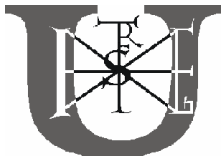

SZENT ISTVÁN UNIVERSITY

STUDIES ON AFRICAN CATFISH SEX BY MOLECULAR GENETIC TOOLS

Ph.D. Thesis

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1 SCIENTIFIC BACKGROUND AND AIMS OF STUDY

The work described in my doctoral thesis was performed in the Laboratory of Fish Molecular Biology at Agricultural Biotechnology Centre (Gödöllő, Hungary). The main scientific project of the research team was to study mechanisms of sex determination and differentiation during ontogenesis of fishes.

The class of fishes is probably the most complex group of vertebrates in terms of sex chromosomal types, sex determination and sex differentiation. When the forms of reproduction are studied in different fish families, protandrous, protogynous, synchronized hermaphrodites and heteroecious species (those with environmental sex determination and gonocronistic reproduction) can be identified, sometimes even within the same family (Devlin and Nagahama, 2002).

The sexual development of fishes and other aquatic species with external ontogenesis shows at least partial dependence on the environment. Gonad development of fish is timed to the first few weeks after hatching. Exposure to high water temperature during this time period usually results in increased ratio of males in the progeny, while temperature below the optimum is leads to the development of females (Uguz et al., 2003). Environmental pH has similar effects in some species as the ratio of females rises at high pH, while the ratio of males increases at low pH in a given population (Rubin, 1985).

There are indications in the literature on similar effects of certain environmentally harmful substances (e.g. communal and industrial sewage, heavy metals, organic phosphates and organic chlorine compounds, substances with hormonal effects, pesticides and herbicides). These might cause sex-conversion, development of intersexual gonad, expression of sex specific proteins, feminization of male gonad, as well as early or delayed sexual maturation (Kime, 1995; Sumpter, 1997).

Normally, the environmental factors listed above are not the primary sex determination factors in fishes. Nonetheless in extreme situations they can take over the control of sexual development in hermaphrodite or gonocronistic individuals, which might result in the development of sex other than the genetically predetermined one (Devlin and Nagahama, 2002).

Sex is genetically encoded in most fish species, though its background mechanisms are hardly known and not uniform. Although the kariotype of more than 1,700 species have been described so far, well-differentiated sex chromosomes were identified only in 176 of them. Some of these species show male heterogametity (XX/XY), others are female heterogametic (ZW/ZZ) types (the former one is twice as frequent then the other). In addition to these species carrying XX/XO and ZZ/ZO sex chromosome types were found as well. Occasionally, several of these chromosome varieties are present within a family. Curiously, sex chromosome-pair has been found in some populations of four species, among the 259 (7 genera) showing some signs of hermaphroditism (Devlin and Nagahama, 2002).

As a result of biochemical and molecular biological analyses, a few enzymes and DNA markers with sex-linked inheritance have been found (most of them in the *Salmonidae* family). None of them are not uniformly applicable to all fish species, i. e. normally they are functional only in the species (rarely in the genus or family) they were identified from (Matsuda et al., 1997; 1998).

Sex determining genes of other species (*Sry* - mammals, *Sxl* - *Drosophila melanogaster*, *Xol* - *Caenorhabditis elegans*) were found not to be sex-linked in fishes. *Dmrt1* is the only gene, for which homologues are present in *D. melanogaster* (*Dsx*), *C. elegans* (*mab3*) and vertebrates among them in fish genomes, and known to have importance in sex development. One of the *Dmrt1* homologues among those isolated from *Oryzias latipe* (*Dmy*) is inherited on the Y chromosome, and its product is the first sex-determining factor identified in fish. At the same time this sex-linked, primary initiator factor is not present in other fish species, except another *Oryzias* species (Nanda et al, 2003; Schartl, 2004).

With the exception of the *Salmonidae* family, the isolation of sex-linked enzymes, DNA markers or sex-determinant genes has not been successful for any fish species farmed in Hungary, despite of the fact, that the importance of such markers in fish biology and breeding is evident. A good example for their utility is molecular sexing, which is a procedure routinely used in mammals. This analysis is valuable for early sex identification, adjusting sex ratio, or creating a sex-biased or monosex group from valuable broodstocks. It might also have some importance for the research on sexes, or might also be used for quick testing of monosex stocks produced by whole genome manipulation. The latter possibility has

economic importance for those species, like the European catfish (*Silurus glanis*), in which the two sexes grow at significantly different rate.

