CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Experimental animals and sample collection

Three families (03, 07, and 10) of Nile tilapia from 12 families, 30 individuals per family including three dam and sire were established. All experiments were carried out the facilities of the Department of Animal Science, at Göttingen University, Germany. The experimental fish, Nile tilapia (*Oreochromis niloticus*) from the Lake Manzala population, were derived from selection of high line (more than 93% males) or low line (less than 60% males) populations after a temperature treatment at 36°C from 10-20 dpf. Genetically all-female (XX) which crosses between a temperature sex-reversed male (ΔXX) from the high line and low line were established (Wessels and Hörstgen-Schwark, 2007, Wessels and Hörstgen-Schwark, 20011, Lühmann *et al*., 2012). Artificially fertilized eggs were incubated at 28 °C for 10 days. The larvae, after yolk sac absorption, were divided into a control and temperature treatment group each consisting 110 fish. The control group was kept 28 °C throughout the experiment, whereas the thermal treatment group was kept at 36 + 0.5 °C, after a gradual increase of temperature, for ten days (10-20 dpf) as previously described by Tessema *et al*. (2006) and Wessels and Hörstgen-Schwark (2007). Subsequently, at 20 dpf of the treatment groups were gradually acclimated to 28°C again. After 90-120 days fish from the different families from control and treatment groups were killed by an overdose of anaesthetics and sexing using aceto-carmine gonad squash technique. Fin clips were collected from all individuals as a source of DNA (n=96). The temperature-dependent treatment pattern was show in **Figure 3.1**.

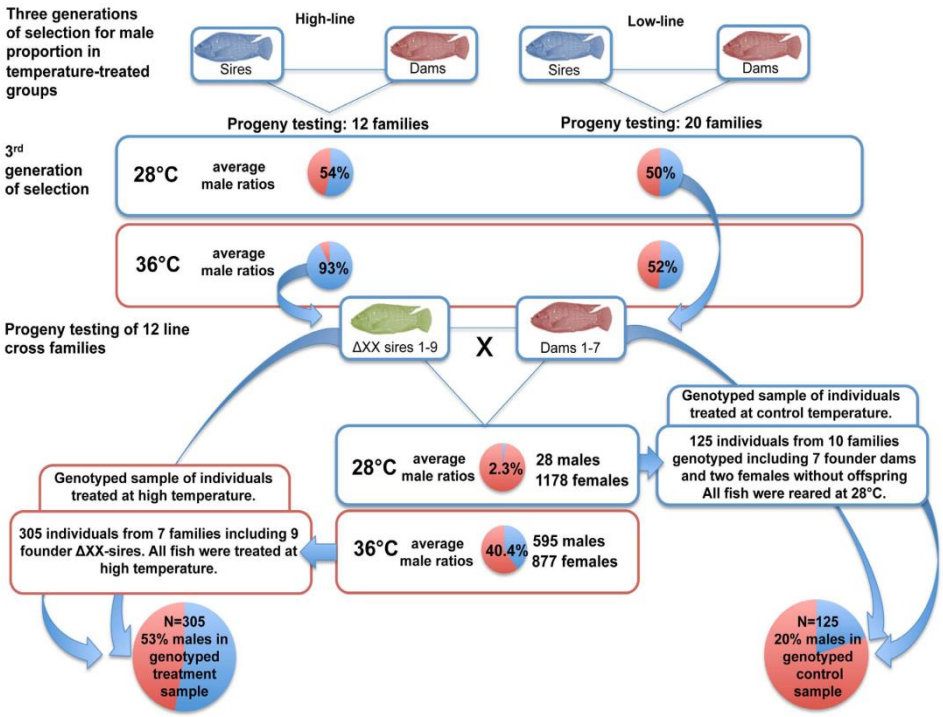

Figure 3.1 The experiment pattern to obtain a genetically female (XX) population to study thermal effect on male proportion (Source: Wessel *et al*., 2014)

