, Wynne *et al*., 2007, Kjøglum *et al*., 2006 and 2008, Johnson *et al*., 2008).

In common carp, there are two paralogous groups of MH class II *B* genes, *Cyca-DAB1*-like and *Cyca-DAB3*-like genes. *Cyca-DAB1*-like genes include *Cyca-DAB1*01* and *Cyca-DAB2*01* sequences (Ono *et al*., 1993), while *Cyca-DAB3*-like genes include *Cyca-DAB3*01* and *Cyca-DAB4*01* sequences (Van Erp *et al*., 1996). In a preliminary study, we examined the allelic polymorphism for *Cyca-DAB1*-like and *Cyca-DAB3*-like genes and found a ubiquitous presence and high polymorphism of *Cyca-DAB1*-like, but not *Cyca-DAB3*-like, genes in different European common carp lines (Rakus *et al*., 2003). This made us focus on the *Cyca-DAB1*-like genes in carp. We recently optimized a technique designated polymerase chain reaction - restriction fragments - single strand conformation polymorphism (PCR-RF-SSCP) to allow for the screening of large numbers of individuals for *Cyca-DAB1*-like genes polymorphism in common carp (Rakus *et al*., 2008a). The advantages of this technique are simplicity, high sensitivity and low costs. In the present manuscript we use the PCR-RF-SSCP method to study associations of *Cyca-DAB1*-like genotypes with resistance of common carp to three different pathogens: the bacterium *Aeromonas hydrophila*, the ectoparasite *Argulus japonicus,* and the blood parasite *Trypanoplasma borreli*. The application of MH genes

as genetic markers for selection aimed at improving disease resistance of carp in semi-intensive pond farming is discussed.

Materials and methods

Fish

European common carp (*Cyprinus carpio carpio* L.) were obtained from the live gene bank at the Institute of Ichthyobiology and Aquaculture in Golysz (Poland), comprising 19 carp lines of various geographical origins. Some of the lines were imported to Golysz as existing closed breeding lines, some were obtained as a result of breeding selection on local carps and on imported fishes from various geographic regions whereas some lines were the result of hybrid crosses between existing lines. Carp taken from commercially exploited lines of Polish (K and R2), Hungarian (R7), Ukrainian (Ur), Israelian (DOR70) and German (N) origin and one reference laboratory cross (R3xR8) have been included in the study. Eggs were collected from three females of each of commercially exploited carp breeding line. Within carp lines, the eggs were pooled together. In the case of males, the milt was collected from 5 donors, and after examination of spermatozoal motility, were used for the fertilization. Eggs were hatched in an incubator and the larvae transferred to the first feeding phase 3 days later. At the time of hatching, fertilized eggs of reference laboratory cross (R3xR8) received from the CBI lab of Wageningen University, were included into setup. The carp progenies were grown in recirculating system of 3 m^3 with three aquaria for each breeding line, supplied with filtered and UV-treated water. For establishing natural survival, larvae of carp lines were stocked in experimental ponds of 632 m^2 surface, in quadruplicate as described below. Carp reared in recirculating system were artemia-fed for the first 14 days post-hatch and subsequently with commercial dry food (ALLER AQUA, Denmark) at a level of 3% of the metabolic body weight per day.

Environmental effect.

Carp larvae of each line (n = 3000 per pond) were divided over n = 4 ponds of 632 m^2 surface. The control group was carp reared in recirculation system. Survival under pond conditions was measured after the first rearing season (September, fish of 5 months of

age) and after the first wintering (April, fish of 11 months of age) according to standard procedures (Guziur *et al,* 2003).

Experimental infections

Carp reared in recirculating system were used to study associations of *Cyca-DAB1-like* genotypes with resistance to three different pathogens, in experimental infections.

Carp (n = 120, mean weight = 119 ± 50 g) received an individual intraperitoneal (i.p.) injection with the bacterium *Aeromonas hydrophila* (3 x 10^7 colony forming units in 100 µl of phosphate buffered saline), as previously described (Das Mahapatra *et al*., 2008, Rakus *et al*., 2008a). During infection clinical signs of disease were noted. Bacteria were re-isolated from kidney of dead fish to confirm infection. Mortality was taken as read-out parameter.

Carp (n = 83, mean weight = 120 ± 35 g) were exposed to n = 150 larval *Argulus japonicus* lice per aquarium and left for a period of 4 weeks for lice to establish themselves (Forlenza *et al*., 2008). Number of parasites at 28 day was taken as read-out parameter.

Carp (n = 263, mean weight = 80 ± 29 g) received an individual i.p. injection with 1 x 10^4 *Trypanoplasma borreli* in PBS, as previously described (Wiegertjes *et al*., 1995a, Jurecka *et al*., 2008). Parasitaemia was taken as read-out parameter and determined at weekly intervals (starting in the second week post infection (p.i.) and terminated in sixth week p.i.) using a Bürker counting chamber.

Cyca-DAB1-like genotyping

Cyca-DAB1-like (*Cyca-DAB1/Cyca-DAB2*) genotyping was performed using PCR-RF-SSCP and confirmed by sequencing, as described by Rakus *et al.* (2008a). In short, genomic DNA was isolated from fin clips by proteinase K digestion and purified using phenol:chlorophorm:isoamyl alcohol (Sambrook and Russell, 2001) or by a Chelex method (Yue and Orban, 2005). Specific primers, DAB12-F (5'-TCTGACATAACTGTAATGCTGC-3') and DAB12-REV (5'-CAGGAGAGATCAGAGTCTTG-3'), were designed to amplify *Cyca-DAB1*-like genes fragments, including the complete exon 2. PCR was carried out in a total volume of 30 µl containing 200-500 ng of template DNA, 200 nM of each primer, 200 µM of each

dNTP, 1.5 mM $MgCl_2$ and 1 unit of GoTaq Flexi polymerase (Promega, Madison, USA). The PCR profile included an initial denaturation step at 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 sec., 55 °C for 30 sec., 72 °C for 50 sec., and a final extension step at 72 °C for 7 min. PCR products were digested by restriction enzyme *Pst* I, denatured at 95 °C for 5 min with the presence of denaturing buffer and loaded on 9% polyacrylamide gel (9%T, 2%C) containing 5% of glycerol. Electrophoresis was performed in 1 x TBE buffer under optimized conditions (600V for 2h 15 min at 6 °C). Gels were silver staining and identification of the *Cyca-DAB1*-like genotypes was performed based on SSCP band patterns of the longer fragment (239 bp) of digested PCR products (Rakus *et al.*, 2008a). For each *Cyca-DAB1*-like genotype PCR products from 2 independent PCR reactions were ligated into pGEM-T Easy vector (Promega, Madison, USA) and cloned into *JM109* competent cells (Promega, Madison, USA) according to the standard protocol. Plasmid DNA was isolated from single positive colonies by alkali lyses and ethanol precipitation. A number of at least eight clones for each PCR product were selected for sequencing using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, USA) and the T7 primer in an ABI 377 automated sequencer (Applied Biosystem, USA). All sequences were examined and corrected using BioEdit 7.0.5.3 software (Hall, 1999) available at http://www.mbio.ncsu.edu/BioEdit/bioedit.html.

Statistical analysis

All statistical analysis were performed by using STATISTICA version 6.0 and MedCalc version 9.3.0.0 software. Differences were considered significant if the *P*-values were less or equal to a level of $\alpha = 0.05$.

In the study on the environmental effect, differences in survival rates between carp lines were tested for significance by one-way ANOVA. Differences in the distribution of *Cyca-DAB1*-like genotypes between carp reared under natural (pond) conditions and carp reared in aquaria conditions were tested for significance using a Chi-square test.

In the challenge experiments, first, differences between carp lines in resistance to selected pathogens measured as survival rate (*A. hydrophila* challenge) or parasitaemia levels (*A. japonicus* and *T. borreli* challenges) were tested. In the last two challenges the parasitaemia level was calculated as the number of lices per fish (at 28^{th} day p.i.) for the

A. japonicus challenge or as Area Under Curve value for the *T. borreli* challenge (based on parasitaemia level recorded for 4 weeks for each individual). In the *A. hydrophila* challenge, differences in cumulative mortality between carp lines were tested for significance by a Kaplan and Meier analysis (Kleinbaum, 2000). In the *A. japonicus* and *T. borreli* challenges, differences in parasitaemia level between carp lines were tested for significance by one-way ANOVA. In all cases there was an absence of significant differences between the carp lines, and association between *Cyca-DAB1*-like genotypes and resistance was tested at the population level (i.e. across all carp lines). Only genotypes that had a representation greater than 15% in the total population were included in the analysis.

Association between the presence or absence of particular *Cyca-DAB1*-like genotypes and survival/mortality during *A. hydrophila* challenge was studied using Cox proportional hazard regressions. Association between the presence or absence of particular *Cyca-DAB1*-like genotypes and parasitaemia level during *A. japonicus* and *T. borreli* challenges were tested by multiple regression. The dependent variable in this analysis was level of parasitaemia. The presence/absence of *Cyca-DAB1*-like genotypes were analysed as dichotomous independent variables, with individual coded 1 for the presence of a particular genotype and 0 for the absence of that genotype. Additionally, for the *T.borreli* challenge, Kruskal-Wallis test was used to compare level of parasitaemia between fish heterozygous or homozygous for the *Cyca-DAB1* gene.

Results

PCR-RF-SSCP reveals the presence of multiple Cyca-DAB1-like genotypes

We used PCR-RF-SSCP to screen a large number of carp individuals for *Cyca-DAB1*-like polymorphism. Genotyping by PCR-RF-SSCP revealed the presence of $n = 9$ different *Cyca-DAB1*-like genotypes (A- K, Fig. 1) in a total number of $n = 745$ carp individuals screened. PCR products representing the individual *Cyca-DAB1*-like genotypes were sequenced. *Cyca-DAB1*-like genes include *Cyca-DAB1* gene and *Cyca-DAB2* gene. Sequencing revealed five different alleles for *Cyca-DAB1* (**02-*06*) (GenBank accession numbers EU203666-EU203669 and EU860997) but only a single allele for *Cyca-DAB2* (**02*) (GenBank accession number EU203670). Some genotypes comprised *Cyca-DAB1* gene only and showed an absence of the *Cyca-DAB2* gene

(Table 1). The number of SSCP bands of each genotype clearly corresponded to the degree of heterozygosity of the *Cyca-DAB1* and *Cyca-DAB2* genes (Fig. 1), confirming the high sensitivity of the PCR-RF-SSCP method.

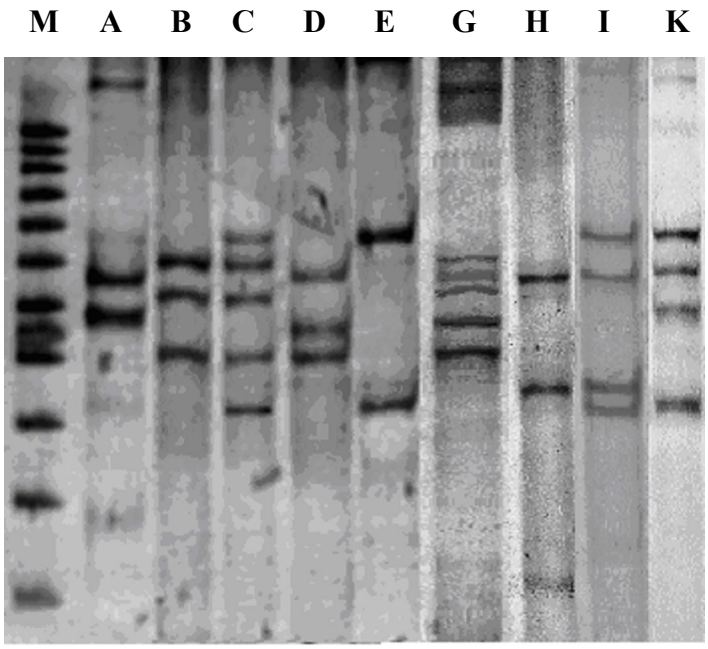

Figure 1. Different *Cyca-DAB1*-like genotypes detected by PCR-RF-SSCP technique and characterized by unique SSCP bands patterns. M-marker (TrackIt 100 bp DNA Ladder, Invitrogen). Genotypes F (Rakus *et al*., 2008a) and J (Rakus *et al*.- in preparation) were not revealed in this study.

Environmental effect

Survival rates of the carp lines raised in earthen ponds did not show significant differences between the carp lines, neither after the first rearing season nor after first wintering (see also Kachamakova *et al*., 2006). The distribution of the different *Cyca-DAB1*-like genotypes was compared between fish raised in ponds, and sampled after the first rearing season (n = 221), and fish raised in an aquarium system (n = 58; no major mortality event observed). No significant differences in distribution of *Cyca-DAB1*-like genotypes were noted between fish reared under pond and fish raised under aquarium conditions ($P > 0.05$). Apparently, association is best studied with defined infections using more than one pathogen. For this reason we chose to study association of *Cyca-DAB1*-like genotypes with resistance to three different pathogens, in experimental infections.

Table 1. Frequency of *Cyca-DAB1*-like genotypes as determined by PCR-RF-SSCP analysis.

Symbol	Genotype		Frequency
	Cyca-DAB1	*Cyca-DAB2*	
A	**04/*04*	not found	2.7%
B	**02/*02*	**02/*02*	47.3%
C	**02/*05*	**02/*02*	6.8%
D	**03/*03*	**02/*02*	18.5%
E	**05/*05*	not found	20.7%
G	**02/*03*	**02/*02*	2.0%
H	**06/*06*	not found	0.7%
I	**05/*06*	not found	1.2%
K	**02/*05*	not found	0.1%

Association between Cyca-DAB1-like genotypes and resistance of fish to selected pathogens

Three genotypes (B, D and E) had sufficient replications (present in >15% of the total population) across challenges with all three pathogens (Fig. 2) to be used for statistical analysis.

Challenge with Aeromonas hydrophila

A total number of $n = 120$ individuals were i.p. injected with *A. hydrophila* and mortality measured as read-out parameter for disease resistance. During the challenge fish showed frequent clinical signs of disease, such us darkened skin, diffuse haemorrhages on the ventral surface of the body and at the base of pectoral and pelvic fins, and ulcerative skin lesions. *A. hydrophila* could be re-isolated from the kidney of dead fish, confirming the mortality was due to the experimental infection. In general, fish died rapidly with a mean survival time (MST) of 3 days post-infection. Mean cumulative mortality over all carp lines was 75.5 ± 10.2% and varied from 65% (Polish carp line K) to 91% (Hungarian carp line R0). There were no significant differences in mortality between the carp lines. Therefore, association studies for the three major *Cyca-DAB1*-like genotypes were performed at the population level (i.e. across all carp lines). No significant associations between particular *Cyca-DAB1*-like genotypes and mortality of fish after *A. hydrophila* infection could be detected ($P = 0.6$) (Fig. 2A).

Challenge with Argulus japonicus

A total number of $n = 83$ individuals were exposed to larval *A. japonicus* and left for a period of 4 weeks for lice to establish themselves (*A. japonicus* take approximately 3-4 weeks to reach maturity at 23°C). The first gross pathological signs of infection were visible as red spots/lesions on the surface of infected fish and appeared between 24 and 48 h post infection. The number of attached lice/fish was not very high and varied between individual fish (mean = 3.8 ± 3.1 S.D.) in all tanks. There were no significant ($P > 0.05$) differences between the carp lines in terms of parasite abundance and infection intensity and association studies for the three major *Cyca-DAB1*-like genotypes were performed at the population level (i.e. across all carp lines). Analysis of variance showed significant correlation between *Cyca-DAB1*-like genotypes and abundance of *A. japonicus* ($F = 2.63$, $P = 0.05$). Association between the presence or absence of particular *Cyca-DAB1*-like genotypes and parasite abundance (number of lice), tested by multiple regression, showed a low regresion fit ($R^2 = 0.07$) but a statistically significant ($F = 6.06$, $P < 0.05$) overall relationship. For one particular genotype (E) we could detect a significant association with the abundance of *A. japonicus* (Fig. 2B). Genotype E was detected in 21 out of 83 fish challenged with *A. japonicus*. The presence of genotype E was associated with a lower number of lice (zero order correlation coefficient $r = -0.26$), indicating increased resistance to *A. japonicus*.