My experiments were performed on members of a very important taxonomic group among teleosts, the siluroid catfishes. The main topic of my research was the study of sex chromosomes in *Clariidae* species. Therefore, major part of my thesis presents the results of experiments on these species. However, I also performed experiments on tench (*Tinca tinca*), a member of the *Cyprinidae* family.

The specific purpose of my study was to identify sex-specific DNA markers, which might be useful for sex-identification at early stage of ontogenesis and for identification of sex chromosome system (XX/XY or ZW/ZZ) in the studied species.

Beside searching for markers, my task also included the isolation and characterization of the identified sex-specific markers. Following the determination of DNA sequence of markers, it was also my task to design a rapid, Polymerase Chain Reaction (PCR) based test, potentially applicable for sex identification during early embryonic stages. I also performed a search for sex markers related to those identified in species closely and distantly related to the one, which the original sex markers were isolated from.

2 MATERIAL AND METHODS

2.1 RESEARCH ANIMALS

African catfish (*Clarias gariepinus*) brooder individuals used in my experiments were purchased from Intenzív Haltenyésztő Ltd (Újszentmargita-Tuka) and Szarvas Fish Ltd. (Szarvas). Caudal fin samples of *Heterobranchus longifilis* were also supplied by the Szarvas Fish Ltd. (Szarvas). DNA samples of *Clarias macrocephalus* were collected by Dr. Graham Mair (Pathumthani, Thailand), whereas samples of *Clarias fuscus* were provided by Prof. Arlo Fast in Hawaii (USA). *Clarias batrachus, Hoplosternum thoracatum*, *H. litorale* and *Corydoras aeneus* individuals were supplied by Pasaréti Gyula (Akvárium Magazin, Budapest).

Caudal fin samples of *Silurus glanis, Heteropneustes fossilis, Ictaulurus punctatus* and *Tinca tinca* were obtained from various Hungarian fisheries, while those of the redtail catfish (*Phractocephalus hemiliopterus*) were supplied by Rainbow Fish Farm in Singapore.

2.2 SAMPLE PREPARATION, DNA ISOLATION

Tissue samples (from caudal fin) were collected from sexually mature individuals after dissection and following visual inspection of reproductive organs.

Tissue samples were digested in SET buffer containing 0,5 μg/ml Proteinase K enzyme. DNA was isolated with phenol-chloroform extraction method described by Sambrook et al. (1989).

Samples for larval-stage molecular sex identification were collected from 1-2 cm long individuals, 3-5 weeks after fertilisation. At sampling, only the caudal fin was cut off without killing the animals. During the period of sex identification, fish were kept individually.

2.3 RAPD ANALYSIS

Female and male DNA pools were created by mixing of sexed samples of individuals. These pools were used lather for comparative RAPD analysis to identify differences between sexes. The differences were confirmed with repeated RAPD analysis on individual samples.

RAPD reactions were prepared according to the method described by Bercsényi et al. (1998).

2.4 FRAGMENTS CLONING AND SEQUENCING

The selected sex-specific and control fragments were isolated with QIAquick Gel Extraction Kit (Qiagen), and were then ligated in the vector of Topo TA Cloning kit (Invitrogen). *E. coli* competent cells were transformed with this ligated product. The successful cloning was confirmed by restriction enzyme digestion and/or PCR amplification.

Sequencing of the clones was initiated with M13 sequencing primers, with the use of BigDye Terminator v2.0 Cycle Sequencing Kit (Applied Biosystems) in an ABI Prism 377 (Applied Biosystems) sequence analyser.

2.5 SOUTHERN AND ZOO HYBRIDISATION

Hybridizations were done with DIG labelling and by using a commercial hybridisation kit (Roche). Three microgrammes of genomic DNA was separated and blotted from each sample. Probes were produced by a special PCR reaction using DIG-dNTP (Roche). Hybridization was done at 42 ºC for at least 16 hours. The membranes were washed at 68 ºC (high stringency or at 65 ^{o}C (zoo hybridization). Chemiluminescent detection was performed according to the recommendations of the manufacturer using Anti-DIG-AP and CSPD or CDP-Star substrates (Roche).

