SEX DETERMINATION AND SEX DIFFERENTIATION IN TILAPIAS

J. F. Baroiller\textsuperscript{1}, H. D’Cotta\textsuperscript{1}, E. Bezault\textsuperscript{1}, J. P. Coutanceau\textsuperscript{2}, C. Ozouf-Costaz\textsuperscript{2}, A. Cnaani\textsuperscript{3}, T. Kocher\textsuperscript{3}, E. Pepey\textsuperscript{1}, A. D’Homt\textsuperscript{4}, & G. Hulata\textsuperscript{5}, B. J. N. Volff\textsuperscript{6}, D. Bienvenu\textsuperscript{1} and B. Chevassus\textsuperscript{7}

1 CIRAD-EMVT, UPR20 Aquaculture et gestion des resources aquatiques Campus International de Baillarguet, TA 30/A, 34398 Montpellier cedex 5, France
2 Museum National d’Histoire Naturelle, Département Systématique et Evolution Service de Systématique moléculaire, IFR 101-CNRS, Case postale n° 26, 57, rue Cuvier, F-75231 Paris Cedex 05, France
3 Hubbard Center for Genome Studies, University of New Hampshire, Durham, New Hampshire 03824, USA
4 CIRAD-CA / UMR PIA, F-34032 Montpellier, France.
5 Institute of Animal Science, Volcani Center, Agricultural Research Organization PO Box 6, Bet Dagan 50250, Israel
6 University of Wuerzburg / Biozentrum, D-97074 Wuerzburg, Germany
7 INRA / Laboratory of Fish Genetics, F-78352 Jouy-en-Josas, France

In tilapias, as in most teleost species, sex chromosomes cannot be recognized by karyotyping. Therefore, indirect approaches (interspecific and intraspecific crosses and backcrosses, progeny testing of hormonally sex-reversed fry and chromosome set manipulation) have suggested that sex determination is predominantly determined by the existence of a major sex-determining gene located on a pair of sex chromosomes. Within the group of tilapias, ZZ/ZW and XX/XY species can be found together within the same genus, i.e. heterogametic XY males in \textit{O. niloticus} (as in mammals), and conversely, heterogametic ZW females in \textit{O. aureus} (as in birds).

Some studies have suggested that the largest pair of chromosomes in \textit{O. niloticus} could be the sex chromosomes (Carrasco, 1999; Bezault \textit{et al.}, 2001; Harvey \textit{et al.}, 2002). Indeed, synaptonemal complex analyses revealed that terminal regions in the largest chromosome pair are unpaired in XY individuals (Foresti \textit{et al.}, 1993), but paired in XX/YY genotypes (Carrasco \textit{et al.}, 1999). Our results have also suggested that due to their chromosome features (high density of heterochromatin, accumulation of retrotransposons and other repeated sequences...) they could be the sex chromosomes (Bezault \textit{et al.}, 2001). \textit{In Situ} hybridization probes have been obtained by chromosome microdissection from this first pair and subsequent degenerated oligonucleotide-primed PCR: comparative hybridization of putative X and Y chromosome-derived probes have suggested the existence of sequence differences between these two chromosomes (Harvey \textit{et al.}, 2002).

Contrary to what happens in some other fish species (i.e. the rainbow trout, carp...), in tilapia the control by a single pair of sex chromosomes appears to be less strict because skewed sex ratios frequently occur and cannot be explained by heterogametic systems.

Parental effects on the sex ratios have been demonstrated in the Nile tilapia, \textit{O. niloticus}: within a defined basal temperature, sex ratios of successive progenies generated by a given couple of breeders are specific (ranging between 5 and 85% males) and stable. Masculinizing and/or feminizing factors can therefore be provided by both parents, and both paternal and maternal influences on the sex ratio have been demonstrated.
Finally, our very recent data on *O. niloticus* and *O. aureus* species (Cnaani et al., submitted) strongly suggests that in tilapia the sex determination system has evolved from an ancestral female heterogametic system (ZZ/ZW) towards a male heterogametic type (XX/XY). In the group of tilapias, the co-existence of both a XX/XY system in *O. niloticus* and a ZZ/ZW system in a sister species, *O. aureus* allows the study of how this evolution occurs and how a gene is recruited and becomes a major sex determinant. Genetic mapping has evidenced that both linkage group 3 (LG3) and LG1 are associated with sex in tilapia. LG3 has been anchored to the large chromosome pair in the tilapia *O. aureus* by FISH and appears to contain a major sex determinant. LG1 has been anchored to a smaller chromosome pair and can contain a minor sex determinant in *O. aureus*. Conversely, in *O. niloticus*, LG3 has lost its importance and the major sex determinant has risen in LG1. Due to their chromosome features (partial suppression of recombination, sex-specific differences in their rate of recombination, accumulation of repeated sequences and retro-transposons, ...), these chromosomes have to be respectively considered as an old and a new sex chromosomes (Cnaani et al, submitted).

However, sex ratios can also be influenced by some specific exogenous factors: whereas salinity has no significant effect on sex ratio, temperature seems to have the most important influence on sex differentiation in *Oreochromis* species.

Low temperatures do not affect the sex ratios in *O. niloticus* when applied during the hormonal-sensitive period (Baroiller et al., 1995). Conversely, high temperature treatments (>32-34°C) covering the sensitive period can increase the percentage of males in some progenies (Baroiller et al., 1995) with functional testes differentiation. All-male populations have been obtained following such treatments in the most sensitive progenies of *O. niloticus* and *O. aureus* species (Baroiller and D’Cotta, 2001), whereas in some other progenies, the proportion of males is not affected by high temperatures in *O. niloticus*.