Challenge with Trypanoplasma borreli

A total number of $n = 263$ individuals were i.p. injected with *T. borreli*. Parasitaemia was monitored weekly for a period of four weeks, starting in the second week p.i.. The individual level of infection with *T. borreli* was calculated as area under the curve (AUC) values. Although the reference laboratory cross (R3xR8) showed the highest parasitaemia level and some mortality, there were no significant differences between the carp lines in terms of level of parasitaemia. Thus, association studies for the three major *Cyca-DAB1*-like genotypes were performed at the population level (i.e. across all carp lines). Kruskal-Wallis test was used to compare level of parasitaemia between fish heterozygous and homozygous for *Cyca-DAB1* gene. Test showed that *Cyca-DAB1* heterozygosity is associated with the low level of parasitaemia ($P < 0.05$).

Study of the association between presence or absence of particular *Cyca-DAB1*-like genotypes and level of parasitaemia, tested by multiple regression analysis, showed a low regresion fit ($R^2 = 0.01$) although statistically significant ($F = 5$, $P < 0.05$) overall relationship. For one particular genotype (D) we could detect a significant association with the level of parasitaemia (Fig. 2C). Genotype D was detected in 39 out of 263 fish challenged with *T. borreli*. The presence of genotype D was associated with a high level of parasitaemia (zero order correlation coefficient $r = 0.14$), indicattive of a lower resistance to *T. borreli.*

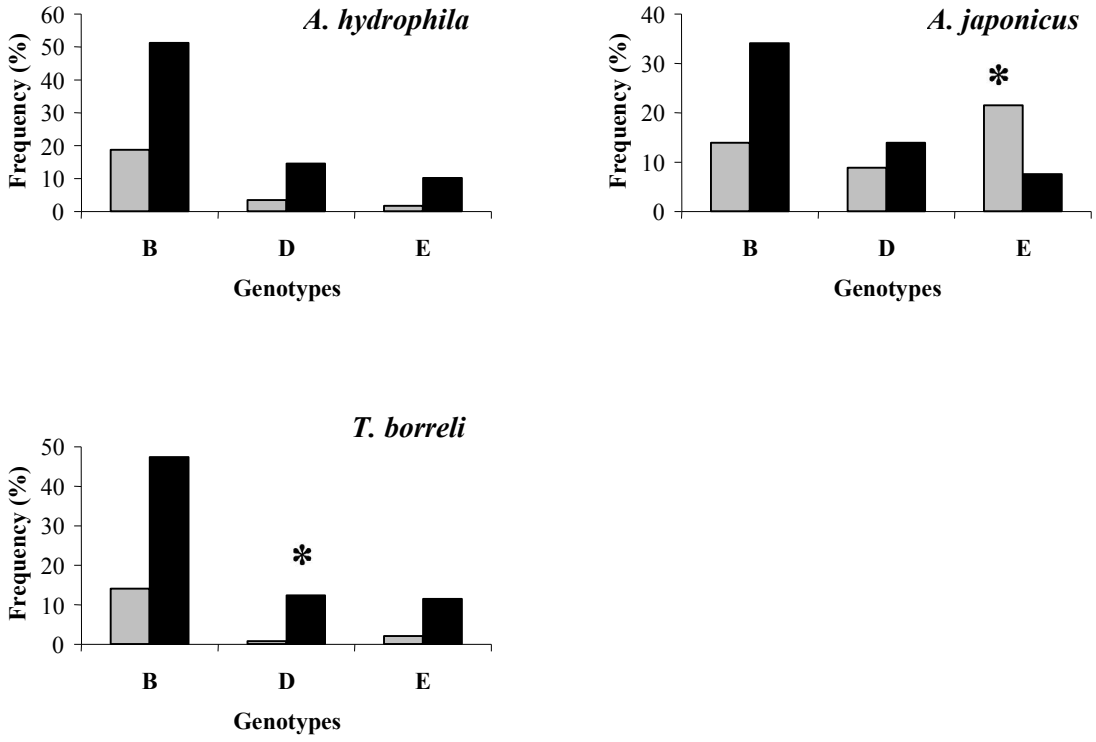

Figure 2. Distribution of *Cyca-DAB1*-like genotypes which had sufficient replication to be included within the statistical analysis in non-infected/survived (grey bars) and infected/dead (black bars) fish challenged with *A. hydrophila*, *A. japonicus*, and *T. borreli*. Asterisks indicate statistically significant ($P < 0.05$) associations between *Cyca-DAB1*-like genotypes and resistance/susceptibility.

Discussion

The ability of MHC molecules to present peptides to T lymphocytes with different effectiveness can influence the immune response of an organism and have an effect on the resistance to pathogenic organisms. In teleost fish species MH class I and class II genes reside on different chromosomes and segregate independently. This has allowed for association studies of only class I or only class II MH genes with disease resistance and given evidence for associations between MH polymorphism and disease resistance in several, but mostly salmonid, fish species. To our knowledge, this is the first association study between MH class II *B* genes and specific disease resistance of common carp. In common carp, there are two paralogous groups of MH class II *B* genes, *Cyca-DAB1*-like and *Cyca-DAB3*-like genes. We specifically examined *Cyca-DAB1*-like genes, which have been linked with high antibody responsiveness (Wiegertjes *et al*., 1996, Rakus *et al*., 2008b), are transcribed in different immune relevant organs (Ono *et. al*., 1993, Rakus *et al*., 2008b) and showed polymorphism among analyzed carp lines (Rakus *et al*., 2003). Nine unique *Cyca-DAB1*-like genotypes, revealed by PCR-RF-SSCP, were sequenced and shown to consist of unique combinations of *Cyca-DAB1* and *Cyca-DAB2* sequences. Three genotypes (B, D and E) were most common and present in frequencies high enough for association studies. Our results indicate that *Cyca-DAB1*-like polymorphism could be linked with resistance of carp to particular pathogens.

Survival of fish under natural conditions is an important parameter in carp production. It is also one of the best criteria for determining the level of resistance because it reflects the cumulative effects of all host-pathogen interactions during production (Wiegertjes *et al*., 1995b, Kachamakova *et al*., 2006). However, the same characteristic makes it very difficult to detect putative associations between MH polymorphism and survival rate. We could not detect statistically significant differences in survival rate among the carp lines reared under pond conditions, and could not detect a difference in distribution of *Cyca-DAB1*-like genotypes between fish reared under pond conditions and fish reared in aquaria. Most likely, the cumulative effects of all pathogens under the pond conditions have been too limited to show clear effects on survival rate. Indeed, no disease outbreaks were noted during the rearing season and regular health status checks proved fish were generally healthy. For these reasons we

subsequently chose not to continue with survival rate under pond conditions, but to examine the effect of particular *Cyca-DAB1*-like genotypes after experimental challenges with defined pathogens.

We studied association of *Cyca-DAB1*-like genotypes with resistance to three different pathogens and could detect an association of *Cyca-DAB1*-like genotype in two cases. The absence of a significant association of *Cyca-DAB1*-like genotypes with resistance or susceptibility to infection with the bacterium *A. hydrophila* could be explained by the apparent high infection dose, resulting in high mortality already at 3 days post infection (p.i.). In experimental infection of Atlantic salmon with *A. salmonicida* the time of death of 50% of infected fish was 15-20 days p.i (Kjøglum *et al*., 2008) and 25-30 days p.i. (Grimholt *et al*., 2003). Major histocompatibility class II restricted antigen presentation to $CD4^+$ T cells and the development of a specific immune response probably requires more time than 3 days only, and a lower infection dose would be required to detect putative associations between particular *Cyca-DAB1*-like genotypes and resistance to the bacterium *A. hydrophila.* However, our experiment did confirm the presence of individual variation in resistance of common carp to *A. hydrophila*, even when a high dose of the bacterium was used. When a disease occurs in a fish population, it does not necessarily affect all fish to the same extent; some fish, sometimes even in large numbers, may not be affected. Even the progeny of a single pair of spawners, reared under the same environmental conditions, do not all possess the same level of disease resistance. In these cases, individual variation may serve as a starting point for selection programmes.

We could detect a significant association of a particular *Cyca-DAB1*-like genotype (E) with increased resistance to ectoparasite *A. japonicus.* Although ectoparasites often are protected from the host's immune system merely by their location on the outside of the fish, a recent study of the immune response of carp against *A. japonicus* revealed up-regulation of several immune-related messengers including TNF- α, IL-1β and CXCa in skin tissue (Forlenza *et al*., 2008). Indeed, association between MH class II genes and susceptibility to the ectoparasitic sea louse *Lepeophtheirus salmonis* in Atlantic salmon has been reported (Glover *et al*., 2007). Ectoparasites have also been found a driving factor for MH polymorphism in European

cyprinid fish (Šimková *et al*., 2006). This supports the idea that MH class II genes can have an impact on the generation of an effective immune response against ectoparasites. The association of genotype E with resistance to the ectoparasite *A. japonicus* is interesting because genotype E comprises a *Cyca-DAB1* gene only and does not seem to comprise a *Cyca-DAB2* gene. In fact, the genotypes that did comprise a *Cyca-DAB2* gene never showed polymorphism for the *Cyca-DAB2* gene. *Cyca-DAB2* is not a pseudogene, since it was originally isolated from cDNA (Ono *et al*., 1993). In addition, we examined by real-time PCR the basal expression of *Cyca-DAB1* and *Cyca-DAB2* genes using gene-specific primers and did not find a major difference in expression level (unpublished data). However, based on polymorphism, our data suggest that of the *Cyca-DAB1*-like genotype the *Cyca-DAB1* gene would be the most important for conferring resistance to pathogens.

We could detect a significant correlation of another *Cyca-DAB1*-like genotype (D) with increased susceptibility to the blood parasite *T. borreli*. We also detected a significantly higher resistance of *Cyca-DAB1* heterozygotes to *T. borreli*. In general, MH heterozygotes are expeected to present a larger diversity of peptide antigens to T cells, which may result in selective advantage regarding disease resistance (Landry *et al*., 2001, Arkush *et al*., 2002). Our finding of significant associations between genotype E and abundance of *A. japonicus* and between genotype D and higher level of parasitaemia after *T. borreli* infection suggest that *Cyca-DAB1*-like polymorphism could be linked with resistance of carp to particular pathogens.

With a few exceptions (Arkush *et al*., 2001, Grimholt *et al*., 2003, Kurtz *et al*., 2004), most of the studies on association between MH polymorphism and resistance of fish have been performed using only one pathogen model (Langefors *et al*., 2001, Palti *et al*., 2001, Miller *et al*., 2004, Kjøglum *et al*., 2006, Zhang *et al*., 2006, Wynne *et al*., 2007, Glover *et al*., 2007, Kjøglum *et al*., 2008, Johnson *et al*., 2008, Xu *et al*., 2008). It has been pointed that resistance to different pathogens is conferred by different MH alleles (Grimholt *et al*., 2003). Or, identical alleles could even have an opposite effect on resistance to different pathogens (Kjøglum *et al*., 2008). Selection based on MH-associated resistance to a single pathogen risks that the population could become more susceptible to other pathogens. We chose to study association of *Cyca-DAB1*-like genotypes with resistance to three different pathogens in experimental infections, and

revealed that different genotypes were associated with resistance or susceptibility to different pathogens. Thus, our study underlines the importance of using more than one pathogen model when examining association between MH genes and disease resistance.

We could detect an association between particular *Cyca-DAB1*-like genotypes and resistance or susceptibility of carp to *A. japonicus* and *T. borreli*, indicating that MH class II *B* genes can be used as potential genetic markers in future breeding programs. The use of a large number of individuals and the use of different infection models have proven informative. At this moment we cannot directly link the associations to a direct effect of *Cyca-DAB1*-like genotype itself or to another locus in which *DAB* genes are in linkage disequilibrium. Thus, there are still additional researches needed to verifying biological significance of the association described in our study as well as to investigate additional genes that may affect the resistance.

Acknowledgements

The authors would like to acknowledge the help of Dr. M.E. Nielsen of the Technical University of Denmark, Danish Research Institute for Fisheries Research, for kindly supplying the original virulent strain of *Aeromonas hydrophila*. In addition, we would like to thank Dr. N. Kachamakova, Dr. D. Onara and K. McNamee for help in parasite counting. This study was supported by the State Committee for Scientific Research in Poland (KBN; grant number 3 P06K 005 25).

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CHAPTER 6

Resistance of common carp (*Cyprinus carpio* L.) to Cyprinid herpesvirus-3 is influenced by major histocompatibility (MH) class II *B* gene polymorphism

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Submitted

Abstract

We analyzed the influence of MH class II *B* (*Cyca-DAB1*-like) genes on resistance of common carp (*Cyprinus carpio* L.) to highly pathogenic virus Cyprinid herpesvirus-3 (CyHV-3), also known as koi herpesvirus (KHV). The material consisting of 934 fish from six carp crosses. Fish were challenged with CyHV-3 at different time points: when fish were 7 and 10 months of age. During challenge experiments the peak of mortality caused by CyHV-3 was observed at days 8-12 p.i. and the overall cumulative mortality reached 79.9%. Among six *Cyca-DAB1*-like genotypes, revealed by PCR-RF-SSCP analysis, one genotype (E) performed significantly better, resulting in carp more resistant to CyHV-3. Three other genotypes (B, H and J) could be linked to higher susceptibility to the virus. Analysis of the alleles that compose the *Cyca-DAB1*-like genotypes linked one particular allele (*Cyca-DAB1*05*) to significantly increased, and two alleles (*Cyca-DAB1*02* and *Cyca-DAB1*06*) to significantly decreased resistance to CyHV-3. Our data indicate that MH class II *B* genes could be used as potential genetic markers in breeding for resistance to this virus.

Introduction

Within the last decade, mass mortality of farmed carp and ornamental domesticated varieties (koi), which have a viral aetiology, has been observed in numerous fish farms in Europe, Israel, USA, Japan, China, Taiwan, Thailand, Indonesia, Malaysia, and South Africa (Neukirch *et al*., 1999, Hedrick *et al*., 2000, Perelberg *et al*., 2003, Haenen *et al*., 2004, Sano *et al*., 2004, Tu *et al*., 2004, Aoki *et al*., 2007, Bondat-Reantaso *et al*., 2007). Outbreaks of disease also have been observed among wild common carp populations in the United Kingdom, Japan and several states in USA (Sano *et al*., 2004, Grimmett *et al*., 2006). The disease-causing virus has been identified as Cyprinid herpesvirus-3 (CyHV-3) (Waltzek *et al*., 2005) also known as Koi Herpesvirus (KHV) (Hedrick *et al*., 2000) and Carp Interstitial Nephritis and Gill Necrosis Virus (CNGV) (Pikarsky *et al*., 2004). CyHV-3 is a member of a new family *Alloherpesviridae* comprising piscine and amphibian herpes viruses, as proposed by McGeoch *et al*., (2006). The major symptoms of disease caused by CyHV-3 are: hyperplasia and necrosis of the respiratory epithelium of the gill (Hedrick *et al*., 2000, Neukirch & Kunz, 2001, Perelberg *et al*., 2003), and focal necrosis of hepatocytes and of glomerular cells in the kidney (Hedrick *et al*., 2000, Perelberg *et al*., 2003). Currently, the losses of farmed carp and koi carp populations caused by CyHV-3 have a very negative impact on production of carp farms over the whole world.