2.6 PREPARATION AND STUDY OF PARTIAL GENOMIC DNA-LIBRARY

Fragments in the size range of the homologue (~4500 bp) that was identified from *Clarias batrachus* genomic DNA by zoo hybridisation with CgaY1 probe, were isolated from gel and they were subsequently ligated in Bluescript KS+ plasmid (Strategene).

The prepared partial genomic DNA-library was transformed to *E. coli* competent cells. The clones were analysed with colony hybridisation using the DIG labelling and hybridisation system of Roche, according to the method described for Southern hybridisation above.

The insert length of the hybridizing colonies was estimated by colony-PCR. PCR products were sequenced according to the formerly described procedure.

On the basis of the comparison to the amplified sequences, 38 clones were selected for further analysis, thus specific primer pairs were designed for them. Their sex-specificity was tested on the DNA pools created as it is described in chapter 3.1. Microsatellite-containing sequences were identified from the sequenced inserts by using Tandem Repeats Finder 2.02 software (Benson, 1999).

2.7 MULTIPLEX PCR REACTIONS

Specific primer pairs were designed for amplification of sex-specific and control fragments, as well. Considering the results from these optimised PCR reactions three primer pairs were selected for multiplexing. These worked at uniform conditions and amplified two sex-specific and one control fragment, respectively. Duplex reactions were set up and optimised with these primers. Results of duplex reactions were compared again and with unification of two reactions triplex PCR reaction was designed (Henegariu et al., 1997).

One of the duplex reactions later has been used for prediction of phenotypic sex of larvae.

Followings of the tested primers were selected and used in further experiments:

Y1-2F: 5'-CTGTTCATTGAAGCGACTTCTG-3';
Y1-1R: 5'-GAACTGAACCCCACATTTTGTC-3';
Y1-5F: 5'-CTGGCTTTTATAGTTAAGGGAC-3'
Y2-1F: 5'-TGACCCTAGACCAGGACGTAAC-3';
Y2-1R: 5'-AACTCAGATCACACTGAATGCG-3';
K1-1F: 5'-AGTACATTGAGGACGAGGACGC-3';
K1-1R: 5'-CATTGTAACAAGAGGAGCCCAC-3'.

The omposition of reaction mix was as follows: 0.8x Promega PCR buffer, 200-200 μM of each dNTP, 500-500 nM Y1-F2 and -R1 primers, 60-60 nM Y2-F1 and -R1 primers, 30-30 nM K1-F1 and -R1 primers, 2 mM $MgCl_2$, 1U Taq DNA polimerase (Promega) and 20 ng genomic template DNA in 25μl final volume. Applicable cycle

parameters for duplex and triplex PCR reactions were: pre-denaturation at 94 °C for 2 min, follows 30 cycles of 94 °C for 20 sec, 61 °C for 20 sec and 72 °C for 60 sec, then a final extension of 72◦C for 5 min.

2.8 TESTING THE APPLICABILITY OF SEX-SPECIFIC MARKERS FOR MOLECULAR SEXING OF LARVAE

Duplex PCR reaction was used to determine the sex of larvae produced by a parent pair. Two fragments (CgaY1 and control) were amplified from the male samples, whereas only one (the control) from the females. The composition of reaction mix and conditions of amplification were identical to the one described above with use of Y1-5F, Y1-1R, K1-1F and K1-1R primers. On the basis of the molecular sexing results the individuals were grouped according to their sex and were grown until five months of age. Phenotypic sex determination was performed at that age by using the histological analysis described by Guerrero and Selton (1974).

3 RESULTS AND DISCUSSION

3.1 SEARCH FOR SEX-SPECIFIC DNA MARKERS IN THE GENOME OF SILUROID AND CYPRINID SPECIES

The genome of five catfish species of *Clariidae* family, and of tench (*Cyprinidae*) was screened for sex-specific DNA markers. DNA samples collected from siblings (if available) or related individuals were pooled according to the phenotype (sex) of individuals and these pools were analyzed. As a consequence of this sampling method individual differences become attenuated, while differences between the phenotypes, that were the criteria of selection are maintained, or enhanced. The differences found between the two groups are more likely to be linked with the selected phenotype (Michelmore et al., 1991). We searched for differences between the created DNA pools by using comparative RAPD analysis.