Thermosensitivity is very stable within successive progenies generated by a same couple of breeders, but again, parental effects (both paternal and maternal effects) are strongly suggested.

Therefore, in this group of tilapias, sex is determined by genetic factors (both major and minor factors), temperature levels, and genotype/temperature interactions.

We have recently investigated the sex determination system of 3 natural populations adapted to extreme thermal regimes: stable temperature regime in Ethiopia (cold temperatures in the highland Koka Lake, or high temperatures in hydrothermal resurgence of Lake Metahara), versus a temperature regime with large seasonal variations in Ghana (Kpandu, Lake Volta). Individual progenies obtained from breeders of these different populations were reared under constant basal (27°C) or high (36°C) temperatures during the 30 days following yolk-sac resorption. Sex ratio analysis of the progenies reared at basal temperature suggests that the three natural populations shared a similar complex genetic sex determination (GSD) system based on a predominant male heterogametic factor (XX/XY) with a possible polymorphism at this locus and/or effects of minor genetic factors. Progenies of the three populations exhibited a clear thermosensitivity of their gonadal sex differentiation, with important familial variations in the sex ratio deviation depending on the parents; this confirms the existence of strong genotype-environment interactions in the temperature induced sex differentiation (TSD) of Nile tilapia. Moreover, the existence of naturally sex-reversed individuals (XX males and/or XY females) is strongly suggested at least in two out of the three natural populations (Kpandu and Koka); however, it was not possible to infer the cause
from minor genetic factors and/or from environmental-induced influences. Therefore, our study brings the first evidence that natural populations of Nile tilapia as well as domestic stocks of this species have a complex sex determination system (SDS) combining polymorphic GSD and TSD components.

Knowledge of the molecular mechanisms implicated in the cascade of sex determination and differentiation is still scarce in vertebrates and particularly in fish. It is still not clear if there is a common underlying sex differentiation pathway in "GSD" fish species (i.e. rainbow trout) as well as in "TSD" fish species (tilapias, hirame, ...). TSD & GSD could be the ends of a continuum rather than two mutually exclusive mechanisms. In fish, most of the genes of the sex determining cascade have been identified through cloning of mammalian orthologue genes. The aromatase gene has been shown to be strongly expressed before and during female differentiation, correlated with high levels of estradiol in the ovaries whereas, the opposite situation is observed in developing testes (D'Cotta et al., 2001a; Baroiller and D'Cotta, 2001). In addition, aromatase enzyme activity has been analyzed in fry heads before and during sex differentiation: during these critical stages, aromatase activity in female heads is 3 times higher than in male brains (D'Cotta et al., 2001a; Baroiller and D'Cotta, 2001). Aromatase expression is strongly repressed with masculinizing temperature treatments (D'Cotta et al., 2001a; Baroiller and D'Cotta, 2001). Aromatase brain activity strongly decreased in temperature-treated females but also in genetic males reared at 35°C (D'Cotta et al., 2001a; Baroiller and D'Cotta, 2001). Lastly, we have isolated by Differential display the MM20C gene (annotation unknown) which has been revealed by semi-quantitative RT-PCR and virtual northern to be upregulated during gonadal differentiation of genetic females treated by masculinizing temperatures (D'Cotta et al., 2001b). In genetically male gonads, the weak expression of MM20C become stronger during masculinizing temperature treatments. Therefore, in tilapia, aromatase and oestrogens play key roles during normal and temperature-induced gonadal sex differentiation of tilapia, O. niloticus. Aromatase repression seems to be required in the gonads (and perhaps in the brain) in order to drive differentiation towards testis development. However, it is clear that other key genes are also involved in the gonadal sex differentiation.

In order to isolate and identify novel sex differentiating genes in tilapia, we have generated several cDNA libraries and combined them to macro and microarrays analysis. The cDNA libraries have been performed using two types of normalisation procedures: 1) suppression subtractive hybridization (SSH) in order to obtain differentially expressed genes that are either XX-specific or XY-specific; 2) a normalisation procedure giving full-length sequences based on the use of a crab duplex-specific nuclease, in order to equalize the mRNAs and thus allow the isolation of rare transcripts. Currently 3000 ESTs have been sequenced from clones randomly picked from the SSH libraries. Annotation of these ESTs was possible for 60% of the sequences. Despite the subtraction system, a large proportion of genes encoded for putative structural and ribosomal proteins. Part of these ESTs were picked and spotted onto nylon macroarrays together with genes specifically cloned in tilapia which have been shown to be related to sex determination or differentiation in mammals (for instance several Sox genes, Amh, FoxL2, Dmrt1). The macroarray containing 1536 spots has been hybridized with differentiating ovaries from XX individuals and differentiating testes from XY individuals, both treated at 27°C as well as differentiating testes from XX individuals which were masculinized by temperature treatment at 35°C. The bioinformatics analysis of the signals is currently being performed. We have simultaneously analysing the
kinetics of expression by real-time PCR of *Amh* and *Sox9* genes cloned in tilapia, both shown to be involved in cascade of mammalian male differentiation.

These recent data and their perspectives will be discussed in this communication.

**REFERENCE**