Even though there is an effective live attenuated vaccine against CyHV-3 ('KoVax') available in Israel, European Union legislation does not allow for the use of such a vaccine. Also, vaccination is an effective approach only in intensive systems and/or high market value species (koi), while traditional carp aquaculture is carried out in semi-intensive pond culture conditions and does not lead to high market value fish. Alternatively, genetic selection for improved carp lines and, to some extent, mass selection can be used to improve aquacultured fish species. For example, it has been shown that hybrid crosses of common carp were less sensitive to diseases than their parental purebred strains (Hines *et al*. 1974; Sovenyi *et al*. 1988). Also, Kirpichnikov *et al*. (1993) developed a disease resistant breed of Krasnodar carp by the use of mass selection of fish for resistance to dropsy, a very serious infectious disease, but this long-term work included breeding for up to 8-9 generations. Recently, Shapira *et al*. (2005) described differential resistance to CyHV-3 among selected common carp strains and

crossbreds, which suggests that resistance of carp to this virus might be affected by genetic factors.

In general, in an immune response to viruses, viral peptides derived from cytosolic virus biosynthesis are presented by major histocompatibility complex (MHC) class I molecules to cytotoxic $CD8^+$ T cells. In addition, MHC class II molecules can present viral peptides derived from degradation in endosomal/lysosomal compartments, to $CD4^+$ T helper cells. The latter process stimulates the production of interferons that can act against virus dissemination and reinfection and stimulates the production of specific antibodies (Neumann *et al*., 2003, Hangartner *et al*., 2006). Although maybe only a minor fraction of these antibodies have direct antiviral activity, antibodies will help to control virus infections by activating the complement system, facilitating phagocytosis and promoting antibody-dependent cellular cytotoxicity (Hangartner *et al*., 2006). Actually, since the final stage of the virion assembly process of herpesviruses, occurs in endosomal compartments, viral peptides can enter the MHC class II pathway very efficiently and be presented to $CD4^+$ T cells (Tomazin *et al*., 1999, Hegde *et al*., 2003, Hegde *et al*., 2005, Wiertz *et al*., 2007). It therefore makes sense to study both major histocompatibility (MH) class I and class II genes in the context of CyHV-3 infection.

The MHC contains some of the most polymorphic genes known to date and each MHC molecule has the ability to bind and present different groups of peptides in more or less successful ways. There are a number of reports that described evidence for associations between MH polymorphism and disease resistance in several, but mostly salmonid, fish species (Langefors *et al*., 2001, Palti *et al*., 2001, Grimholt *et al*., 2003, Miller *et al*., 2004, Kjøglum *et al*., 2006, Wynne *et al*., 2007, Johnson *et al*., 2008). We recently optimized a simple, but highly sensitive technique based on single strand conformation polymorphism (SSCP) that allows us to screen large numbers of individuals for MH class II *B* polymorphism (Rakus *et al*., 2008). We are in the process of optimizing this technique for MH class I polymorphism. In common carp, there are two paralogous groups of MH class II *B* genes; *Cyca-DAB1*-like genes, and *Cyca-DAB3*-like genes. Previous studies have indicated a ubiquitous presence and high polymorphism of *Cyca-DAB1*-like, but not *Cyca-DAB3*-like, genes (Rakus *et al*., 2003). For this reason we decided to focus on the *Cyca-DAB1*-like genes. Association between

particular MH class II *B* (*Cyca-DAB1*-like) genotypes and higher resistance to CyHV-3 would allow us to use MH genes in future selection for increased resistance to disease in common carp. Such genetic selection would present a sustainable approach to disease control in semi-intensive carp pond farming.

This study has the following objectives: (*i*) to estimate the genetic differences in resistance to CyHV-3 infection between selected common carp crosses; and (*ii*) to determine the association between major histocompatibility (MH) class II *B* genes (*Cyca-DAB1*-like) polymorphism and ability of carp to resist infection with CyHV-3.

Materials and methods

Fish

European common carp (*Cyprinus carpio carpio* L.) used in this study included 12 reciprocal crosses obtained at the Institute of Ichthyobiology & Aquaculture in Gołysz (Poland) by diallelic crosses of four carp lines: Polish 'K' and 'R6', Hungarian 'R7' and French 'F' (Table 1). From each line one female and one male were randomly taken for propagation. All matings were performed on the same day. Eggs of one female from each line were divided into three equal portions and each portion fertilized with milt of one male from the appropriate carp line (Table 1). Separate incubation and hatching were used for 12 reciprocal crosses. At the swim-up stage, fish from each of reciprocal cross were moved into a system with UV treated recirculating water and bio-filters and were grown in separate tanks (500 fish per tank) in triplicate. Total capacity of the system was 3 m^3 and the temperature of water was maintained at 21 ± 2 ^oC. Daily feeding with carp commercial pellets (Bestfeed) at 1 % body weight was applied. Before challenge experiments, carp from the 12 reciprocal crosses were randomly sampled, marked by fin clipping, mixed and transported to the Laboratory of Fish Pathology and Immunology IFI, Żabieniec (Poland). Fish were allowed to acclimatize to the new recirculating system for a period of 14 days. No mortalities were observed during the acclimatization period.

Table 1. Experimental design. For each challenge experiment (n = 2) fish from the 12 reciprocal crosses were mixed together in one tank, in duplicate, and infected by i.p. injection. The third tank was the control.

♀ \ ♂	K	R6	R7	F
K	-	K x R6	K x R7	K x F
R6	R6 x K	-	R6 x R7	R6 x F
R7	R7 x K	R7 x R6	-	R7 x F
F	F x K	F x R6	F x R7	-

Virus cultivation

Koi fin cell (KFC) cultures, kindly supplied by professor Moshe Kotler from the Department of Pathology, Hebrew University-Hadassah Medical School in Jerusalem (Israel) were used for virus propagation. Cells were grown in culture medium containing 60 % Dulbecco's modified Eagle's medium, 20 % Leibovitz (L15) medium, 10 % fetal bovine serum (Sigma), 10 % tryptose phosphate (Difco) and supplemented with 1 % HEPES and antibiotics to form of monolayer over a period of 10 – 14 days in a 22 ^{o}C incubator supplemented with 5 % CO_2. The monolayer cultures were trypsinized and transferred into new flasks with fresh medium. The KFC cultures were inoculated with supernatants prepared from gills and kidney of CyHV-3 infected fish. The infected KFC cultures were incubated at 22 ^{o}C for 10 days and examined for cytopathic effect (CPE). The virus was released into the culture medium during the appearance of a CPE, but a significant amount remained associated with the cell. When CPE was observed in the KFC cultures, CyHV-3 viral identification was confirmed by PCR. Purification of virus from culture medium was done according to protocol (Pikarsky *et al*., 2004). Virus concentrations were estimated by $TCID_{50}$ using the method of Reed and Meunch (1938) as presented for fish herpesvirus by Hedrick *et al*. (2000). Identification of CyHV-3 from gills and kidneys of experimental infected carps was confirmed by PCR.

Challenge test

Two challenge experiments were performed at different time points: when fish were 7 months and when fish were 10 months of age. For both challenges fish were kept in three 500L tanks. Each tank (approximately 240 fish) consisted of 12 reciprocal crosses, represented by 20 individuals per cross (Table 1). Tanks were supplied with aerated and

filtrated water at 22°C. Two tanks were used to challenge fish and the third tank served as negative control group. A total number of *n* = 934 fish were infected with CyHV-3. Fish were infected by intraperitoneal (i.p.) injection with 0.2 ml of isolated virus suspension at concentration 4 $TCID_{50}$ /ml as previously described by Pikarsky *et al*. (2004). The control group was intraperitoneally injected with 0.2 ml of medium harvested from uninfected KFC culture (no virus). At day 7 after experimental infection *n* = 10 fish from the experimental and control groups were lethally anesthetized by the use of Propiscin (5 ml/l) and were sampled for identification of CyHV-3 by PCR. Kidneys and gills from *n* = 10 fish after mortality were isolated for identification of the virus by PCR. The main outbreak caused by CyHV-3 started after day 8 and the study was terminated at day 33 after infection. Dead fish were sampled daily.

Detection of viral DNA by PCR

Viral DNA was detected in infected fish using a CyHV-3-specific PCR according to Bercovier *et al*. (2005). DNA was extracted from gills and kidneys using thermal lysis. PCR amplification was performed using specific primers for the CyHV-3 thymidine kinase gene (TK-fw 5'-GGGTTACCTGTACGAG-3' and TK-rev 5'-CACCCAGTAGATTATGC-3') and amplified a 409 bp product. Amplification of target DNA was carried out in 20 µl reaction using 1 µl of total DNA, 10x Taq Buffer, 25 mM $Mg(OA_C)_2$, 200 µM of each dNTP, 1 µM each of the primers and 2 units of Taq polymerase (Eppendorf, AG Hamburg Germany). The PCR profile included an initial denaturation step at 95 °C for 5 min, followed by 35 cycles under following conditions: denaturation 95 °C for 30 sec, annealing 52 °C for 30 sec, extension 72 °C 1 min, and a final extension step at 72 °C for 10 min.

PCR products were detected in 1% agarose gel with ethidium bromide staining and UV illumination (MINI-SUB CELL GT, PowerPac Basic, Molecular Imager Gel Doc TM, Biorad Laboratories Inc.).

Cyca-DAB1-like genotyping

In common carp, there are two paralogous groups of MH class II *B* genes. *Cyca-DAB1*-like genes, which include *Cyca-DAB1* and *Cyca-DAB2* genes, and *Cyca-DAB3*-like genes, which include *Cyca-DAB3* and *Cyca-DAB4* genes. *Cyca-DAB1*-like genotyping

was performed using PCR-RF-SSCP and confirmed by sequencing, as described by Rakus *et al.* (2008). Specific primers, DAB12-fw (5'-TCTGACATAACTGTAATGCTGC-3') and DAB12-rev (5'-CAGGAGAGATCAGAGTCTTG-3'), were designed to amplify the *Cyca-DAB1*-like genes fragments including the complete exon 2. PCR products were digested by restriction enzyme *Pst* I, denatured at 95 °C for 5 min with the presence of denaturing buffer and loaded on 9% polyacrylamide gel (9%T, 2%C) containing 5% of glycerol. Electrophoresis was performed in 1 x TBE buffer under optimized conditions (600V for 2h 15 min at 6 °C). Gels were silver-stained and identification of the *Cyca-DAB1*-like genotypes was performed based on SSCP bands patterns of the longer fragment (239 bp) of digested PCR products. For each *Cyca-DAB1*-like genotype PCR products from 2 independent PCR reactions were cloned and sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, USA) and the T7 primer in an ABI 377 automated sequencer (Applied Biosystem, USA). All sequences were examined and corrected using BioEdit 7.0.5.3 software (Hall, 1999) available at http://www.mbio.ncsu.edu/BioEdit/bioedit.html.

Statistical analysis

The main variable in this study was time until death and this variable was graphically expressed using Kaplan and Meier survival plots (Kleinbaum, 2000). The results are presented as proportional Hazard Ratio (HR) with 95% confidence intervals (CI) and as cumulative mortality (%). The Hazard Ratio compares mortality in a group with a particular genotype or allele versus a group without this genotype or allele. The proportional HR was calculated using Cox proportional hazard regressions. All statistical analyses were performed using STATISTICA version 6.0 and MedCalc version 9.3.0.0 software. Differences were considered significant if the *P*-values were found less or equal to a level of $\alpha = 0.05$.

Results

Common carp crosses show genetic differences in resistance to CyHV-3

During challenge experiments the peak of mortality caused by CyHV-3 was observed at days 8-12 p.i. and the overall cumulative mortality reached 79.9%. Presence of CyHV-3 was confirmed by PCR in fish from infected groups sampled at day 7 p.i. and in selected infected fish which died during the experiment. There were no significant differences in performance of reciprocal crosses (cumulative mortality) between the duplicate aquaria (no tank effect). There also were no significant differences in performance of reciprocal crosses between the two replicate challenge experiments (no time effect). Thus, the mortalities for each reciprocal cross as measured in each experiment in duplicate tanks were taken together and used as one large dataset for statistical analysis. This analysis did not show a significant parental effect on cumulative mortality, with the exception of the cross between carp from the Hungarian R7 line and carp from the Polish R6 line ($P < 0.05$). However this parental effect was observed only once and not confirmed in other crosses when the same individual females and males were used. Therefore, the data for the reciprocal crosses were combined and used as one large dataset on six crosses (F x R6, F x R7, K x F, K x R6, K x R7, and R6 x R7) for further statistical analysis (see also Table 1).

First, the effect of different carp crosses on cumulative mortality was analysed. A highly significant difference in mortality between carp crosses ($P < 0.0001$), indicated the involvement of a genetic factor in resistance to CyHV-3 (Fig. 1, Table 2). The range between the crosses with highest and lowest mortality was 26%. Post-hoc analysis showed a significant genetic effect on reduced mortality in case of crosses K x R7 and K x F and on increased mortality in case of crosses F x R6, F x R7, and R6 x R7 during experimental challenge with CyHV-3. In general, crosses obtained using spawners of Polish breeding line K showed a significantly lower mortality, indicating a direct line effect.

Cross	Mortality
F x R6	89%
F x R7	90%
K x F	65%
K x R6	76%
K x R7	64%
R6 x R7	89%

Table 2. Overall cumulative mortality of each carp crosses after two challenge experiments with CyHV-3. Crosses with significant increased or reduced mortality are presented in bold.

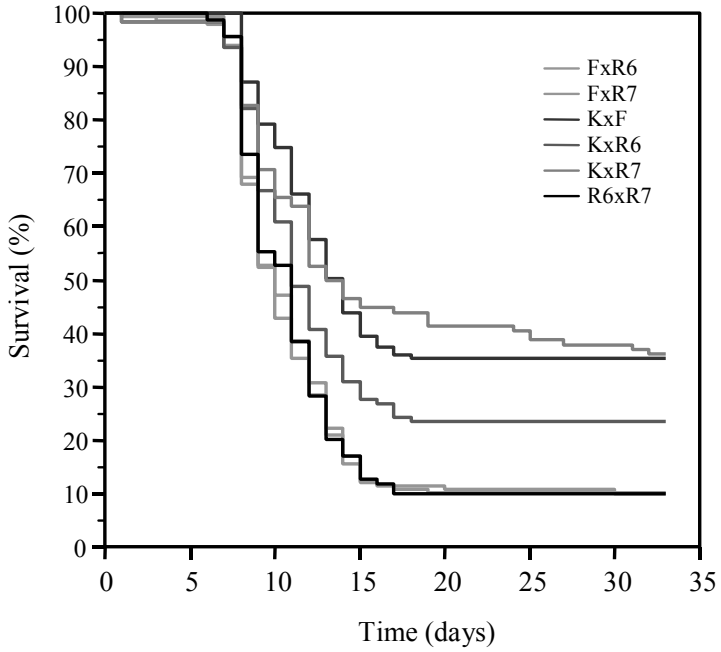

Figure 1. The time in days from the challenge to the death expressed by Kaplan and Meier survival plots for different carp crosses.

Cyca-DAB1-like genotypes are associatied with resistance or susceptibility to CyHV-3

We used PCR-RF-SSCP to screen a large number of carp individuals for *Cyca-DAB1*-like polymorphism. Genotyping by PCR-RF-SSCP revealed the presence of n = 6 different *Cyca-DAB1*-like genotypes (B - J) in a total number of n = 934 carp individuals infected with CyHV-3 (Table 3).