All the RAPD primers, which amplified a differential pattern from the two pools (sexes), were selected for more detailed analysis. The so-called "potential markers" were further analyzed on individual DNA samples to test the sex-specificity. On the basis of the results of these assays, two markers were found to be linked strongly with maleness (Y chromosome). Their Y-linkage was later confirmed by dissection as well. Both of them were detected in males of *Clarias gariepinus*, while no sex-specific marker was isolated from the genome of the other five species investigated.

The low number of markers found and the low efficiency in different species might be the result of several factors. On one hand, sex-determination is highly plastic in fish and can be modified even by secondary factors (modification genes, environmental effects), which might result in conversion of phenotypic sex (Devlin and Nagahama, 2002). Presence of sex-converted individuals in the experimental stock could make the identification of sex-specific markers with the method used in my experiments difficult or even impossible. On the other hand, there is more and more evidence for an extra genome duplication, that had occurred in the common ancestor of teleosts following its separation from the ancestor of land vertebrates (Aparicio, 2000; Nonaka, 2001). Some

segments of the duplicated genome persisted and translocated to various chromosomes during the rearrangement of the genome, lowering the chance of success in such searches.

3.2 CHARACTERISATION OF THE ISOLATED MARKER SEQUENCES, CONSTRUCTION OF MOLECULAR SEXING METHOD

Two sex-specific (CgaY1, CgaY2) and one control (CgaK1) fragments were isolated, cloned and sequenced from RAPD patterns of the analysis. The latter fragment was present in each individual of both sexes. Total DNA sequence of the marker was published in the GenBank (reference numbers: CgaY1 - AF332597; CgaY2 - AF332598; CgaK1 - AF332599). Accurate length of CgaY1, CgaY2 and CgaK1 markers were found to be 2584 bp, 458 bp and 697 bp, respectively.

When public databases were searched with the two sex-specific sequences no significant homologies were found at the DNA level. However, on protein level, 3' end of the CgaY1 marker showed some homology with tyrosine phosphatase (PTP or PTEN) genes, which are hormonally controlled in mammals (Mauro et al., 1994) and some of them are expressed specifically in the testis (Tokuchi et al., 1999). These genes are supposed to have potential role in the development of the testis.

The CgaY2 marker has shown poor homology on a 470 bp fragment with a reverse transcryptase gene of *Takifugu rubripes* (Poulter et al., 1999). Such viral or transposon-derived, degraded sequences are relatively common in eukaryote genomes. Most of them are not functional, they are "genetic fossiles of the evolution" (Goodier and Davidson, 1994).

The sex-specific and control fragments were converted to SCAR markers. Specific PCR reactions of these markers were used to set up duplex and then triplex PCR reactions after several optimisation steps. These multiplex PCR reactions allow easy and user-friendly molecular sexing of *C. gariepinus* individuals.

Before the beginning of our work only one sex-specific RAPD marker has been reported in the literature, it was isolated from rainbow trout by Iturra et al. (1997). Therefore, our markers are the second and the

third sex-specific markers isolated from pooled fish DNA samples. Furthermore, CgaY1 and CgaY2 were the first two sex-specific DNA markers isolated in *Siluridae*, although later several sex-linked microsatellite markers were described from channel catfish (*Ictalurus punctatus*) by Waldbieser et al. (2001).

3.3 STUDY OF THE SEX-SPECIFIC MARKERS WITH SOUTHERN HYBRIDISATION

Southern hybridization analysis was prepared to confirm the sex-specificity of the isolated markers and assessing their copy number in the genome. Nine restriction enzymes were used to digest genomic DNA before hybridization analysis with the two sex markers, respectively.

The CgaY1 marker has shown strong hybridization to the male genome, whereas no hybridization was found to the female genome. Several fragments were produced in the male genome, although four of the used restriction enzymes (Bgl II, BstE II, Not I and Pac I) did not have cutting site within CgaY1 fragment. This implies that this sequence is present in several copies in the male genome of *C. gariepinus*.