3.1.2 Equipments

1) Bench centrifuge 5415R (Eppendorf AG, Germany)
2) Bench centrifuge 5424 (Eppendorf AG, Germany)
3) Cool centrifuge 5417 (Eppendorf AG, Germany)
4) DNA Analyzer ABI PRISM 3100 (Applied Biosystems GmbH, Germany)
5) Electrophoresis (horizontal) (Bio-Rad Laboratories GmbH, Germany)
6) Electrophoresis power supply (Bio-Rad Laboratories GmbH, Germa
7) Gel-document system (PEQLAB Biotechnologic GmbH, Germany
8) Incubator (Heraeus Instruments GmbH, Germany)
9) Nanodrop ND-1000 (PEQLAB Biotechnologic GmbH, Germany)
10) Pipettes 10-100 µl; 100-1000 µl (Eppendorf AG, Germany)
11) Pipettes 1-10 µl; 2-20 µl (Gilson, U.S.A.)
12) Themocycler T-Gradient (Biometra GmbH, Germany)
13) Thermocycler T-3000 (Biometra GmbH, Germany)
14) Vortex Genie 2 (Scientific Industries, U.S.A)

15) Water bath 5 liter (GLF-Labortechnik GmbH, Germany)

3.1.3 Chemicals and Kits

1) ExoSAP-IT PCR clean-up kit (Affymetrix/USB, Germany)
2) Big Dye Terminator V3.1 Cycle Sequencing kit (Applied Biosystems GmbH, Germany) Agarose (Biozym Scientific GmbH, Germany)
3) Ethidium bromide (Roth, Germany)
4) Ethanol (Echter Nordhäuser Spirituosen GmbH, Germany)
5) DNA Ladder Mix (Fermentas GmbH, Germany)
6) Q-Solution (Qiagen, Germany)
7) 2U FastStart Taq DNA Polymerase kit (Roche Applied Science, Germany)
8) Oligonucleotide primers (Sigma-Aldrich Biochemic GmbH, Germany)
9) dNTPs (Roche Applied Science, Germany)
10) Roti-aqua-phenol (Roth,Germany)
11) Chloroform (Roth, Germany)
12) Phenol (Roth,Germany)
13) Fast *Taq* DNA polymerase (Roche, Germany)
14) Deionized water

3.1.4 Solutions and Buffers

All solutions and buffers used in this study were prepared with double distilled Millipore water

1) 96% Ethanol
2) Lysis buffer
3) Proteinase K solution
4) 1x TBE buffer
5) 10x TBE buffer
6) TE buffer pH 8.

3.1.5 Primers design

The sequence of the *amh* gene was derived from of *amh* Genebank of Nile tilapia reference accession No. EF512167.1 (www.ncbi.nlm.nih.gov). The primer were design by primer 3 software with primer length 20-23 bp for amplify more than 500 base pairs of a consensus *amh* gene. All primers cover the region of *amh* from exon 2 to exon 7. Primers were designed to cover the whole sequence of *amh* (except 3'→5'UTR) gene and a M13 universal primer was add to the 5' end of primer to enable bidirectional amplification on an 3130xL Genetic Analyzer (Applied Biosystems, Germany) using the Big Dye terminator Kit (Applied Biosystems). The 4 pairs of primers used in this study are listed in **Table 3.1.**

Table 3.1 Primers used for SNP discovery and genotyping

Primer name	Primer sequence (5'-3')	Amplified region	T_{Am} (°C)
Amh-F227	FW: ACTCACTCCCATCAGCGAAG	Exon 2,3 and Intron 3	60
Amh-R892	RV:AAACAAGTGCAATTTGTACACCA		
Amh-F946	FW:GATGTCTCAGTGATGAAATGTGC	Exon 4,5 and Intron 3	60
Amh-R1517	RV: AAGCACATCTGGGAAAGCAA		
Amh-F1688	FW: CACAGTCTTTGGCTTCACGA	Exon 6 and Intron 6	60
Amh-R2374	RV: CAGCCAAGCTCACACACACT		
Amh-F2156	FW: CGGTCCCAGTGACCTATGAG	Exon 7 and Intron 6	60
Amh-R3060	RV: GCATCTGGCTTGATGGAGAT		