Table 3. Frequency of *Cyca-DAB1*-like genotypes as determined by PCR-RF-SSCP analysis.

Symbol*	Genotype		Frequency
	Cyca-DAB1	*Cyca-DAB2*	
B	**02/*02*	**02/*02*	6.5%
C	**02/*05*	**02/*02*	5.4%
E	**05/*05*	not found	61.4%
H	**06/*06*	not found	6.5%
I	**05/*06*	not found	14.7%
J	**02/*06*	**02/*02*	5.2%

*Genotypes A, D, F, G were not revealed in the present study. They are present in Fig. 1 in chapter 3 and Fig. 1 in chapter 5 this thesis.

A significant variation in mortality between fish with different *Cyca-DAB1*-like genotypes was detected ($P < 0.0001$) (Fig. 2). The range of mean mortality between *Cyca-DAB1*-like genotypes performing best and worst varied by 34%. Of the six *Cyca-DAB1*-like genotypes four genotypes significantly affected resistance or susceptibility of carp to CyHV-3. The analysis based on presence or absence of particular *Cyca-DAB1*-like genotypes, identified one genotype (E) with a significantly reduced hazard ratio (HR), and three genotypes (B, H, and J) with a significantly increased HR (Table 4). The hazard ratio compares probability of mortality in groups with or without a particular genotype.

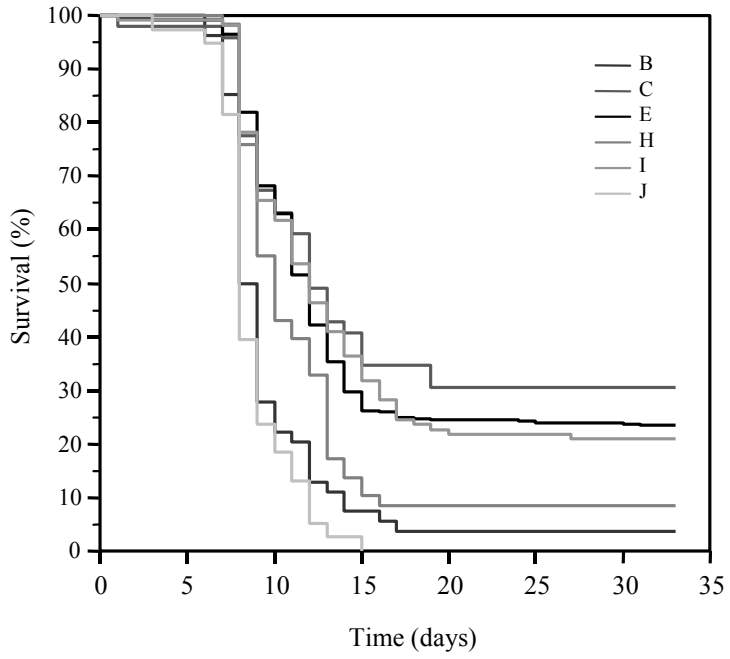

Figure 2. The time in days from the challenge to the death expressed by Kaplan and Meier survival plots for groups having different *Cyca-DAB1*-like genotypes.

Table 4. Hazard ratio and mortality in groups with different *Cyca-DAB1*-like genotypes. Significant HR are presented in bold.

Genotype (symbol)	Hazard ratio (95% CI)	Mortality
B	**2.126** (1.625, 2,781)	95%
C	0.726 (0.515, 1.023)	66%
E	**0.760** (0.657, 0.878)	76%
H	**1.472** (1.122, 1.932)	92%
I	0.897 (0.735, 1.095)	82%
J	**2.092** (1.566, 2.794)	100%

Taking into account that significant differences in mortality between families were detected, we performed a within cross analysis to detect a possible bias in the estimate of *Cyca-DAB1*-like genotype effect. Such a bias may be caused by random associations of *Cyca-DAB1*-like genotypes with better or worse performing families, or by interactions with background genes in particular crosses. Within cross analysis confirmed that genotype E was the best performing genotype while genotypes B, H, and J were worst performing genotypes (data not shown). Cox regression analysis confirmed that in the susceptible crosses F x R7 and R6 x R7, genotype E remained the genotype with a significantly reduced HR ($P < 0.05$).

Cyca-DAB1 alleles are associated with resistance or susceptibility to CyHV-3

Cyca-DAB1-like genotypes include *Cyca-DAB1* and *Cyca-DAB2* genes. In order to study effect of single alleles, PCR products representing different *Cyca-DAB1*-like genotypes were sequenced. Sequencing analysis confirmed the presence of three different *Cyca-DAB1* alleles (*Cyca-DAB1*02, Cyca-DAB1*05,* and *Cyca-DAB1*06*) (GenBank accession numbers EU203666, EU203669 and EU860997) but only a single allele for *Cyca-DAB2* (*Cyca-DAB2*02*) (GenBank accession number EU203670). Some *Cyca-DAB1*-like genotypes comprised a *Cyca-DAB1* gene only. If the *Cyca-DAB2* gene was present, it was always present in homozygous form (Table 3).

We included only *Cyca-DAB1* allelic polymorphism in the analysis of association between *Cyca-DAB1*-like alleles and resistance to CyHV-3. The analysis was based on the presence or absence of *Cyca-DAB1* alleles and identified one particular allele (*Cyca-DAB1*05*) with a significantly reduced HR, and two alleles with a significantly increased HR (*Cyca-DAB1*02* and **06*). Fish with the *Cyca-DAB1*05* allele performed significantly better with lower mortality than fish without the presence of the *Cyca-DAB1*05* allele. Fish with the allele *Cyca-DAB1*02* or *Cyca-DAB1*06* performed significantly worse with higher mortality than fish not expressing these alleles (Table 5).

Table 5. Hazard ratio and mortality in groups with different *Cyca-DAB1* alleles. Significant HR are presented in bold.

Allele	Hazard ratio (95% CI)	Mortality
*Cyca-DAB1*02*	**1.526** (1.271, 1,832)	87%
*Cyca-DAB1*05*	**0.497** (0.417, 0.591)	74%
*Cyca-DAB1*06*	**1.233** (1.053, 1.442)	88%

Discussion

We analyzed the association between major histocompatibility (MH) class II *B* genes and resistance of common carp to CyHV-3. To this end we used the PCR-RF-SSCP technique, optimized for studying in an efficient manner polymorphism of carp *Cyca-DAB1*-like genes. We could detect a significant association between the presence of particular *Cyca-DAB1*-like genotypes and resistance or susceptibility of carp to CyHV-3. Among six *Cyca-DAB1*-like genotypes detected by PCR-RF-SSCP analysis, one genotype (E) performed significantly better, resulting in carp more resistant to CyHV-3. Three other genotypes (B, H and J) could be linked to higher susceptibility to the virus. Analysis of the alleles that compose the *Cyca-DAB1*-like genotypes linked one particular allele (*Cyca-DAB1*05*) to significantly increased, and two alleles (*Cyca-DAB1*02* and *Cyca-DAB1*06*) to significantly decreased resistance to CyHV-3. Our data indicate that MH class II *B* genes could be used as potential genetic markers in breeding for resistance to this virus. The genotype E especially, which has also been associated with increased resistance to *Argulus japonicus* (Rakus *et al*., chapter 5 this thesis), could be linked to increased resistance to CyHV-3 and could serve as a potential marker for improved disease resistance of carp.

The *Cyca-DAB1*-like genes in common carp include *Cyca-DAB1* and *Cyca-DAB2* genes (Ono *et al*., 1993). In our study, the *Cyca-DAB2* gene was either homozygous or absent. We also found a very high frequency (about 80%) of *Cyca-DAB1* genes that were homozygous. For the genotypes that were heterozygous for the *Cyca-DAB1* gene (about 20% of the animals), only the combination of two susceptible *Cyca-DAB1* alleles (**02/*06*) had a significant effect on performance, resulting in the susceptible genotype J. The combination of resistant and susceptible *Cyca-DAB1* alleles in the other heterozygous genotypes (C and I; **02/*05* and **05/*06*, respectively) had no significant effect on performance, although the hazard ratio in both cases was below one. We could detect no significant heterozygote advantage with respect to increased resistance to CyHV-3. Absence of significant heterozygote advantage for MH class II *B* genes in fish has also been noted in other laboratory-based disease challenge experiments (Langefors *et al*., 2001, Miller *et al*., 2004, Wynne *et al*., 2007).

The limited heterozygosity of the carp MH class II *B* genotypes could be connected to a high level of inbreeding combined with bottleneck effects, typical for

breeding populations and usually considered unfavorable effects of closed breeding programmes. Interestingly, however, homozygous MH class II haplotypes, more than heterozygous ones, could be associated with increased resistance towards the infectious salmon anaemia virus (ISAV) and *Aeromonas salmonicida* causing furunculosis in Atlantic salmon (Grimholt *et al*. 2003). In our study homozygous genotype E was found to be significant associated with increased resistance of carp to CyHV-3. This suggest that for MH class II genes homozygosity may be an advantageous in respect to higher resistance of fish at least under laboratory-based challenge tests.

Studies in three-spined stickleback indicated that fish, which have an intermediate number of MH class II B alleles, showed the lowest parasite load when exposed to different parasites in natural conditions (Wegner *et al*., 2003) as well as showed the highest resistance to *Schistocephalus solidus* and microsporidians *Glugea anomal* during laboratory challenge tests (Kurtz *et al*., 2004). This suggests that in three-spined stickleback optimal rather than maximal MH class II diversity is advantageous with respect to higher resistance against parasites. In general, when more than one locus exists, different numbers of alleles per individual can be the result of both heterozygosity at existing loci and differing numbers of loci. One of the highest variations in number of MH class II *B* loci has been described for cichlid fish, where the number of transcribed loci varied from 1 to 13 (Malaga-Trillo *et al*., 1998). In common carp there are at least two transcribed MH class II *B* loci including *Cyca-DAB1*-like and *Cyca-DAB3*-like sequences, respectively. We did not study allelic polymorphism of the *Cyca-DAB3*-like genes. However, Ottová *et al*. (2005) studied overall polymorphism of *DAB* genes (MH class II *B*) in 11 cyprinid species from central Europe and found that the total number of distinct *DAB* transcripts observed per individual fish was 1-3. When three different transcripts were detected in a single individual, either two *DAB1* and one *DAB3* or one *DAB1* and two *DAB3* sequences were present. This observation, together with the hypothesis developed for sticklebacks of optimal MH diversity indicate that it is not necessary to maintain heterozygosity at each MH class II *B* locus in fish. Polymorphism of *DAB* genes can be therefore maintained by the presence of variable number of loci.

It is not surprising to find that MH class II *B* genes of carp can have an effect on immunity against CyHV-3 infection, based on the fact that viral peptides derived from

endogenous proteins of other herpesviruses can be efficiently presented by MHC class II molecules to $CD4^+$ T cells (Wiertz *et al*., 2007). In humans and mouse several reports suggest that $CD4^+$ T cells are essential in the control of herpesvirus infection (Heller *et al*., 2006, Wiertz *et al*., 2007). Studies of mouse herpesvirus MHV-68 revealed that $CD4^+$ T cells could control an infection *in vivo,* independent of $CD8^+$ T cells and B cells (Christensen *et al*., 1999). It has been also shown that efficient presentation of Epstein Barr virus glycoproteins by MHC class II molecules can result in direct killing of EBV-infected B cells by $CD4^+$ T cells (Landais *et al*., 2004, Heller *et al*., 2006). Little is known about the immune response of carp against CyHV-3. Electron microscopy analysis revealed the presence of CyHV-3 virions in immune organs such as gill and liver, including both hepatocytes and branchial epithelial cells, and also in lymphocyte-like cells in the capillaries of both gill and hepatic blood vessels (Hedrick *et al*., 2000). Expression of MH class II genes in fish can be found in immune organs, including gills and liver (Rodrigues *et al*., 1995, Koppang *et al*., 1998, Chen *et al*., 2006). Thus, it is likely that peptides derived from CyHV-3 can be presented by MH class II molecules to $CD4^+$ T cells in carp. The sequence for CD4 of carp has recently been published (Sun *et al*., 2007). Different MH class II molecules could present different peptides in more or less successful ways, also in carp. Therefore, the immune response of carp against CyHV-3 can be influenced by the MH genotype.

Of course, our study cannot answer whether the association between MH class II *B* genotypes or alleles and higher resistance to CyHV-3 is due to the presence of a particular genenotype (E), or allele (*Cyca-DAB1*05*), or due to an association of this genotype/allele with other closely linked genes on the same chromosome. Nevertheless, our results indicate that MH class II *B* genes are potential gene markers which can be used in selection aimed at increasing resistance of common carp to CyHV-3.

In conclusion, we have shown the presence of differences in resistance of various carp crosses to CyHV-3 under laboratory challenge circumstances. We described an association between resistance to CyHV-3 and MH class II *B* genes. It would make sense to study both MH class I and class II genes in this context. To also be able to study the association between carp MH class I diversity and resistance to CyHV-3, we are developing a fast and sensitive SSCP-based method to screen for MH class I polymorphism in large numbers of fish. We believe that comprehensive studies

including both MH class I and class II genes would provide the required knowledge for implementation of MH polymorphism as markers of resistance to CyHV-3. Such genetic selection would present a sustainable approach to disease control in semi-intensive carp pond farming.

Acknowledgements

We thank Mrs Barbara Kazuń and dr. Krzysztof Kazuń for help with challenge experiments and Ms Aldona Nikiel for help with DNA isolation. We are grateful to Prof. Moshe Kotler from the Department of Pathology, Hebrew University-Hadassah Medical School in Jerusalem (Israel) for providing Koi fin cells (KFC) culture. This study was supported by the MNiSW in Poland (grant no.: N N308 097834).

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CHAPTER 7

General discussion

7. General discussion

7.1. Major Histocompatibility genes as markers for disease resistance in fish

The common carp (*Cyprinus carpio* L.) is one of the main aquacultured species in the world. Domesticated in ancient times (Balon, 1995), carp have undergone selective breeding for centuries (Vandeputte, 2003). Although several reports have described genetic improvement of common carp by selective breeding, crossbreeding, hybridisation and genome manipulation (Wohlfarth, 1986, Kirpichnikov *et al*., 1993, Bakos and Gorda, 1995, Hulata, 1995, Vandeputte, 2003), so far, notable success has been achieved mostly for improvement of productive traits (body shape, scaliness type, growth rate ect.) (Moav and Wohlfarth, 1966 and 1976, Białowąs, 1991, Wohlfarth, 1993). Limited effort has gone into a genetic approach to reduce the impact of diseases, caused by a wide range of pathogens (viruses, bacteria and parasites).

Genes of the MHC are candidate genes for disease resistance because they are crucial elements of adaptive immunity, represent the most polymorphic genes in the vertebrate genome and have been associated with resistance to various diseases in many vertebrate species, including fish (**chapter 1**). The most notable differences in the organization of the major histocompatibility genes in teleost fish with the organization in e.g. human and mouse are the nonlinkage of class I and class II regions (Bingulac-Popovitc *et al*., 1997, Sato *et al*., 2000), and the wide dispersion of genes that otherwise reside in the mammalian MHC, across several different linkage groups (Nonaka *et al*., 2001, Sambrok *et al*., 2002, and 2005). In line with these findings, Stet *et al.* (2003) proposed to use, for teleost fish, as terminology major histocompatibility (MH) genes instead of major histocompatibility complex (MHC) genes. In teleost, two distinct MH class I lineages ('U' and 'Z/ZE') have been reported (Kruiswijk *et al*., 2002, Stet *et al*., 2003) and showed not to be linked with MH class II lineages. However, recent study revealed presence in cyprinid and salmonid fishes a third MH class I lineage ('L') that was shown to be linked with MH class II loci in zebrafish (Dijkstra *et al*., 2007). The L lineage consists of extensively diversified nonclassical (Ib) MH genes and was present in Ostariophysi and Protacanthopterygii but was not in Neoteleostei. The linkage of one zebrafish L locus with MH class II loci is presumably a remnant of the ancestral MHC organization. Overall, the fact that in teleost fish the MH class I and class II genes are not found in a single complex but on different chromosomes, allows for separate

association studies of only class I or only class II genes with disease resistance (Grimholt *et al*., 2003, Stet *et al*., 2003).