Clear hybridization patterns were obtained with the CgaY2 probe to both the male and female genome. However, no difference was found between the hybridization pattern. On the basis of the hybridization patterns, a single copy of CgaY2 seems to be present in the genome of both *C. gariepinus* males and females. However, using RAPD analysis and specific primers, amplification of this fragment could only be amplified from the male genome. A possible explanation for this controversy is, that the homologue of the male-specific fragment could be present in female genome, but one end of it could be deleted. This is why difference has occurred among PCR amplification products, but no difference has shown in Southern hybridisation pattern, when deletion covers the attachment site not only for the of RAPD primer, but for one of the specific primers, as well. (ca. 25 bp length) However, deleted sequence can not be too long, as no difference was detected between the hybridization patterns of the fragments.

3.4 SEX CHROMOSOME SYSTEM OF AFRICAN CATFISH

The African catfish was used earlier in several studies on genome manipulation and hormonal sex-inversion, but these provided only indirect data on the sex chromosome system of these species. The conclusions drawn by these studies are often contradictory. Some of them indicates an XX/XY chromosome system (Liu and Yao, 1995; Galbusera et al., 2000), while others presume a ZZ/ZW system (Ozouf-Costaz et al.,1990; Váradi et al.,1999). The situation is further complicated by the fact, that both male heterogametic and female heterogametic species can be found among the siluriods (review: Devlin and Nagahama, 2002).

The isolation and characterization of our two male-specific DNA markers, however prove convincingly that the sex of African catfish is inherited according to an XX/XY sex chromosomal system, and not to a ZW/ZZ one. This conclusion is supported by the chromosome hybridization analysis („Fluorescent *In Situ* Hybridization") performed by our collaborator, Catherine Ozouf-Costaz (Museum of Science, Paris, France) with our male-specific CgaY1 marker as a probe. Her results showed that the CgaY1 marker hybridizes only to one metacentric chromosome (in the same position on both chromosome arms) but not to its chromosome pair in the male chromosome set of 56 chromosomes (no hybridization was observed to the female chromosome set.)

3.5 SEARCH FOR HOMOLOGUES OF SEX-SPECIFIC MARKERS IN OTHER CATFISH SPECIES

According to the literature only one of the known teleost-derived sex-specific DNA markers turned out to be sex-linked in several species. This sequence was isolated from a „Chinook" salmon (Du et al., 1993) and it seems to be Y-linked exclusively in genera of *Oncorhynchus* (Forbes et al., 1994; Nakayama et al., 1999) only.

The conservation of the two isolated sex-specific markers were examined in related catfish species. For this purpose, the markers were used as hybridization probes in a so-called "zoo hybridisation" analysis to identify homologous DNA sequences. Both sexes of twelve catfish species from five *Siluroid* families (*Clariidae, Callichthyidae, Siluridae, Ictaluridae, Pimelodidae)* were examined by "zoo hybridization" at high and low stringency hybridization conditions with both probes.

The CgaY2 marker has hybridized with samples of both sexes of each inspected species and, similarly to *C. gariepinus,* no difference was found between the two sexes.

"Zoo hybridization" with the CgaY1 probe at low stringency hybridisation conditions showed potential homologs in the genomes of three closely related species, but the results were different. There was no difference between the patterns of the two sexes in *C. fuscus*, while in *C. batrachus* sole homologous fragment was found, but only in males. Hybridization patterns of the two sexes for *H. longifilis* were different only in one fragment, which might be a potential marker, as it was present only in male samples. It is of importance, that strong signals were obtained only in male samples of *C. gariepinus* even at low stringency conditions. This seems to indicate that the sequence similarity of the marker with the presumed homologs from the other species is low.

3.6 IDENTIFICATION OF MICROSATELLITE SEQUENCES BY THE HOMOLOG SEARCH

The isolation of the identified homologs might produce additional useful sex-specific markers, witch could provide further interesting information. Therefore, a attempt was made out to isolate the homolog(s) of CgaY1 marker from *Clarias batrachus*. A partial genomic plasmid library was designed for this purpose, which was tested with colony hybridisation at same conditions as described for "zoo hybridization". According to the hybridization results, 96 colonies were selected, and the sequences of their inserts were determined.

The clone with the highest sequence homology to CgaY1 showed only 57% similarity. However, neither this, nor the other 38 tested clones (for which specific primers were designed) have shown any difference between sexes in PCR reactions run on pooled DNA samples of *Clarias batrachus*. On the other hand sequence analysis identified 21 microsatellite- type repeats in the insert sequences and 18 of them were suitable for designing primers to the flanking regions to generate microsatellite markers.