3.1.6 Softwares

1) Blast program http://www.ncbi.nih.gov/blast
2) Primer 3 http://www.primer3plus.com/cgibin/dev/primer3plus.cgi
3) DNASTAR Lasergene V6 (DNASTAR,Inc., Germany)
4) Unipro UGENE 1.12 http://ugene.unipro.ru/

3.2 Methods

3.2.1 DNA extraction

The DNA was isolated from finclips by phenol-chloroform extraction (Chomczynski and Sacchi, 1987).

1) Cut off the finclips into small pieces and placed in 1.5 ml tube.
2) Add 350 μl of lysis buffer.
3) Add 150 μl of protienase K.
4) Vortex and centrifuge the sample gentlely for few minutes.
5) Incubate the sample at 56 °C in water bath overnight.
6) After overnight incubation, vortex and centrifuge few minutes and add 500 μl of Phenol (Do under fume hood).
7) Vortex few minutes and centrifuge at room temperature (14,000 rpm) for 7 minutes.
8) Put the upper layer in new 1.5 ml. tube which has 500 μl of chlorophorm inside.
9) Vortex few minutes and centrifuge at room temperature (14,000 rpm) for 5 minutes.
10) Put the upper layer in new 1.5 ml. tube which has 500 μl of ethanol 96% inside.
11) Vortex few minutes and centrifuge at 4 °C (14,000 rpm) for 5 minutes.

12) Get rid off the supernatant ethanol and centrifuge with speed vacuum.

13) Dilute DNA dried pellet with 1 x TE (pH 7.5) 50 μl and store at -20°C.

3.2.2 Polymerase chain reaction (PCR) of *amh* gene

The *amh* sequences were amplified by PCR with the optimum annealing temperature. DNA pool (20 ng/µl) was used as DNA template. PCR reaction was performed with the following conditions (**Figure** 3.3):

1) PCR Mixture prepared in a 0.2 ml microcentrifuge tube on ice in 25 µl reaction volume containing,

DNA (20 ng/µl.)	2.00 µl.
1xTBE containing Mgcl2	2.50 µl.
1x Q-solution (Roche)	5.00 µl.
Forward primer (10 pmol/ µl.)	1.00 µl.
Reverse primer (10 pmol/ µl.)	1.00 µl.
10mM dNTP mix (Roche)	0.50 µl.
2U FastStart Taq DNA polymerase (Roche)	0.30 µl.
$MgCl_2$ (Roche)	2.50 µl.
add ddH_2O	10.2 µl.

2) Place the tube in a Biometra T-3000 Thermocycler (Biometra GmbH, Germany) and perform PCR,

Initial denaturation	95 °C	10 min.
Denaturation	92 °C	30 sec.
Annealing	60 °C	30 sec.
Extension	72 °C	1 min.
Final extension	72 °C	5 min.

Cycling 34 cycles of deturation, annealing and extension time.