This thesis addressed possible implementations of molecular genetic information with regard to the MH genes for genetic selection aimed at improving disease resistance of common carp in semi-intensive pond farming. We described the polymorphism of MH class II *B* genes in different carp lines of various geographical origins and revealed associations between particular MH class II *B* genotypes/alleles and resistance of carp to selected pathogens in laboratory-based challenge tests. The selection of carp for particular MH class II *B* genotypes or alleles could allow for an increased survival upon challenge with selected pathogens and possibly, an increased survival rate under pond conditions. The application of MH class II *B* genes as genetic markers of disease resistance is discussed here.

7.2. Cyca-DAB1-like genotypes as potential genetic markers to study association with disease resistance in common carp

In common carp, two paralogous groups of MH class II *B* genes (*Cyca-DAB1*-like and *Cyca-DAB3*-like) are present. The *Cyca-DAB1*-like group includes at least two genes, *Cyca-DAB1* and *Cyca-DAB2* genes (Ono *et al*., 1993). The *Cyca-DAB3*-like group includes at least *Cyca-DAB3* and *Cyca-DAB4* genes (Van Erp *et al*., 1996). In a preliminary study, we examined the polymorphism of *Cyca-DAB1*-like and *Cyca-DAB3*-like genes and found a ubiquitous presence and high polymorphism of the first but not the second group of MH class II *B* genes in different European common carp lines (**chapter 2**). It has been reported that some carp individuals could express only *Cyca-DAB1*-like genes or only *Cyca-DAB3*-like genes whereas others can express both (Stet *et al*., 1997, Ottova *et al*., 2005). We extensively analyzed the transcription of *Cyca-DAB1*-like and *Cyca-DAB3*-like genes by real-time quantitative PCR and found an equally high constitutive transcription level for both groups of MH class II *B* genes (**chapter 4**), not different from what would be expected for functional MH class II *B* in normal lymphoid tissues (Rodrigues *et al*., 1995, Koppang *et al*., 1998, Chen *et al*., 2006). However, we could show that the presence of *Cyca-DAB1*-like, but not *Cyca-DAB3*-like genes, preferentially led to a high DNP-specific antibody response in carp (**chapter 4**). The allelic polymorphism and apparent functional advantage made us

focus primarily on the *Cyca-DAB1*-like genotypes as potential genetic markers to study association with disease resistance in common carp.

7.3. Infection models and genotyping technique

The ability to induce experimental infections is the first and very important step to study possible association between MH genes and disease resistance in fish. To detect possible associations between MH class II *B* genes with respect to resistance to a specific pathogens, different infection models were applied. We performed a series of challenge experiments using a large number of individuals of different genetic backgrounds. These carp were challenged with either the bacterium *Aeromonas hydrophila*, the ectoparasite *Argulus japonicus,* the blood parasite *Trypanoplasma borreli,* or the virus CyHV-3 (also known as Koi Herpesvirus- KHV) (**chapters 5 and chapter 6**).

Different challenge methods can result in different challenge pressures and can cause deviations from the expected performance. In general, the challenge method should closely mimic the natural exposure to the specific pathogen. Especially with respect to bacterial and viral pathogens, applying bath and co-habitant challenge methods fulfil this requirement best. However, these methods are also much more difficult to control and standardise, than injection methods (Nordmo, 1997). Indeed, for the challenges with ectoparasite *A. japonicus*, carp were successfully exposed to parasites contained in the water, representing the natural way of infection. For the other experimental infections we used intraperitoneal injection to infect carp with *A. hydrophila, T. borreli* and CyHV-3. In case of infection with *T. borreli*, it has been shown that both intraperitoneal (i.p.) and intramuscular (i.m.) injections mimic the natural infection route by leeches, however the infectious dose can be controlled more precisely during i.p. injection (Jones et al, 1993). In the case of bacterial and virus challenges the i.p. method allowed us to tightly control the dose of pathogen for each individual fish.

A second but not less important step in association studies is the development of a typing technique that allows for the screening of large numbers of individuals, to obtain sufficient statistical power. In order to be able to screen large numbers of individuals of common carp for the polymorphic *Cyca-DAB1*-like genes, we optimized a technique designated Polymerase Chain Reaction - Restriction Fragments - Single

Strand Conformation Polymorphism (PCR-RF-SSCP) (**chapter 3**). This technique allowed us to differentiate between the various *Cyca-DAB1*-like genotypes based on different SSCP band patterns. Each genotype was confirmed by DNA sequencing to identify unique combinations of *Cyca-DAB1*-like alleles. The advantages of PCR-RF-SSCP technique are simplicity, high sensitivity and low costs.

7.4. The Cyca-DAB2 gene is monomorphic which limits its applicability for association studies

As explained in **chapter 4**, cyprinid fish have two paralogous groups of MH class II *B* genes, *Cyca-DAB1*-like and *Cyca-DAB3*-like. The *Cyca-DAB1*-like genes appeared to be present ubiquitously and display a high degree of polymorphism, unlike the *Cyca-DAB3*-like genes. The *Cyca-DAB1*-like gene group includes at least two genes; the *Cyca-DAB1* gene and the *Cyca-DAB2* gene, which are the result of a recent duplication event (Ono *et al.*, 1993, van Erp *et al.*, 1996). We found that among the genes composing the *Cyca-DAB1*-like genotypes, the *Cyca-DAB2* gene was either homozygous or absent. In the latter case this thus affected the number of MH class II *B* genes expressed per animal.

Variation in number of expressed MH loci has been described for several fish species (Malaga-Trillo *et al.*, 1998, Sato *et al.*, 1998, Figueroa *et al.*, 2001). Extensive studies in three-spined sticklebacks have shown a variable number of MH class II *B* loci presented per individual (up to six) (Sato *et al.*, 1998, Binz *et al.*, 2001) with a possible effect on disease resistance. Kurtz (Kurtz *et al.*, 2004) reported a relationship between the infestation level of the tapeworm *Schistocephalus solidus* and microsporidians *Glugea anomal* and the MH allele number in stickleback. The same authors suggested that optimal rather than maximal MH diversity confers the highest resistance to parasites. This supported previous observation of natural populations of three-spined sticklebacks, which revealed that lowest parasite load was observed within fish having intermediate (optimal) number of MH class II *B* alleles (Wegner *et al.*, 2003).

When more than one locus exists, different numbers of alleles can be the result from both heterozygosity at existing loci and from differences in the number of duplicated loci (Reusch *et al.*, 2004). Thus, polymorphism can be maintained by a fitness advantage of individuals possessing different gene products at multiple,

functional equivalent loci, even if they are homozygous at the individual locus (Wegner *et al.*, 2003). A more recent study showed that there is a correlation between the level of transcription of MH class II *B* genes and the number of alleles in three-spined sticklebacks (Wegner *et al.*, 2006). The authors suggest that a negative correlation exists between individual MH diversity (i.e. the number of MH class II *B* sequence variants per individual) and the level of MH class II *B* transcription, implying compensatory up-regulation in fish with low (i.e. suboptimal) MH sequence variability.

We examined by real-time PCR the basal expression of *Cyca-DAB1*-like and *Cyca-DAB3*-like genes using gene-specific primers and did not find a major difference in expression level (**chapter 4**). In light of the findings in sticklebacks, it could be interesting to compare, in carp, the expression level of *Cyca-DAB1*-like genes between genotypes which have *i)* heterozygous *Cyca-DAB1* and homozygous *Cyca-DAB2* genes, *ii)* homozygous *Cyca-DAB1* and homozygous *Cyca-DAB2* genes, and *iii)* homozygous *Cyca-DAB1* and no *Cyca-DAB2* gene. In order to be able to do this, allele-specific RQ primers must be applied, which could distinguish between highly similar MH alleles of common carp.

The absence of a *Cyca-DAB2* gene in high numbers of individual fish was confirmed by PCR with newly designed primers on conserved regions of *Cyca-DAB1* and *Cyca-DAB2* genes. Also using this strategy we consistently were unable to find a *Cyca-DAB2* sequence, even after sequencing more than 40 clones per individual. We conclude that due to the high similarity between *Cyca-DAB1* and *Cyca-DAB2* genes it will also be difficult to design useful probes for Southern blot analysis, limiting the possibilities for further studies on the genomic presence of *Cyca-DAB2* genes. Overall, the lack of variation within the *Cyca-DAB2* gene and its absence in high number of individuals, limits its applicability for association studies. For this reason we concentrated our studies on associations between the *Cyca-DAB1* alleles and disease resistance in carp.

7.5. The Cyca-DAB1 gene is polymorphic and may influence disease resistance

The *Cyca-DAB1*-like gene group includes at least two genes, of which the *Cyca-DAB2* gene was either present in a homozygous form or absent. The *Cyca-DAB1* gene was polymorphic and our study showed the presence of five alleles (**02* - **06*). The

degree of variation at the *Cyca-DAB1*-like genes that we have examined is lower than has generally been found for MH class II *B* genes in other fish species (Dixon *et al.*, 1996, Miller and Withler, 1996, Dorshner *et al.*, 2000, Langefors *et al.*, 2001, Kruiswijk *et al.*, 2005, Chen *et al.*, 2006). This could be explained by the combined effects of inbreeding and genetic bottlenecks, experienced by the breeding populations of common carp during their history of selection. Indeed, a reduction of genetic variability in farmed populations of common carp compared to wild populations was confirmed in studies using microsatellite markers. The genetic variability of carp populations, measured as average number of alleles per locus at four microsatellite loci, was about 2 times higher in wild than in farmed stocks (Kohlman *et al.*, 2005, Lehoczky *et al.*, 2005, Memis and Kohlmann, 2006). Also in Atlantic salmon, genetic typing of Norwegian farmed salmon (Stet *et al.*, 2002, Consuegra *et al.*, 2005) showed a reduced number of MH alleles present in the farmed populations, compared to their wild counterparts. In addition, all MH alleles found in farmed fish were already present in the wild populations. In common carp, polymorphism of *Cyca-DAB* genes can still be maintained by the presence of variable number of loci, even if these *Cyca-DAB* genes are homozygous at an individual locus.

Interestingly, in our study we found a very high frequency (about 80%) of *Cyca-DAB1*-like genotypes with a homozygous *Cyca-DAB1* gene. One obvious explanation is inbreeding, typical for carp breeding populations and usually considered as unfavorable effects of closed breeding programs. In a population that is inbred, the frequency of homozygotes is increased, and the frequency of heterozygotes is reduced relative to random mating proportions. Heterozygote advantage in MH class II *B* genes, however, was not confirmed when related to higher survival of the Atlantic salmon challenged with *A. salmonicida* (Langefors *et al*., 2001), but a specific allele was found at higher frequency in uninfected and surviving fish, which supported frequency dependent selection rather than heterozygote advantage. Also, Miller *et al*. (2004) reported no statistically significant relationship with heterozygosity at MH class I (*UBA*) and class II *A* (*DAA*) and II *B* (*DAB*) loci and resistance to infectious hematopoietic necrosis virus (IHNV) in Atlantic salmon, but they did find an association with presence of one particular allele (*Sasa-B-04)* and higher resistance. However, in another study on Chinook salmon, Arkhus *et al*. (2002) investigated the effect of MH class II genes on

disease resistance to three different pathogens, and showed different results from those on Atlantic salmon in several ways. A higher average survival of heterozygotes than homozygotes were observed in fish infected with IHNV and, there was no evidence for an effect of specific MH genotypes on resistance to all tested pathogens. It has been however suggested by Grimholt *et al.* (2003) for Atlantic salmon that homozygosity for MH class II genes may actually be advantageous. Homozygous MH class II haplotypes showed significantly stronger association with increased resistance towards the infectious salmon anaemia virus (ISAV) and to *A. salmonicida* causing furunculosis in Atlantic salmon, in controlled laboratory-based challenges, than heterozygous haplotypes (Grimholt *et al*. 2003). In our study, the homozygous genotype E especially, could be linked to increased resistance of common carp to *Argulus japonicus* (**chapter 5**) and to CyHV-3 (**chapter 6**). We showed that the presence of this genotype could be advantageous with respect to a profound disease resistance of carp under selected laboratory-based challenges. The strong association of genotype E especially, with the resistance to CyHV-3, could indicate its suitability for genetic selection of carp.

*7.6. The Cyca-DAB1*05 allele is associated with higher resistance of common carp*

Genotype E consisted of a homozygous *Cyca-DAB1*05* allele and did not comprise the *Cyca-DAB2* gene. Phylogenetic analysis of all *Cyca-DAB1* alleles showed that the **05* allele represented the oldest allele in our study, since the branching order suggested its earliest diversification from the common ancestral sequence (Fig. 1). It has been suggested by Klein and O'hUigin (1994) that the great majority of non-synonymous PBR substitutions which differentiate HLA alleles and lineages in humans are generally old. The major influence on this polymorphism has come from parasites which co-evolved with their host in the far distant past, rather than newly emerged pathogens that affect the host only recently and have become very virulent. The driving force for this positive selection is widely accepted to be the mechanism of the MHC polymorphism. However, it has been suggested that under frequency-dependent selection new MHC alleles would have a selective advantage because pathogens could not adapt to such new alleles (Takahata and Nei, 1990). But old rare alleles may also impose high fitness. Such old alleles, which were common in the past, could loose their fitness advantage because the pathogens would have evolved resistance to these particular alleles and, as a consequence, these alleles might decline in frequency due to

negative selection. Subsequently, the pathogen adaptation may also decline or disappear and such old rare allele may also become adaptive and increase in frequency (Slade and McCallum, 1992, Hedrick, 2002). The *Cyca-DAB1*05* allele was found to be present with intermediate frequency in all examined carp lines except the experimental laboratory cross R3 x R8, in which this allele was not present (**chapter 4 and 5**).

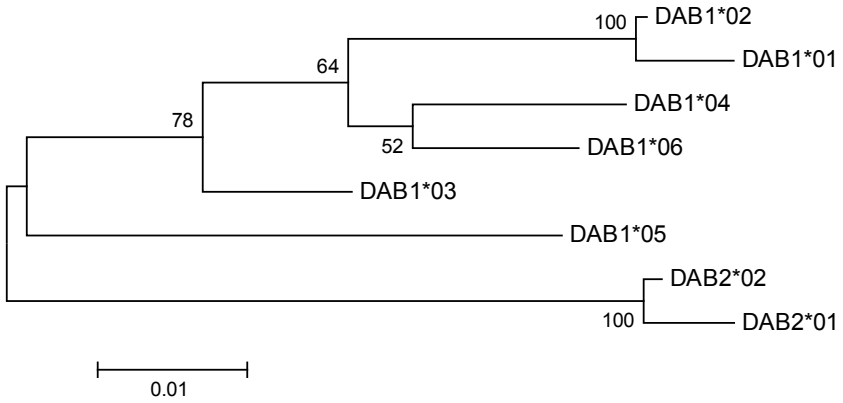

Figure 1. Neighbor-joining tree based on *Cyca-DAB1* and *Cyca-DAB2* nucleotide sequences. The topology of the tree is supported by bootstrap *P* values (1000 iterations).