The performance of these microsatellite markers was tested in *Clarias batrachus* and other catfish species from three different families

by our collaborator, Genhua Yue (Temasek LifeSciences Laboratory, Singapore). Fifteen of the primer pairs designed for the flanking regions of the microsatellites have amplified specific fragments at least in one related species, while thirteen of them amplified specific fragments at least in three of the inspected species.

These isolated microsatellite sequences seem to be useful for the genetic analysis of various *Clarias* species. They are applicable for genetic mapping, for pedigree and inheritance analysis, genetic analysis of populations, differentiation of varieties and hybrid lines, but could also used for identifying individuals, or determining of degree of evolutionary relationship between species within a family.

3.7 THE EXPERIENCE FROM PRACTICAL USE

The efficiency of the molecular sexing method was tested for predicting the adult sex phenotype of individuals during the larval period. In this study phenotypic sex of individual progenies was predicted at three weeks post fertilisation. Duplex PCR reaction using the CgaY1 and CgaK1 markers was used for this purpose. Following the analysis the two sexes were grown separately until five months of age, when histological analysis of the genitals was performed on 87 male and 92 female individuals to identify the gonad type unambiguously.

By comparing the results of histological analysis with the data from molecular sexing, the latter was found to predict phenotypic sex with 96.6% fidelity. Interestingly the predicted genotypic sex and the observed phenotypic sex were different in 3.4% of the animals, while in a former experiment, using the CgaY1 marker on 80 individuals from different populations, no disagreement was found between genotypic and phenotypic sex. The possible explanations are as follows: One possible explanation is the sex chromosomal recombination within region between the marker and the sex-determinant factor. If this is the case, the distance of marker and sex-determinant factor should not be more than 3.4 cM. However, further experiments are needed to confirm this theory.

Another reason for the above mentioned mismatch could be the sensitivity of sex determination and early differentiation, which allows social or other environmental factors to override the genetically determined sex. Such natural sex reversal was observed in a number of

teleost species (Badura and Friedman, 1988; Shapiro, 1992; Harry et al., 1992; Pandian and Koteeswaran, 1998).

A repeated PCR test on the "mismatched" individuals confirmed the sexual genotype predicted for all individuals, except three of them. Thus only 1.7% of the original marker tests have shown a false result. Such errors might arise from mis-evaluation of test results or accidental migration between the two groups during the growing period.

4 NEW SCIENTIFIC RESULTS

1) Comparative RAPD analysis on five catfish species of *Clariidae* family and tench (*Tinca tinca*) has found that RAPD patterns produced OPO-01 and OPM-08 primers are linked to detected phenotypic sex in African catfish. No sex-specific pattern was amplified from the genome of the five other species studied.

2) The sex-specific fragments were isolated and cloned into plasmid vector; then the full nucleotide sequence of these fragments was determined. The length of CgaY1 and CgaY2 markers is 2,584 bp and 458 bp, respectively.

3) Southern hybridization was used to verify that CgaY1 marker is present only in male genome of *C. gariepinus* (presumably in several copies), while it is absent in females' genomes. The hybridization patterns of CgaY2 marker did not shown differences between the genomes of two sexes.

4) Specific primers were designed for two isolated sex-specific and one control fragments and they were converted to SCAR markers. They were employed to set up user-friendly molecular sexing methods, based on duplex and then triplex PCR reactions. The earlier amplify two (one sex-specific and one control) and the latter three (two sex-specific and one control) fragments in males, while only one fragment in females.

5) The detailed analysis of the isolated male-specific markers proves convincingly, that the African catfish has an XX/XY sex chromosomal system.

6) In a "zoo hybridization" study on twelve catfish species from five families, the two sex markers hybridized only to the genome of species from the *Clariidae* family. CgaY2 showed strong hybridization in both sexes of the studied species in this family (*Clarias gariepinus; Clarias macrocephalus; Heterobranchus longifilis*) but no difference was detected between the two sexes. On the other hand "zoo hybridizations" using the CgaY1 probe indicated the presence of a potentially Y-linked homolog(s) of the in *Heterobranchus longifilis* and *Clarias batrachus,* whereas an autosomal one in *C. fuscus*.