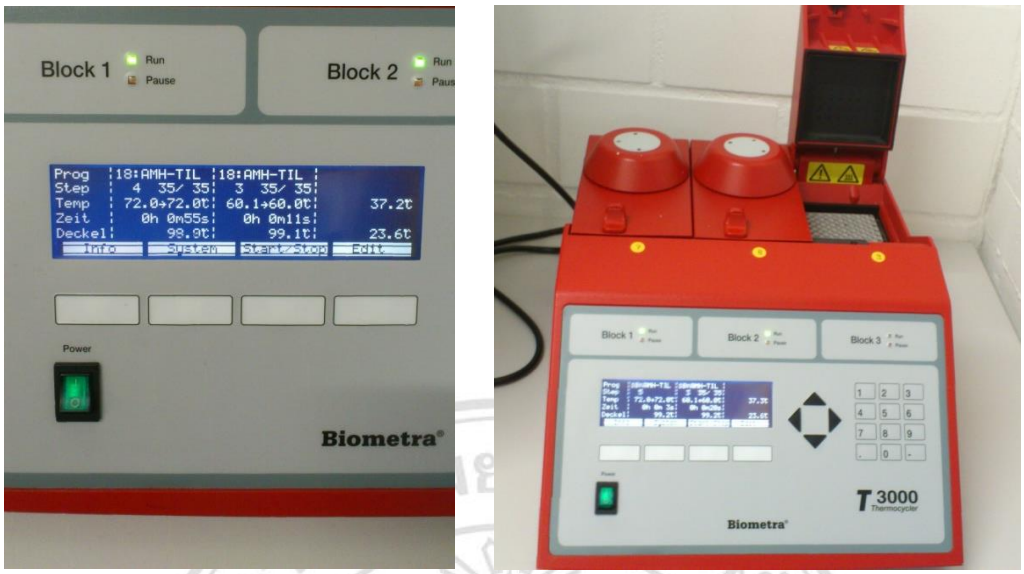

Figure 3.2 Amplifiedf amh gene in Biometra T-3000 Thermocycler (Biometra Gmb) Germany)

3.2.3 Gel electrophoresis

PCR product was load on 2.0% agarose gel with 0.1% Ethidium bromide in 1x TBE buffer. Then, the gel was photographed under the gel system. (**Figure** 3.3)

Figure 3.3 Gel electrophoresis with 2.0% agarose gel

3.2.4 PCR product purification with Exo-SAP-IT

PCR products were cleaned up by ExoSAP- IT kit (Affymetrix/USB, Germany) in 7 µl. reaction volumes containing,

PCR product	5.00 µl.
ExoSAP- IT kit (Affymetrix/USB)	2.00 µl.

The mixture was put in the Biometra T-3000 Thermocycler (Biometra GmbH, Germany) to incubate at 37°C for 15 min. followed by 80 °C for 15 min.

3.2.5. DNA amplify sequencing

1) After purification, amplifications were performed in a Biomettra T-3000 thermocycler to sequencing. The amplicons were subjected to bi-directional sequencing on an ABI Prism 3100 DNA analyzer by using the Big Dye terminator kit in 7 reaction volume containing,

Purified PCR product	2.00 µl.	
10 µM of M13 primer (Forward or reverse)	1.00 µl.	
BigDye Terminator (V.3.1)	1.00 µl.-	5x
sequencing buffer	1.00 µl.	
H_2O	2.00 µl.	

2) Amplifications was performed in a Biomettra T-3000 thermocycler to sequencing,

Initial denaturation	96°C	30 min.
Denaturation	96°C	10 sec.
Annealing	53.7°C	10 sec.
Extension	60°C	4 min.

Cycling 27 cycles of deturation, annealing and extension time.

3.2.6 Sequence analysis

The sequences of the DNA fragments were trimmed and SNPs were trimmed and were manually identified using the program software suit DNASTAR Lasergene 6 (DNASTAR, Inc., Germany) compare with *amh* sequence reference accession no. EF512167.1 (www.ncbi.nlm.nih.gov).

3.2.7 Statistical analysis

The genotype (allele) frequencies of segregating SNPs (N=3) were analyzed using chi-square analysis with statistic program package. SNPs haplotypes of 3 families Nile tilapia which treated at high temperature including dams and sires were measured of linkage disequilibrium (LD) in r^2- value (Naomi, 2005) between segregating SNPs. The measure of LD was calculated in R-Statistic program. Moreover, the analysis was carry out to detect associations between the SNP genotype code as 0 = CC, 1=GC, and 2 = GG and binary trait in sex code as 0 = male and 1 = female.