Pathogens of common carp exists with a long evolutionary history, possibly even with a different host specificity than carp. At some point in history they infected carp species and caused a lethal disease. At the beginning high virulence is essential for spreading of infectious agents. The innate immune defense might have been effective in killing many, but not all pathogens. However, due to natural selection the pathogens might becomes less lethal later on, when carp cultures become better protected and more resistant.

Cultured carp populations are under joint action of inbreeding and natural selection, which is due to the fact that fish are grown in an open environment, and thus exposed to natural pathogens. Therefore the distribution of genotypes and allelic diversity observed in carp breeding lines is a result of a strong environmental effect, which might act in the direction of maintaining rare alleles when they are advantageous during disease outbreaks. In other words, low-frequency alleles found in a population

may have been selected against, but are still maintained at low frequencies as potential candidates for handling selective pressures exerted by emerging pathogens. Thus, the maintenance of the MH genetic diversity is an important issue for the preservation of carp populations from future disease outbreaks.

7.7. Implication and future perspectives of the carp live gene bank.

This thesis described the first study on MH genes as potential gene markers associated with disease resistance or susceptibility in common carp. In Europe there are three major carp live gene banks, maintained in Poland (Gołysz), Hungary (Szarvas) and Czech Republic (Vodnany). To our knowledge, in the above mentioned carp breeding centers, marker assisted genetic selection has not been used in any aquaculture breeding scheme to date. So far, most of the selection work in carp was based on selective breeding and crossings aimed at increasing productive traits.

Our findings of an association of particular MH genotypes or alleles with higher resistance of common carp to specific pathogens showed that MH genes can be used as potential gene markers in selection aimed at production more resistant fish. The next step should be the evaluation of the performance of selected genotypes using experimental challenges on larger numbers of individual fish and investigating how these genotypes perform in a natural environment. It could also be interesting to study the performance of specific MH genotypes using carp lines originating from different live gene banks. In addition, the presence of newly identified, different MH genotypes may be revealed when comparing carp from these different live gene banks. Knowledge obtained on the effects of MH class II genes and gene combinations could be used to breed new, stronger and more robust strains of common carp.

We are aware that in addition to MH genes, other genes of the background genome could also affect resistance against infectious diseases. However, as yet not many candidate genes other than the MH genes have been identified (Kjøglum *et al.*, 2005). The use of specific gene information is not a panacea, but could help to increase rates of genetic improvement, and open opportunities for using additive and non-additive genetic effects. Improvement based on MH genes of increased resistance should be combined with so-called ‘traditional' or ‘conventional' methods based on phenotypic and genealogical information.

However, it is difficult to predict how selected MH genotypes will perform against newly emerging and probably upcoming diseases. For example, it has been shown for Atlantic salmon that identical alleles could even have an opposite effect on resistance to different pathogens (Kjøglum *et al.*, 2008). Selection based on MH-associated resistance to a single pathogen puts the population at risk, such that this population could become more susceptible to other pathogens. It is reasonable to suggest that, together with the creation of new breeding schemes based on individual genotypes, the maintenance of high variability among MH genes should be an important issue for the preservation of carp populations from future disease outbreaks. This could be achieved through existing programs of protection of carp genetic resources, where the information on MH variability could be incorporated, with special attention to maintenance of rare alleles. Alternatively, particular MH alleles could be selected, to produce populations with high frequencies of disease-specific resistant alleles which can then be included in genetic selections directed towards geographical regions where specific diseases caused by specific pathogens occur. Or, as suggested by Kjøglum *et al.* (2008), long-term studies could also end in the identification of useful all-round performing (MH) alleles that could be maintained at high frequencies in breeding programs. Additional information is required not only on the genes that affect a trait relevant to disease resistance, but also on the interactions between these genes. Further information on gene interactions may be obtained from gene expression studies.

Our study could also contribute to the rational design of new vaccines, for example against CyHV-3. In Atlantic salmon a single allele (*SasaUBA*0301*) was found associated with higher resistance to infectous salmon anaemia virus (ISAV) (Grimholt *et al.*, 2002, 2003, Kjøglum *et al.*, 2006). Subsequent analysis of the peptide-binding motif for this allele was performed using recombinant proteins *β_2m/SasaUBA*0301* to screen a phage display library (Zhao *et al.*, 2008). The prediction of the binding motifs for the particular resistant allele is suggest to have an important implication on the development of vaccine strategies designed to induce immunological responses against viruses in fish and therefore may have future application in studies aimed at developing sufficient vaccine against CyHV-3 virus for carp.

In our study we performed multiple challenges using various pathogens and revealed significant association between particular MH class II *B* genotypes as well as alleles and resistance of carp to selected pathoges. We now have to proceed to investigate the underlying biological meaning of the associations found by studying the immune system of particular MH-typed carp carrying different *Cyca-DAB* alleles. We expect that selection of carp for particular MH class II *B* genotypes or alleles could allow for an increased survival upon challenge with selected pathogens and possibly, increased survival rate under pond conditions. There is great optimism that this will lead to a successful application of marker assisted selection for resistance to diseases in common carp.

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Summary

The impact of diseases caused by a wide range of pathogens (viruses, bacteria and parasites) is the most important problem in aquaculture of common carp (*Cyprinus carpio* L.). Genetic selection aimed at obtaining population of more resistant common carp is potential and sustainable approach to disease control in semi-intensive carp pond farming. Genes of the major histocompatibility complex (MHC) are candidate marker genes for studies on association with disease resistance. The MHC contains some of the most polymorphic genes known to date and are considered crucial to adaptive immunity. MHC molecules bind both self and foreign peptides and present them to T lymphocytes (T cells). MHC class I molecules present endogenously derived peptides to $CD8^+$ T cells, while MHC class II molecules present exogenously derived peptides to $CD4^+$ T cells. Each MHC molecule has the ability to bind and present different groups of peptides in more or less successful ways. This can influence the immune response of an organism since the peptides derived from a certain pathogen may either not be presented by specific MHC molecules, which can result in higher susceptibility or, may be bound with a high affinity by specific MHC molecules which could lead to increased resistance to the pathogenic organism.

In teleosts, unlike to humans, tetrapods and cartilaginous fish, class I and class II genes are not linked and segregate independently, which allows for association studies of only class I or only class II MH genes with disease resistance. MHC class II molecules generally have a broader spectrum of action in the immune system than the MHC class I genes. There are also observations that suggest a more intense selection pressure and a more rapid evolution of MH class II than class I alleles in fish. Although the expression of both MH class II chains is equivalent, the beta chain generally has a higher degree of polymorphism than the alpha chain. This thesis addressed possible implementations of MH class II *B* genes for selection aimed at improving of a common carp resistance in semi-intensive pond farming.

In common carp there are two paralogous groups of MH class II *B* genes, *Cyca-DAB1*-like and *Cyca-DAB3*-like genes. In a preliminary study, we examined the polymorphism for the *Cyca-DAB1*-like and *Cyca-DAB3*-like genes in different European common carp lines (**chapter 2**). These carp lines were of various geographical origins and part of carp live gene bank, which is maintained at the Institute of Ichthyobiology and Aquaculture in Gołysz. Previous observations over a period of at

least 15 years revealed significant differences between lines in survival rate and parasite load under natural conditions. Also, differences in resistance to atypical *Aeromonas salmonicida* in laboratory based challenge tests was observed, suggesting genetic differences in resistance between the carp lines. Analysis of polymorphism of MH class II *B* genes in selected carp lines revealed a ubiquitous presence and high polymorphism of *Cyca-DAB1*-like but not *Cyca-DAB3*-like genes. The observed allelic polymorphism for *Cyca-DAB1*-like genes rather than *Cyca-DAB3*-like genes stimulated further studies into the association of *Cyca-DAB1*-like allelic polymorphism and disease resistance of common carp.

In order to study association between *Cyca-DAB1*-like gene polymorphism and resistance of common carp we optimized a technique designated polymerase chain reaction -restriction fragments- single strand conformation polymorphism (PCR-RF-SSCP) to be able to screen and type large numbers of individual carp (**chapter 3**). The advantages of this technique are simplicity, high sensitivity and low costs. PCR-RF-SSCP analysis of n = 79 carp individuals from 8 lines challenged with *Aeromonas hydrophila* revealed the presence of different genotypes consisting of unique combinations of *Cyca-DAB1* and *Cyca-DAB2* sequences. We found four alleles for the *Cyca-DAB1* (**02-*05*) gene but only one allele for *Cyca-DAB2* (**02*). We noted that the *Cyca-DAB2* gene was either homozygous or absent. The degree of heterozygosity of the *Cyca-DAB1* and *Cyca-DAB2* genes clearly correlated with the number of SSCP bands. Thus, we proved that PCR-RF-SSCP is a reliable technique that can be used for screening large number of individuals for investigating the *Cyca-DAB1* and *Cyca-DAB2* genes polymorphism in common carp.

Previously, we performed a long-term divergent selection of common carp for antibody production, which successfully resulted in carp lines with a different immune response. We studied the segregation of *Cyca-DAB* genes with the DNP-specific antibody response and we showed that the presence of *Cyca-DAB1*-like, but not *Cyca-DAB3*-like genes, preferentially leads to a high DNP-specific antibody response in carp (**chapter 4**). Background genes other than *Cyca-DAB* genes also influenced the level of antibody response. We also studied the transcription of both *Cyca-DAB1*-like and *Cyca-DAB3*-like genes in different organs of carp. The constitutive transcription for both *Cyca-DAB1*-like and *Cyca-DAB3*-like genes was high, although *Cyca-DAB1*-like genes

consistently showed slightly higher mRNA transcription than *Cyca-DAB3*-like genes in some immunological relevant organs. Sequence information, constitutive transcription levels and co-segregation data indicated that both paralogous *Cyca-DAB1*-like and *Cyca-DAB3*-like groups represent functional MH class II *B* genes.

We then proceeded to study association of *Cyca-DAB1*-like genotypes with resistance to four different pathogens; the bacterium *Aeromonas hydrophila*, the ectoparasite *Argulus japonicus,* and the blood parasite *Trypanoplasma borreli* (**chapter 5**) and the viral pathogen Cyprinid herpesvirus-3 (CyHV-3) (**chapter 6**). We used a large number of individuals of different carp lines and revealed, using PCR-RF-SSCP, the presence of $n = 9$ unique *Cyca-DAB1*-like genotypes, of which three genotypes (B, D, and E) were most common (**chapter 5**). In general, *Cyca-DAB2* was often homozygous or absent while allelic polymorphism was detected in *Cyca-DAB1* gene. We could detect significant associations between genotype E and abundance of *A. japonicus* and between genotype D and higher level of parasitaemia after *T. borreli* infection. We also observed a significant association between *Cyca-DAB1* heterozygosity and lower level of parasitaemia after *T. borreli* infection. In **chapter 6**, we showed a strong association between *Cyca-DAB1*-like genotypes and resistance or susceptibility to CyHV-3. One genotype (E) performed significantly better, resulting in carp more resistant to CyHV-3, while three other genotypes (B, H and J) could be linked to higher susceptibility to the virus. Subsequent analysis of the alleles that compose the *Cyca-DAB1*-like genotypes linked one particular allele (*Cyca-DAB1*05*) to significantly increased, and two alleles (*Cyca-DAB1*02* and *Cyca-DAB1*06*) to significantly decreased resistance to CyHV-3. The resistant genotype E did not comprise the *Cyca-DAB2* gene and consisted of a homozygous *Cyca-DAB1*05* allele. Phylogenetic analysis of all *Cyca-DAB1* alleles showed that the *Cyca-DAB1*05* allele represents the oldest allele in our study (**chapter 7**). We discussed the possibility for using *Cyca-DAB1* allelic polymorphism as a potential genetic marker in future breeding programs of common carp (**chapter 7**). We expect that selection of carp for particular MH class II *B* genotypes or alleles could allow for an increased survival upon challenge with selected pathogens and possibly, increased survival rate under pond conditions.

Samenvatting

Het meest belangrijke probleem in de aquacultuur van karper (*Cyprinus carpio* L.) is de invloed van ziektes, veroorzaakt door een grote verscheidenheid aan pathogene organismen (virussen, bacteriën en parasieten). Genetische selectie gericht op het verkrijgen van een meer resistente populatie heeft zeker potentie als een duurzame oplossing om ziektes te beheersen in semi-intensieve karperkweek. Genen van het major histocompatibility complex (MHC) zijn mogelijk geschikte kandidaat merker genen voor ziekteresistentie. Het MHC bevat enkele van de meest polymorfe genen beschreven tot op heden welke als cruciaal worden beschouwd voor de adaptieve tak van het afweersysteem. MHC moleculen binden zowel eigen als vreemde peptiden en presenteren deze aan T-lymfocyten (T-cellen). MHC klasse I moleculen presenteren endogeen geproduceerde peptiden aan $CD8^+$ T-cellen, in tegenstelling tot MHC klasse II moleculen die exogeen geproduceerde peptiden presenteren aan $CD4^+$ T-cellen. Ieder MHC molecuul heeft het vermogen tot het meer of minder succesvol binden en presenteren van verschillende groepen peptiden. Dit kan van invloed zijn op de immuunrespons van een organisme omdat de peptiden van bepaalde pathogenen misschien niet gepresenteerd worden door specifieke MHC moleculen. Dit kan leiden tot hogere vatbaarheid voor het pathogeen, ofwel de peptiden kunnen juist met een hoge affiniteit gebonden worden aan specifieke MHC moleculen, hetgeen zou kunnen leiden tot verhoogde weerstand tegen het pathogene organisme.

In beenvissen zijn, in tegenstelling tot mensen, viervoeters en kraakbeenvissen, klasse I en klasse II genen niet aan elkaar gekoppeld maar juist onafhankelijk en van elkaar gescheiden. Hierdoor kunnen associatie studies voor ziekteresistentie gedaan worden voor alleen klasse I of alleen klasse II MH genen. MHC klasse II genen hebben over het algemeen een breder actiespectrum in het immuunsysteem dan MHC klasse I genen. Er zijn ook waarnemingen die suggereren dat MH klasse II genen in vis, onder invloed van een intense selectiedruk, een snellere evolutie door hebben gemaakt dan de klasse I genen. Hoewel de expressie van beide MH klasse II ketens gelijkwaardig is, is de bèta keten over het algemeen meer polymorf dan de alfa keten. Dit proefschrift beschrijft een onderzoek naar de mogelijke implementatie van MH klasse II *B* genen als merker voor genetische selectie, met als doel het verhogen van de resistentie van karper in semi-intensieve karperkweek.

Er zijn in karper twee groepen paraloge MH klasse II *B* genen, *Cyca-DAB1*-achtig en *Cyca-DAB3*-achtige genen. In voorgaande studies hebben we het polymorfisme voor de *Cyca-DAB1*-achtig en *Cyca-DAB3*-achtige genen in verschillende Europese karperstammen onderzocht **(hoofdstuk 2)**. Deze karperstammen zijn afkomstig uit verschillende Europese gebieden en zijn onderdeel van een 'levende genenbank' op het Instituut voor Ichtyobiologie en Aquacultuur in Gołysz, Polen. Eerdere observaties over een periode van ten minste 15 jaar lieten significante verschillen zien in overlevingskans en parasiet aantallen onder natuurlijke omstandigheden. Ook zijn verschillen in resistentie tegen atypische *Aeromonas salmonicida* onder laboratorium test condities waargenomen. Deze waarnemingen suggereren de aanwezigheid van genetische verschillen in resistentie tussen de verschillende karperstammen. Uit de analyse van polymorfisme van MH klasse II *B* genen in de geselecteerde karperstammen kwam naar voren dat *Cyca-DAB1*-achtige genen, in tegenstelling tot *Cyca-DAB3*-achtige genen, alom aanwezig waren en een hoog polymorfisme vertonen. Dit allelische polymorfisme van *Cyca-DAB1*-achtige genen, in tegenstelling tot *Cyca-DAB3*-achtige genen, leidde tot vervolgstudies naar een mogelijke associatie van het allelisch polymorfisme van *Cyca-DAB1*-achtige genen en ziekteresistentie in karper.