7) According the results of an experiment performed with the progeny group of a breeder pair, the molecular sexing method using duplex PCR with the CgaY1 Y-specific DNA marker has 96.6% fidelity in practice for predicting the phenotypic sex.

8) Twenty-one microsatellite repeats were identified from a partial genomic library created for the islation of the CgaY1 marker homologue from *Clarias batrachus.* Eighteen of these sequences were suitable for designing microsatellite markers, which might be used for genetic analysis not only in *Clarias batrachus*, but also in several other species of *Clariidae* family.

5 SUGGESTIONS

Sex-specific markers have wide range of potential utilization both in applied and fundamental research. *In vivo* sampling allows to collect samples at very early stage of ontogenesis, or to collect representative samples at embryonic/larval stage to determine the sex ratio of a population even just a few hours after fertilization. Molecular sexing provides a method to confirm andro- or gynogenesis and success of hormone-induced sex-reversal at early stages of development, without the necessity to wait until sexual maturation of the progeny. This might shorten the time frame needed for such experiments even by a whole generation.

A significant difference has been observed between the growth rates of sexes in African catfish, where the males are growing faster (Henken és mtsai., 1987). An all-male population for increased meat production could be produced by creating so-called “supermales” carrying a YY sex chromosome pair by androgenesis or by crossing sex-reversed XY neo-females with males. In this process the early identification of genotypic sex might provide substantial advantage, thus molecular markers have great potential for the aquaculture industry. Sex markers allow for the study of male and female development from the early stages. Such markers might also be useful starting points for identification of genes involved in sex determination and genital development or of genes with divergent activity in the two sexes. They also have the potential to be helpful in studies of hormonal processes in the two sexes during ontogenesis.

The use of sex-specific DNA markers allows detailed investigation on the effects of environmental factors (temperature and pH) with potential effect on sexual development. Especially significant is their utility to describe the effect of various herbicides and other hormone-like pollutant as well as to determine their environmental saturation level and critic concentration. These substances can cause abnormal sexual development at very low concentration in animals developing in aqueous environment, outside of their mother’s body. An analytical method based on sex-specific markers has already been designed for measuring the effect on sex development of such pollutants in salmon. The test methods designed for African catfish allows the use this species for similar investigations in natural aqueous environment of poor quality and oxygen saturation, at which salmonids would not be able to survive.

6 PUBLICATIONS CONNECTING TO THE PH.D. ACTIVITY

Papers:

Kovács, B., Egedi, S., Bártfai R., and Orbán L. (2001) Male-specific DNA markers from African catfish *(Clarias gariepinus)*. Molecular sexing of African catfish by novel male-specific DNA markers, GENETICA **110**: 267-276 **IF2001: 0,916**

Bártfai, R., Egedi, S., Yue, G.H., **Kovács B**., Urbányi, B., Tamás G., Horváth, L. and Orbán L. (2003*)*: Genetic analysis of two common carp broodstocks by RAPD and microsatellite markers, *Aquaculture,* **219(1-4)**: *157-167* **IF2003:1,507**

Yue, G.H., **B. Kovacs** and L. Orban. (2003): Microsatellites from *Clarias batrachus* and their polymorphism in seven additional catfish species. *Molecular Ecology Notes*. **3**: 465-468. **IF2003: 1,145**

Presentations:

Hill, J. Hill, J.A., G. Yue, Y. Li, F. Chen, **B. Kovacs**, R. Bartfai, S. Egedi, C.H. Goh and L. Orban (2000): Identification of sex-related genomic markers and genes involved with sex determination in fish. Oral presentation at "Genetics in Aquaculture VII", Townsville, Australia, July 15-22 (2000) Proceedings p52.

Yue, G.H., Y. Li, F. Chen**, B. Kovacs**, D. Ong, Bartfai, R., J.A. Hill, L.C. Lim and L. Orban (2001): Molecular sexing of fish: potential for basic research and aquaculture. Invited lecture at the Aquarama 2001, Singapore, June 1-5 (2001)

Posters:

Orbán, L., Egedi, S., Bártfai, R., Kobolák, J., **Kovács, B.** and Horváth, L. (1998): Searching for sex-linked DNA markers in cyprinid and siluroid fish species. Poster at the 1998 Meeting on Zebrafish

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