Om de associatie tussen *Cyca-DAB1*-achtig gen polymorfisme en resistentie van karper beter te kunnen onderzoeken hebben we de techniek 'polymerase chain reaction - restriction fragments- single strand comformation polymorphism (PCR-RF-SSCP)' geoptimaliseerd, zodat we grote aantallen individuele karpers konden analyseren en typeren **(hoofdstuk 3)**. De voordelen van deze eenvoudige techniek zijn hoge gevoeligheid en lage kosten. PCR-RF-SSCP analyse van $n = 79$ individuele karpers van 8 karperstammen blootgesteld aan *Aeromonas hydrophila* toonde de aanwezigheid van verschillende genotypes aan, bestaande uit unieke combinaties van *Cyca-DAB1* en *Cyca-DAB2* sequenties. We vonden vier allelen voor het *Cyca-DAB1* (*02-*05) gen en maar één allel voor *Cyca-DAB2* (*02). Hieruit concludeerden we dat het *Cyca-DAB2* gen òf homozygoot òf afwezig was. De mate van heterozygotie van *Cyca-DAB1* en *Cyca-DAB2* genen correleerde duidelijk met het aantal SSCP banden. Hiermee hebben we bewezen dat PCR-RF-SSCP een betrouwbare techniek is, die gebruikt kan worden

voor het onderzoeken van grote aantallen individuen voor *Cyca-DAB1* en *Cyca-DAB2* gen polymorfisme in karper.

Eerder hebben we een lange termijn divergente selectie van karper gedaan voor antilichaam productie die succesvol resulteerde in karperstammen met verschillende immuunrespons. In dit proefschrift bestudeerden we de segregatie van *Cyca-DAB* genen tesamen met een DNP-specifieke antilichaamrespons en konden we aantonen dat de aanwezigheid van *Cyca-DAB1* en niet *Cyca-DAB3*-achtige genen bij voorkeur leidde tot een hoge DNP-specifieke antilichaam- respons in karper **(hoofdstuk 4)**. Achtergrondgenen anders dan *Cyca-DAB* genen beïnvloedden mede de sterkte van de antilichaamrespons. We bestudeerden ook de transcriptie van *Cyca-DAB1*-achtig en *Cyca-DAB3*-achtige genen in verschillende organen van de karper. De basale transcriptie voor *Cyca-DAB1*-achtig en *Cyca-DAB3*-achtige genen was hoog, hoewel de *Cyca-DAB1*-achtige genen in een aantal immunologisch relevante organen, consequent een iets hogere mRNA transcriptie lieten zien dan de *Cyca-DAB3*-achtige genen. Sequentie informatie, basale transcriptie en co-segregatie gegevens lieten zien dat *Cyca-DAB1*-achtig én *Cyca-DAB3*-achtige groepen beide functionele MH klasse II B genen hebben.

Vervolgens bestudeerden we de associatie van *Cyca-DAB1*-achtige genotypen met resistentie tegen vier verschillende ziekteverwekkers; de bacterie *Aeromonas hydrophila*, de ectoparasiet *Argulus japonicus* en de bloedparasiet *Trypanoplasma borreli* **(hoofdstuk 5)** en het virus Cyprinus herpesvirus-3 (CyHV-3) **(hoofdstuk 6)**. We gebruikten een groot aantal individuen van verschillende karperstammen en ontdekten, gebruik makende van PCR-RF-SSCP, de aanwezigheid van n = 9 unieke *Cyca-DAB1*-achtige genotypen, waarvan drie genotypen (B,D en E) het meest voorkwamen **(hoofdstuk 5)**. In het algemeen was *Cyca-DAB2* homozygoot of zelfs afwezig, terwijl allelisch polymorfisme wel werd waargenomen voor het *Cyca-DAB1* gen. We konden een significante associatie zien tussen genotype E en de hoeveelheid *A. japonicus* en tussen genotype D en hogere aantallen parasieten na *T. borreli* infectie. In **hoofdstuk 6** laten we een sterke associatie zien tussen *Cyca-DAB1*-achtige genotypes en hun resistentie tegen of vatbaarheid voor CyHV-3. Één genotype (E) scoorde significant beter, hetgeen resulteerde in karpers die meer resistent waren tegen CyHV-3, terwijl drie andere genotypes (B, H en J) gekoppeld konden worden aan een hogere

vatbaarheid voor het virus. Verdere analyse van de allelen die de *Cyca-DAB1*-achtige genen vormen koppelde één specifiek allel (*Cyca-DAB1**05) met een significante verhoging en twee allelen (*Cyca-DAB1**02 en *Cyca-DAB1**05) met een significante verlaging aan resistentie tegen CyHV-3.

Het resistente genotype E bevatte niet het *Cyca-DAB2* gen en bevatte een homozygoot *Cyca-DAB1**05 allel. Fylogenetische analyse van alle *Cyca-DAB1* allelen liet zien dat *Cyca-DAB1**05 het oudste allel in onze studie is **(hoofdstuk 7)**. We verwachten dat de selectie van karper voor, in het bijzonder MH klasse II *B* genotypen of allelen, kan leiden tot een verhoogde overlevingskans na blootstelling aan de geselecteerde pathogenen, en mogelijk ook kan leiden tot een verhoogde overlevingskans in de aquacultuur van karper.

Streszczenie

Jednym z głównych problemów, jakie dotyka dzisiejsze rybactwo karpiowe to straty wynikające z niskiej przeżywalności obsad. Główną tego przyczyną są choroby ryb wywoływane przez szerokie spektrum patogenów (wirusy, bakterie, pasożyty). Jedną z metod ograniczenia strat spowodowanych chorobami jest odpowiednio prowadzona selekcja zmierzająca do produkcji bardziej odpornego materiału obsadowego. Selekcję tą można prowadzić z wykorzystaniem markerów genetycznych związanych z odpornością. Wśród genów-markerów szczególną rolę pełnią geny **głównego układu zgodności tkankowej** (**MHC,** *ang. Major Histocompatibilty Complex*). Geny MHC odgrywają kluczową rolę w układzie odpornościowym kręgowców. Kodują dwa typy (MHC klasy I i klasy II) funkcjonalnie i strukturalnie różniących się białek. Zadaniem cząsteczek MHC jest prezentacja własnych oraz obcych peptydów limfocytom T, dzięki czemu dochodzi do zapoczątkowania nabytej odpowiedzi immunologicznej. Cząsteczki MHC klasy I biorą udział w odpowiedzi immunologicznej przeciwko zakażeniom wewnątrzkomórkowym (np. zakażeniom wirusowym) oraz w odrzucaniu przeszczepów. Prezentują peptydy pochodzenia wewnątrzkomórkowego limfocytom T cytotoksycznym (Tc, $CD8^+$). Z kolei cząsteczki MHC klasy II biorą udział w odpowiedzi immunologicznej przeciwko zakażeniom zewnątrzkomórkowym (np. infekcjom bakteryjnym czy inwazjom pasożytniczym) i prezentują peptydy pochodzenia zewnątrzkomórkowego limfocytom T pomocniczym (Th, $CD4^+$). Różne cząsteczki MHC posiadają zdolność przyłączania i prezentowania różnorodnych peptydów w sposób bardziej lub mniej efektywny, co z kolei wpływa na efektywność odpowiedzi immunologicznej. Odpowiedź organizmu na zakażenie patogenem może być zatem genetycznie uwarunkowana przez posiadany haplotyp MHC.

Szczegółowe badania organizacji genów MHC u ryb doskonałokostnych (Teleostei) wykazały, iż w przeciwieństwie do pozostałych grup kręgowców, w tym także ryb chrzęstnoszkieletowych, ich geny MHC klasy I i klasy II nie tworzą jednego kompleksu, lecz zlokalizowane są na różnych chromosomach i segregują niezależnie od siebie. Odkrycie to sprawiło, że niektórzy z autorów zaproponowali nazwę genów zgodności tkankowej (**MH**, *ang. Major Histocompatybility*), jako trafniejsze określenie dla genów MHC u ryb doskonałokostnych. Taka organizacja umożliwia także niezależne badanie związku pomiędzy genami MH klasy I lub MH klasy II, a

odpornością na choroby. Szereg obserwacji wskazuje, iż u ryb geny MH klasy II są ewolucyjnie młodsze i znajdują się pod silniejszą presją selekcyjną aniżeli geny MH klasy I. Geny MHC klasy II kodują łańcuch α (geny MHC II *A*) i łańcuch β (geny MHC II *B*) cząsteczek MHC klasy II. Geny MHC klasy II *B* charakteryzują się jednak znacznie większym polimorfizmem aniżeli geny MHC klasy II *A* i należą do najbardziej polimorficznych genów w genomie kręgowców.

Obecna praca opisuje możliwość wykorzystania polimorfizmu genów MH klasy II *B* karpia (*Cyprinus carpio* L.) jako markerów genetycznych w selekcji karpia zmierzającej do otrzymania bardziej odpornego materiału obsadowego.

Zakład Ichtiobiologii i Gospodarki Rybackiej PAN w Gołyszu posiada unikatową na skalę Europy kolekcję linii hodowlanych karpia pochodzących z różnych regionów geograficznych i wchodzących w skład tzw. „żywego banku genów karpia". Prowadzone na przestrzeni kilkunastu lat obserwacje przeżywalności linii hodowlanych karpia w warunkach stawowych wykazały istotne różnice w przeżywalności pomiędzy badanymi liniami. Dodatkowo, przeprowadzone w warunkach laboratoryjnych testy z zakażeniem wybranych linii hodowlanych karpia bakterią *Aeromonas salmonicida* wykazały, iż linie różnią się odpornością na zakażenie tym patogenem. Dane te sugerują istnienie genetycznie uwarunkowanych różnic w odporności pomiędzy poszczególnymi liniami hodowlanymi karpia.

U karpia opisano dwie paralogiczne grupy genów MH klasy II *B*: grupę *Cyca-DAB1* oraz grupę *Cyca-DAB3*. W **rozdziale 2** przedstawiono wyniki analizy polimorfizmu genów obydwu grup w wybranych liniach hodowlanych karpia. W przeprowadzonych badaniach wykazano, iż geny grupy *Cyca-DAB1* występowały u wszystkich badanych osobników, podczas gdy geny grupy *Cyca-DAB3* występowały tylko u nielicznych osobników. Dodatkowo wykazano polimorfizm w obrębie genów grupy *Cyca-DAB1*. Otrzymane wyniki wskazują na to, iż to geny grupy *Cyca-DAB1* aniżeli *Cyca-DAB3* powinny być rozpatrywane w badaniach nad związkiem pomiędzy genami MH klasy II *B* a odpornością karpia na choroby.

W celu zbadania związku pomiędzy występowaniem genów grupy *Cyca-DAB1,* a odpornością karpia opracowano szybką i dokładną metodę analizy polimorfizmu genów grupy *Cyca-DAB1* (**rozdział 3**). Technika ta, określona w skrócie PCR-RF-

SSCP pozwala na przetestowanie dużej liczby próbek w stosunkowo krótkim czasie i przy niewielkim nakładzie finansowym. Z wykorzystaniem metody PCR-RF-SSCP przeprowadzono genotypowanie osobników karpia pochodzących z 8 linii hodowlanych i biorących udział w teście z zakażeniem bakterią *Aeromonas hydrophila*. Przeprowadzona analiza pozwoliła wyodrebnić 6 genotypów różniących się składem allelicznym genów *Cyca-DAB1* i *Cyca-DAB2*. Opisano 4 allele genu *Cyca-DAB1* i tylko jeden allel genu *Cyca-DAB2*. Dodatkowo wykazano, że gen *Cyca-DAB2* występuje w formie homozygotycznej lub nie występuje w badanych osobnikach. Nie wykazano istotnego związku pomiędzy wyodrębnionymi genotypami a śmiertelnością karpia po zakażeniu *A. hydrophila*. Wykazano natomiast istotny związek pomiędzy obecnością genu *Cyca-DAB2* a poziomem przeciwciał określonym za pomocą testu aglutynacyjnego podczas trwania eksperymentu.

W **rozdziale 4** opisano wyniki analizy segregacji genów *Cyca-DAB* z produkcją przeciwciał skierowanych przeciwko cząsteczką haptenu DNP-KHL (*ang. dinitrophenyl-keyhole limpet haemocyanin*). W badaniach tych wykorzystano otrzymane wcześniej eksperymentalne linie karpia wyselekcjonowane w kierunku wysokiej bądź niskiej produkcji przeciwciał przeciwko DNP-KHL, krzyżówkę między tymi liniami oraz krzyżówki wsobne. Wykazano, iż większa produkcja przeciwciał skierowanych przeciwko cząsteczkom DNP-KHL była związana z obecnością genów z grupy *Cyca-DAB1* aniżeli grupy *Cyca-DAB3*.

W dalszych eksperymentach przeprowadzono analizę transkrypcji genów grupy *Cyca-DAB1* i *Cyca-DAB3* w różnych narządach karpia. Stwierdzono, iż poziom transkrypcji genów obydwu grup był wysoki, jednakże w niektórych narządach limfatycznych (grasica, nerka głowowa, śledziona) poziom transkrypcji genów grupy *Cyca-DAB1* był istotnie wyższy aniżeli genów grupy *Cyca-DAB3*. Wyniki przeprowadzonych badań sugerują, iż obie grupy genów *Cyca-DAB* stanowią funkcjonalnie czynne geny MH klasy II *B* u karpia.

Zbadano również związek pomiędzy genotypami *Cyca-DAB1,* a odpornością karpi na zakażenie bakterią *Aeromonas hydrophila*, ektopasożytem *Argulus japonicus*, wiciowcem *Trypanoplasma borreli* (**rozdział** 5) oraz wirusem Cyprinid herpesvirus-3 (CyHV-3) (**rozdział 6**). Zakażeniu poddano ryby pochodzące z wybranych linii hodowlanych karpia oraz krzyżówek pomiędzy tymi liniami. Wszystkie ryby zostały

przebadane w kierunku posiadanych genotypów *Cyca-DAB1* z wykorzystaniem metody PCR-RF-SSCP. Wykazano związek pomiędzy genotypem E, a większą odpornością karpi na zakażenie *A. japonicus,* oraz pomiędzy genotypem D, a zwiększoną podatnością karpi na zakażenie *T. borreli*. Dodatkowo wykazano związek pomiędzy heterozygotycznością genu *Cyca-DAB1,* a niższym poziomem parazytemii po zakażeniu *T. borreli* (**rozdział 5**). W **rozdziale 6** opisano związek pomiędzy genotypami *Cyca-DAB1,* a odpornością bądź podatnością karpi na zakażenie wirusem CyHV-3. Wykazano związek pomiędzy genotypem E, a zwiększoną odpornością karpi, podczas gdy występowanie genotypów B, H i J było związane z większą podatnością na zakażenie wirusem i większą śmiertelnością. Przeprowadzona analiza na poziomie alleli wykazała, iż allel *Cyca-DAB1*05* był związany z większą odpornością, zaś allele *Cyca-DAB1*02* i *Cyca-DAB1*06* były związane z większą podatnością na zakażenie. Wykonane eksperymenty pozwoliły wyodrębnić jeden genotyp (E), który może być związany z większą odpornością u karpia. Genotyp E zawiera homozygotyczny allel *Cyca-DAB1*05* i nie zawiera genu *Cyca-DAB2*. Analiza filogenetyczna wyodrębnionych alleli genu *Cyca-DAB1* wykazała, iż allel *Cyca-DAB1*05* jest najstarszym z opisanych alleli (**rozdział 7**).

Przedstawione w pracy wyniki po raz pierwszy wskazują na możliwość wykorzystania polimorfizmu genów *Cyca-DAB1* w selekcji karpia zmierzającej do otrzymania bardziej odpornego materiału obsadowego. Selekcja karpia z wykorzystaniem genów MH jako markerów odporności może zwiększyć odporność karpi na zakażenie patogenami, oraz zwiększyć przeżywalność ryb w warunkach stawowych obniżając tym samym straty spowodowane chorobami

Acknowledgements

This PhD thesis is the result of research work, which I have done at the Institute of Ichthyobiology & Aquaculture in Gołysz (Poland) and at the Cell Biology and Immunology group, Wageningen University (The Netherlands). It was a great honor and pleasure for me to work with many people who have encouraged, supported and helped me a lot with my research.

First of all I would like to thank my Promotors, Prof. dr. ir. Huub Savelkoul (CBI, WUR) and Dr. Andrzej Pilarczyk (IIA, Gołysz), for their advice and useful comments during this PhD work.
To my co-promotors and daily supervisors, Dr. Geert Wiegertjes (CBI, WUR) and Dr. Ilgiz Irnazarow (IIA, Gołysz), thank you very much for your guidance throughout this work, continued support and valuable time. Geert, your energy, efficiency of work and ability to correct the manuscripts in so short a time are admirable! Thank you for being always helpful and for organizing all 'short visits', which helped me a lot in completing research at CBI lab. Ilgiz, thank you for all the discussions we held (not only of scientific nature), useful advices and for having been open to all my ideas and suggestions!
I owe special thanks to Prof. William van Muiswinkel who started a co-operation between Gołysz and Wageningen about 30 years ago. You were always enthusiastic about my work and very helpful.
I also wish to thank Prof. René Stet (in memoriam) who, together with Geert, supervised my work during my stay in Wageningen and was always available for scientific discussions.

I would like to thank my colleagues from Gołysz and Wageningen. Thank you for all insightful discussions, tea breaks and positive energy.

From Gołysz: I am especially grateful to my roommates: Maciek (thanks for direct help with lab and pc problems), Asia, Magda and Mikołaj for being such good fellows and for all private talks and help. It is a pleasure to share a room with you! Special thanks to Patrycja for seamlessly working together at the lab and support towards the finish of our PhD work. I am also grateful to Heniek for providing scan images of *Cyprinus carpio L.*, which are found in the General Introduction of this Thesis. I wish to thank Dalia, Neli, Beata (*min. za długie rozmowy o 'życiu'*), Vitaliy, and Patrik for sharing nice time in the lab and guesthouse. I also wish to thank technical staff in Gołysz: Danka, Jola, Basia, and Ula. *Dziękuję za wszelką pomoc w laboratorium oraz przy rybach, szczególnie Uli za utrzymywanie ryb w dobrej kondycji, a także za niezapomnianą atmosferę rozmów w „kawowym".* I would like to thank Mirek and dr. Elżbieta Brzuska for useful talks and Prof. Maria Szumiec for help with writing the history of carp farming in Poland. *Składam także serdeczne podziękowania Panu Józefowi Związkowi (dyrektorowi Zakładu Doświadczalnego Gospodarki Stawowej PAN w Gołyszu), oraz Panu Janie Brodzie (byłemu dyrektorowi) za umożliwienie mi zamieszkania w Gołyszu w hotelu należącym do ZDGS.*

From Wageningen: I wish to thank my paranymphs: Trudi and Maria for the big involvement in organization of my PhD defense. Thank you Trudi for introducing me to the lab techniques and for assisting me with numerous plasmid isolations at the beginning of my work in Wageningen. Maria, as close colleague and friend, we share a very special time together at the beginning of our PhD projects in Wageningen. I am grateful to you for all your help and great hospitality during my 'short visits' in Wageningen. I also wish to thank Maaike and Jörn for nice time, help in the lab and joined dinners. I would like to acknowledge all the people from CBI for creating a friendly working atmosphere, having nice talks with, and making me feel like at home: Lidy, Jan (for very useful talks and help), Beja, Adrie, Anja and Nico (for the technical support), Hilda (for help with administrative procedures), Carla, Corine, Heidi, Ellen, Ola, Joop, Mark (for your help in the lab, useful discussions and sharing nice time). Bedankt!

I wish to thank Dr. Zsigmond Jeney and Dr. Galina Jeney for giving me the opportunity to work in the DNA laboratory at the HAKI Research Institute, Szarvas (Hungary). I visited Szarvas several times during my PhD project and always found there a great atmosphere and fantastic hospitality. Very warm thanks to Istvan, Laszlo, Peter and Zoltan for being great colleagues and for introducing me to the Hungarian style of enjoying life. Köszönöm!

To Dr. Magdalena Chadzińska I would like to express my gratitude for having reviewed the Polish Summary (Streszczenie) of this Thesis and for being always available for scientific discussions through the Internet.

I would like to thank the students who worked with me during their summer practice in Gołysz, especially to Marcin, Marysia, Aldona (*dziekuję za pomoc w przygotowaniu rozprawy doktorskiej do druku*) and Natalia. Thank you for your hard work!

Special thanks go to my Parents, Sister and Mother-in-Low for their understanding, support and helping me in pursue what I wanted to do. *Dziękuję moim Rodzicom, Siostrze oraz mojej Teściowej za ich pomoc oraz wsparcie.*

Last but not least, thanks to my Wife Joanna for always supporting me in following my dreams and for always believing in me. *Dziękuję za wyrozumiałość, troskę i miłość.*

Training and Supervision Plan

Training and Supervision Plan **Graduate School WIAS**

Name PhD student	Krzysztof Łukasz Rakus
Project title	Major histocompatibility (MH) polymorphism of common carp: Link with disease resistance
Group	Cell Biology and Immunology
Daily supervisors	Dr. ir. Geert F. Wiegertjes, Dr. Ilgiz Irnazarow
Supervisors	Prof. dr. ir. Huub F.J. Savelkoul, Dr. Andrzej Pilarczyk
Project term	Sept. 2002- Sept. 2008
Submitted	25 August 2008

THE BASIC PACKAGE	**Year**	**ects**
- WIAS Introduction Course, Wageningen, The Netherlands, Jan. 20-24	2003	1.5
- Course on philosophy of science, Katowice, Poland	2005-2006	1.5
SCIENTIFIC EXPOSURE		
International conferences		
- Genetic Days: Current problems of Animal Genetics, Brno, Czech Rep., Sept. 12-13	2002	0.6
- ISDCI, StAndrews, Scotland, 29. June- 04. July	2003	1.5
- EAFP, St Julians, Malta, Sept. 21-26	2003	1.5
- Conference on Fisheries and Aquaculture, Szarvas, Hungary, May 4-5	2005	0.6
- Problems of Aquaculture, Kyiv, Ukraine, Sept. 26-30	2005	1.5
- Biotechnology 2006, Ceske Budejovice, Czech Rep., Feb. 15-16	2006	0.6
- 7th Nordic Symposium on Fish Immunology, Stirling, Scotland, June 19-22	2007	1.2
Seminars and workshops		
- WIAS science day, Wageningen, The Netherlands, Mar. 14	2002	0.3
- WIAS seminar plus: Host-Parasite Interaction in the Genomics Era, Wageningen, The Netherlands, Sept. 05-06	2002	0.6
- Parasite Immunology Workshop, Gołysz, Poland, July 12	2002	0.3
- PARITY Mid – term Review Meeting, Ustroń, Poland, Aug. 25	2004	0.3
- SCOFDA Workshop, Copenhagen, Denmark, Nov. 3-4	2004	0.6
- Meeting of the Committee of Agricultural Sciences PAS, Szczyrk, Poland, Oct. 4-5	2005	0.6
- Health protection in fish culture, Puławy, Poland, May 22-23	2007	0.6
- Characterization of Common Carp Genetic Resources, Szarvas, Hungary, Dec. 4-6	2007	0.9
- Meeting of the Committee of Agricultural Sciences PAS, Szczyrk, Poland, Sept. 23-24	2008	0.6
Poster presentations		
- Genetic Days, Brno, Czech Rep., Sept. 12-13	2002	1.0
- ISDCI, StAndrews, Scotland, 29. June- 04. July	2003	1.0
- EAFP, St Julians, Malta, Sept. 21-26	2003	1.0
- EAFP, Copenhagen, Denmark, Sept. 11-16	2005	1.0
- Biotechnology 2006, Ceske Budejovice, Czech Rep., Feb. 15-16	2006	1.0
- 7th Nordic Symposium on Fish Immunology, Stirling, Scotland, June 19-22	2007	1.0
Oral presentations		
- Meeting of the Scientific Board of the IIA PAS Gołysz, Poland, Feb 25	2004	1.0
- Problems of Aquaculture, Kyiv, Ukraine, Sept. 26-30	2005	1.0
- Meeting of the Committee of Agricultural Sciences PAS, Szczyrk, Poland, Oct. 4-5	2005	1.0
- Health protection in fish culture, Puławy, Poland, May 22-23	2007	1.0
- Characterization of Common Carp Genetic Resources, Szarvas, Hungary, Dec. 4-6	2007	1.0
- Meeting of the Committee of Agricultural Sciences PAS, Szczyrk, Poland, Sept. 23-24	2008	1.0

IN-DEPTH STUDIES		
Disciplinary and interdisciplinary courses		
- Fish Vaccination Workshop, Wageningen, The Netherlands, Apr. 22-26	2002	1.5
- Molecular Biology Techniques Course, Poznan, Poland, June 24-28	2002	1.5
- Advanced statistics; design of animal experiments, Wageningen, The Netherlands, Nov. 25-27	2002	1.0
- Advanced Immunology Training Course, Utrecht, The Netherlands, Jan. 6-10	2003	1.5
- Fish Immunology Workshop, Wageningen, The Netherlands, Apr. 21-25	2003	1.5
- DNA Sequencing Course, Warszawa, Poland, Dec. 5-7	2005	0.9
- AQUALABS- Aquatic Animal Disease Diagnostic, Stirling, Scotland, Jan. 15-20	2006	1.5
Statutory Courses		
- Health and Safety-at-Work Legislation Course, Gołysz, Poland,	2006	0.6
PROFESSIONAL SKILL SUPPORT COURSES		
- Writing and Presenting Scientific Papers, Wageningen, The Netherlands, Mar. 28-31	2006	1.2
- Project and Innovations Management, Bielsko-Biała, Poland	2008	5.0
RESEARCH SKILL TRAINING		
- Composing sandwich PhD research proposal, Wageningen, The Netherlands, Sept. 2002- Feb. 2003	2002-2003	2.0
- External training period, HAKI Szarvas, Hungary, (Dec. 2003, Dec. 2004, July 2005)	2003	2.0
DIDACTIC SKILLS TRAINING		
Supervising MSc theses		
- Mikołaj Adamek (MSc student)	2005	2.0
- Patrik Frost (MSc student)	2006	2.0
EDUCATION AND TRAINING TOTAL *(minimum 30, maximum 60 credits)*		**51.5**

List of publications

Adamek M., Huebner A., **Rakus K.Ł.,** van der Marel M., Dauber M., Irnazarow I., Steinhagen D. Does interferon type I expression modulate the resistance of common carp cells for Cyprinid herpesvirus-3? *Submitted*

Białowąs H., Irnazarow I., **Rakus K.Ł.,** Jurecka P., Pilarczyk A. Carp breeds of Poland. *Submitted*

Irnazarow I., Adamek M., **Rakus K.Ł.,** Frost P., Kazuń K., Kazuń B., Głąbski E., Lepa A., Siwicki A.K. Analysis of a diallel cross to estimate effects of crossing on resistance to CyHV-3 in common carp (*Cyprinus carpio* L.). *Submitted*

Rakus K.Ł., Wiegertjes G.F., Siwicki A.K., Lepa A., Adamek M., Savelkoul H.F.J., Pilarczyk A., Irnazarow I. Resistance of common carp (*Cyprinus carpio* L.) to Cyprinid herpesvirus-3 is influenced by major histocompatibility (MH) class II *B* gene polymorphism. *Submitted*

Rakus K.Ł., Wiegertjes G.F., Jurecka P., Walker P.D., Pilarczyk A., Irnazarow I. Major histocompatibility (MH) class II *B* gene polymorphism influences disease resistance of common carp (*Cyprinus carpio* L.). *Submitted*

Jurecka P., Wiegertjes G.F., **Rakus K.Ł.,** Pilarczyk A., Irnazarow I. (2008). Genetic resistance of carp (*Cyprinus carpio* L.) to *Trypanoplasma borreli*: Influence of transferrin polymorphism. *Vet. Immunol. Immunopathol.* Available as OnlineEarly article. doi:10.1016/j.vetimm.2008.09.006. (IF: 1,957)

Rakus K.Ł., Irnazarow I., Forlenza M., Stet R.J.M., Savelkoul H.F.J., Wiegertjes G.F. (2008). Classical crosses of common carp (*Cyprinus carpio* L.) show co-segregation of antibody response with major histocompatibility class II *B* genes. *Fish Shellfish Immunol.* Available as OnlineEarly article. doi:10.1016/j.fsi.2008.08.011. (IF: 3,160)

Rakus K.Ł., Wiegertjes G.F., Adamek M., Bekh V., Stet R.J.M., Irnazarow I. (2008). Application of PCR-RF-SSCP to study major histocompatibility class II *B* polymorphism in common carp (*Cyprinus carpio* L.). *Fish Shellfish Immunol.* 24: 734-744. (IF: 3,160)

Onara D.F., Forlenza M., Gonzalez S.F., **Rakus K.Ł.,** Pilarczyk A., Irnazarow I., Wiegertjes G.F. (2008). Differential transcription of multiple forms of alpha-2-macroglobulin in carp (*Cyprinus carpio*) infected with parasites. *Dev. Comp. Immunol.* 32: 339-347. (IF: 3,155)

Rakus K.Ł., Wiegertjes G.F., Stet R.J.M., Savelkoul H.F.J., Pilarczyk A., Irnazarow I. (2003). Polymorphism of MHC class II *B* genes in different lines of the common carp (*Cyprinus carpio* L.). *Aquat. Living Resour.* 16: 432-437. (IF: 0.768)

Curriculum vitae

Krzysztof Łukasz Rakus was born on October 14th, 1977 in Cieszyn, Poland. He studied Biology (specialization in Biotechnology) at the Department of Biology and Environmental Protection, University of Silesia in Katowice, Poland. While completing his 5th year of study, he spent five-month long research training period at the Department of Food Science and Technology, Agricultural University of Athens, Greece (Socrates/Erasmus scholarship). He completed his Masters Degree in 2001 and joined the Institute of Ichthyobiology & Aquaculture in Gołysz, Poland. In 2002 he was awarded a six-month Marie Curie scholarship at the Cell Biology and Immunology Group, Wageningen University, The Netherlands. After that he spent additional six months at CBI, WUR, starting his PhD project on correlation between MH genes and disease resistance of common carp. The PhD project was a "sandwich" construction financed by WUR and IIA, Gołysz. Part of the research was carried out at CBI, WUR; however most of it was done at IIA, Gołysz. The two combined efforts resulted in the Thesis presented herein.

Printed by Wöhrmann Print Service, Zutphen, The Netherlands.